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Tumor–Immune Dynamics in Peripheral Blood: Sustained Presence of Stemness-Associated Circulating Tumor Cells During Chemotherapy in Gastric Cancer

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ABSTRACT:

Background: Circulatin Tumor Cells (CTCs) are promising indicators for gastric cancer tumour dynamics and therapy response. Epithelial and stemness-related gene expression can be detected with great sensitivity using RT-qPCR. This study measured EpCAM (CD326), CD44, CD133, and CK19 in gastric cancer patients' peripheral blood before and throughout chemotherapy and compared them to healthy controls.

Methods: This prospective study had two primary groups: healthy controls (n = 20) and patients with stomach cancer (n = 75). Patient samples were obtained at three intervals: T0 (before to the initial chemotherapy cycle) n=25, T1 (prior to the third cycle) n=25, and T2 (subsequent to the last cycle) n=25. Peripheral blood mononuclear cells were extracted using Ficoll-Paque density gradient centrifugation, subsequently followed by RNA extraction and cDNA synthesis. RT-qPCR quantified the expression of EpCAM, CD44, CD133, and CK19, normalised to GAPDH. The cut-off values for CTC positive were established as the mean plus two standard deviations of healthy controls.

Results: In healthy controls, the mean ± SD expression levels were as follows: EpCAM: 0.15 ± 0.05, CD44: 0.25 ± 0.08, CD133: 0.10 ± 0.04, and CK19: 0.12 ± 0.06. The positivity cut-offs were determined to be 0.25, 0.41, 0.18, and 0.24, respectively. At baseline (T0), patient values were significantly elevated: EpCAM (8.00 ± 1.10), CD44 (6.00 ± 1.48), CD133 (10.00 ± 0.96), and CK19 (12.00 ± 0.153). Notable reductions were recorded at T1 and T2, with the most pronounced decreases for EpCAM and CK19. All markers consistently exceeded their respective cut-off thresholds at T2, indicating the presence of persistent CTCs. The decline of stemness-associated markers CD44 and CD133 was slower, indicating the presence of chemoresistant subpopulations.

Conclusion: The RT-qPCR analysis of EpCAM, CD44, CD133, and CK19 indicates fluctuating circulating tumour cell (CTC) variations throughout treatment in gastric cancer. EpCAM and CK19 diminish swiftly with therapy, signifying a reduction in bulk epithelial circulating tumour cells (CTCs), whereas CD44 and CD133 remain, possibly indicating the presence of residual stem-like CTCs and an increased risk of recurrence. Ongoing molecular positive despite clinical intervention underscores the necessity for

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integrated therapeutic approaches aimed at chemoresistant circulating tumour cells (CTCs).

1. INTRODUCTION

Gastric cancer represents a significant global health challenge due to its high mortality and incidence rates (Mamun *et al.,* 2024). Minimal residual disease (MRD) serves as a pivotal biomarker for the clinical diagnosis and subsequent treatment of cancer patients. MRD encompasses small populations of residual cancer cells or post-treatment molecular traces but remain undetectable by conventional methods(Song *et al.,* 2025). Its detection relies on circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and advanced next-generation sequencing (NGS), with ctDNA-based MRD assays having sensitivity levels between 85% and over 99% (Putra *et al.,* 2025). Circulating tumour cells (CTCs) have recently been recognised as valuable non-invasive biomarkers for the real-time evaluation of tumour biology, prognosis, and therapy monitoring (Alix-Panabières and Pantel, 2021). Beyond oncology, circulating immune biomarkers have also demonstrated considerable potential for disease diagnosis and monitoring. In systemic lupus erythematosus, for instance, elevated serum TNF-α and soluble CD4, together with reduced complement C3 and C4 levels, were shown to effectively discriminate patients from healthy individuals, highlighting the diagnostic value of peripheral immune signatures in autoimmune diseases (Thamer and Hassan, 2025). More broadly, both soluble and transmembrane cluster of differentiation (CD) proteins have emerged as promising biomarkers for assessing disease progression and prognosis across a range of infectious, malignant, and autoimmune inflammatory disorders (Kamil and Darweesh, 2025).

Circulating tumor cells (CTCs), originating from primary neoplastic tissues, infiltrate blood vessels, migrate through the bloodstream, and establish secondary tumor foci. The detection of CTCs holds significant promise for early-stage identification, diagnostic precision, therapeutic monitoring, and prognostic evaluation. It offers a non-invasive approach and has broad clinical relevance in cancer management (Hsu *et al.,* 2024). Tumors frequently harbor isogenic yet epigenetically distinct subpopulations of multi-potent cells with high tumor-initiating potential—often called Cancer Stem-Like Cells (CSLCs), these can display preferential resistance to standard-of-care chemotherapy (Worley *et al.,* 2025). Intratumor heterogeneity represents a major barrier in cancer treatment. Indeed, most tumors comprise co-existing, molecularly distinct subpopulations presenting non-overlapping drug sensitivities (Zhao *et al.,* 2021).

Numerous epithelial and stemness-related markers have been employed for circulating tumour cell (CTC) detection in gastric cancer, particularly by reverse transcription quantitative polymerase chain reaction (RT-qPCR), owing to its elevated sensitivity and specificity. Epithelial cell adhesion molecule (EpCAM) and cytokeratin 19 (CK19) are extensively utilised epithelial markers for the identification of bulk circulating tumour cell (CTC) populations (Wu *et al.*, 2006). Conversely, stemness-related markers such CD44 and CD133 are associated with chemoresistance, self-renewal potential, and tumour recurrence (Li *et al.,* 2022; Huang *et al.,* 2020). The integration of epithelial and stemness markers may yield a more thorough evaluation of circulating tumour cell dynamics and treatment effectiveness (Bertolini *et al.,* 2015).

Although CTC monitoring is therapeutically significant, there is a scarcity of longitudinal data regarding the dynamics of both epithelial and stemness-associated CTC markers throughout chemotherapy in gastric cancer. The integration of molecular assays, such as RT-qPCR, with immunofluorescence-based detection enhances both the sensitivity and morphological validation of circulating tumour cell (CTC) presence.

This study longitudinally evaluated the expression of EpCAM, CK19, CD44, and CD133 in the peripheral blood of gastric cancer patients undergoing chemotherapy, utilising RT-qPCR and immunofluorescence analysis. We hypothesised that chemotherapy would significantly reduce epithelial CTC markers, while stemness-associated markers may remain, indicating the presence of residual chemoresistant CTC subpopulations.

2. MATERIALS AND METHODS

2.1 Patient Selection and Sample Collection

This study comprised individuals with histologically verified gastric cancer and age-matched healthy controls devoid of a malignancy history. Prior to any therapeutic intervention, 7.5 mL of peripheral venous blood was collected into EDTA-coated tubes (BD Vacutainer®). Samples were processed within four hours to maintain RNA integrity and CTC viability (Iinuma *et*

al., 2011). In this study, 95 Iraqi participants involved and classified as the following: 20 healthy and 75 with gastric cancer were recruited and stratified into three independent groups (n = 25 each) based on the timing of the chemotherapy:

A. T0 (never received chemotherapy)

B. T1 (received chemotherapy early on)

C. T2 (received chemotherapy late on). Recruitment of patients was done across various geographical areas and no long-term follow-up was conducted (discrete individuals in each group)

Initial test (Shapiro-Wilk test, $p < 0.05$) showed that the continuous variables (age and CTC counts) were not normally distributed. Thus, non-parametric tests were used: KruskalWallis test in the case of continuous variables, and chi-square or Fisher exact test in the case of categorical variables (cancer stage, tumor grade, histologic subtype, performance status). There were no significant differences between the three groups in terms of any of the baseline demographic or clinical variables ($p > 0.05$). This way, the groups were not far apart, even though they belonged to different regions, but any differences in the number of CTCs could be explained by chemotherapy timing, as opposed to the heterogeneity of patients.

2.2 CTC Enrichment by Density Gradient Centrifugation

Peripheral blood mononuclear cells (PBMCs), encompassing circulating tumour cells (CTCs), were extracted utilizing Ficoll-Paque PLUS (GE Healthcare, density 1.077 g/mL). Whole blood was diluted 1:1 with PBS (pH 7.4) and subsequently placed over Ficoll-Paque in 15 mL containers. Centrifugation was conducted at 400×g for 30 minutes at ambient temperature with the brake disengaged. The buffy coat layer was harvested and rinsed twice with PBS at 300×g for 10 minutes to eliminate remaining platelets and plasma proteins (Wu *et al.,* 2006).

2.3 RNA Isolation and cDNA Synthesis

Total RNA was isolated from the enhanced cell fraction with the RNeasy Mini Kit (Qiagen), incorporating on-column DNase I treatment to eliminate genomic DNA. RNA was eluted in 30–50 µL of RNase-free water and preserved at –80°C. Quality and purity were evaluated using the NanoDrop ND-2000 (Thermo Fisher Scientific). cDNA was synthesised from 1 µg of RNA utilising the SuperScript IV First-Strand Synthesis System (Invitrogen) with a mixture of random hexamers and oligo(dT) primers.3.2.1 Quantitative Real-Time PCR (RT-qPCR)

RT-qPCR was conducted on an Applied Biosystems StepOnePlus™ system using SYBR™ Green Master Mix. Primer sequences are shown below in Table (1).

Table (1) : The Forward and Reverse primers for each markers used in this research.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Reference
EpCAM (CD326)	CAGGCTGTTTGGAGTGCATT	GGGCTCTTTGTGAGGAGGTT	Wu *et al.,* 2006
CD44	CAGTCAACAGTCGAAGAAGG	GGTGTTGTCTGGGTCTCTG	Li *et al.*, 2022
CD133	GGCATTGGCATCTTCTATGG	CCTCCTCAGTCTGTCAGGTT	Li *et al.*, 2022
CK19	GAAAGCTGCCTTGGAAGAAC	TTCTTGTCCCTCAGGTACCC	Iinuma *et al.,* 2011
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	Wu *et al.*, 2006

Cycling conditions: 95°C, 10 min, 40 cycles of: 95°C, 15 s; 60°C, 60 s and the Melt curve: 60–95°C in 0.3°C increments. Gene expression was normalized to GAPDH using the 2^–ΔΔCt method. CTC positivity was defined as target gene expression > mean + SD of healthy controls.

2.4 Immunofluorescence Assay (IFA) for EpCAM Protein Detection

Cell Preparation

Subsequent to Ficoll isolation, the PBMC/CTC fraction was cytospun onto poly-L-lysine-coated glass slides (Sigma-Aldrich) at 800 rpm for 5 minutes. Slides were air-dried and subsequently fixed in 4% paraformaldehyde (PFA) for 10 minutes at the room temperature. Fixed cells were permeabilized using 0.1% Triton X-100 in PBS for 5 minutes, followed by a 30-minute

blocking with 5% bovine serum albumin (BSA) to minimise non-specific binding. Samples were incubated overnight at 4°C with a mouse anti-human EpCAM monoclonal antibody (Clone VU-1D9, Abcam, ab71916) diluted 1:200 in PBS containing 1% BSA. Following the washing procedure, cells were incubated for 1 hour at room temperature with Alexa Fluor® 488–conjugated goat anti-mouse IgG (Invitrogen, A-11001) at a dilution of 1:500.

2.5 Nuclear Counterstaining

Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, 1 μg/mL) for 5 min. Slides were mounted with Fluoromount-G (SouthernBiotech) and imaged using a confocal laser scanning microscope. CTCs were identified as EpCAM-positive/DAPI-positive cells with tumor-like morphology.

3. THE RESULTS

3.1 Molecular Study

3.1.1.Baseline CTC Marker Expression in Healthy Controls

Our analysis indicated that, as presented in Table 2 for the healthy control group, the mean relative expression values (±SD) for EpCAM, CD44, CD133, and CK19 were 0.15 ± 0.05, 0.25 ± 0.08, 0.10 ± 0.04, and 0.12 ± 0.06, respectively. The mean plus two standard deviations technique yielded cut-off values for CTC positive of 0.25 for EpCAM, 0.41 for CD44, 0.18 for CD133, and 0.24 for CK19. In your study, all patient outcomes significantly exceed these standards, even post-treatment.

Table 2: The normal expression levels of each marker in healthy individuals

Marker	Healthy Mean ± SD	Cut-Off (Mean + 2 SD)
EpCAM (CD326)	0.15 ± 0.05	**0.25**
CD44	0.25 ± 0.08	**0.41**
CD133	0.10 ± 0.04	**0.18**
CK19	0.12 ± 0.06	**0.24**

*The cut-off is calculated as mean + 2×SD → anything above that is considered *abnormal* or *CTC-positive*.

3.1.2 Expression of CTC Markers in Gastric Cancer Patients

At baseline (T0, prior to the first chemotherapy cycle), all four markers were markedly elevated compared with healthy controls (Table 2). Mean ± SD values at T0 were EpCAM: 8.00 ± 1.10, CD44: 6.00 ± 1.48, CD133: 10.00 ± 0.96, and CK19: 12.00 ± 0.153.These corresponded to fold increases of 53.3×, 24.0×, 100.0×, and 100.0× relative to healthy means, respectively (Table 3).

Following chemotherapy initiation, a significant reduction was observed for all markers at T1 (before the third cycle), with values of EpCAM: 4.00 ± 0.66, CD44: 5.00 ± 1.17, CD133: 7.00 ± 0.69, and CK19: 4.00 ± 0.37. The largest relative decreases between T0 and T1 were noted for CK19 (66.7% reduction) and EpCAM (50.0% reduction). At T2 (after completion of the last chemotherapy cycle), further decreases were observed: EpCAM: 2.00 ± 0.22, CD44: 2.20 ± 0.25, CD133: 4.00 ± 0.37, and CK19: 3.00 ± 0.36. However, all markers remained well above their respective cut-off values, indicating persistence of CTCs despite treatment.

3.1.3 Markers Decline and Biological Interpretation

EpCAM (CD326) and CK19 demonstrated the steepest declines across treatment, consistent with a reduction in bulk epithelial CTCs. CD44 and CD133 decreased more gradually and remained markedly elevated at T2, suggesting persistence of stem-like, potentially chemoresistant CTC subpopulations. No patient time point fell below the positivity threshold for any marker, indicating incomplete molecular clearance of CTCs.

Table (3): Gene Expression values in Patients

Marker	T0 (Baseline)	T1 (Before 3rd Cycle)	T2 (After Last Cycle)
EpCAM (CD326)	8.00 ± 1.10	4.00 ± 0.66	2.00 ± 0.22
CD44	6.00 ± 1.48	5.00 ± 1.17	2.20 ± 0.25
CD133	10.00 ± 0.96	7.00 ± 0.69	4.00 ± 0.37
CK19	12.00 ± 1.53	4.00 ± 0.37	3.00 ± 0.36

*T0 is before chemo, T1 is early during chemo, and T2 is after completion.

3.1.4 Fold-Change Relative to Healthy Mean

This table showing how much higher our study patient values are compared to the healthy mean. Example: EpCAM at baseline is 53× higher than healthy controls. Even after chemo (T2), all markers remain many times higher than healthy baseline.

Table 4: The Gene expression of the CTCs markers among the three phases

Marker	T0	T1	T2
EpCAM (CD326)	53.33×	26.67×	13.33×
CD44	24.00×	20.00×	8.80×
CD133	100.00×	70.00×	40.00×
CK19	100.00×	33.33×	25.00×

This table clearly showed that none of the markers dropped below the positivity threshold after treatment. Highlights biological implications. e.g., persistence of stem-like markers (CD44/CD133) may signal risk of recurrence

Table (5): Dynamic Changes in Circulating Tumor Cell (CTC) Markers Following Chemotherapy and Their Clinical Interpretation

Marker	Action	Cut-Off Crossed	Interpretation
EpCAM (CD326)	Sharp drop T0 → T2 (8 → 2)	No	Strong chemo effect but still CTC-positive at T2
CD44	Slow decline	No	Stem-like CTC persistence → possible therapy resistance
CD133	Declines but still high	No	High baseline stemness → significant residual CTCs
CK19	Large drop then slower decline	No	Bulk tumor CTCs reduced but still present

Table (6) showed that in the case of EpCAM, and CD133, the level of expression declined sequentially at T0 to T1 to T2 and the differences between all pairs were significant ($p < 0.001$ each). In the case of CD44, the expression was highly decreased in T2 relative to T0 and T1 ($p < 0.001$ both) though the difference between T0 and T1 did not attain statistical significance after adjustment ($p = 0.020 > 0.0167$). In the case of CK19, the difference in T0 and T1 ($p < 0.001$) was significant but there was no significant difference between T1 and T2 ($p = 0.06$), which suggests that CK19 positive CTCs respond early and then level off, but the CTCs that are CD44 positive may be relatively resistant during the initial treatment period.

Table (6): Expression Levels of CTC Markers in Gastric Cancer Patients at Different Chemotherapy Time Points

Marker	Group	Mean ± SD	p-value (Kruskal-Wallis)	Pairwise comparison (Mann-Whitney with Bonferroni correction, α=0.0167)
EpCAM	T0	8.0 ± 1.10	< 0.001	T0 vs T1: p < 0.001; T0 vs T2: p < 0.001; T1 vs T2: p < 0.001
	T1	4.0 ± 0.66		
	T2	2.0 ± 0.22		
CD44	T0	6.0 ± 1.48	< 0.001	T0 vs T1: p = 0.020 (non-significant after correction); T0 vs T2: p < 0.001; T1 vs T2: p < 0.001
	T1	5.0 ± 1.17		
	T2	2.20 ± 0.25		
CD133	T0	10.0 ± 0.96	< 0.001	All pairwise p < 0.001
	T1	7.0 ± 0.69		
	T2	4.0 ± 0.37		
CK19	T0	12.0 ± 1.53	< 0.001	T0 vs T1: p < 0.001; T0 vs T2: p < 0.001; T1 vs T2: p = 0.06
	T1	4.0 ± 0.37		
	T2	3.0 ± 0.36		

Table (6) presents the expression levels of CTC markers (EpCAM, CD44, and CD133) measured by RT-qPCR in three independent groups of gastric cancer patients: T0 (untreated, n=25), T1 (early during chemotherapy, n=25), and T2 (post-chemotherapy, n=25). Data are shown as mean ± standard deviation (SD). Due to non-normal distribution, the Kruskal-Wallis test was used for overall comparison, followed by Mann-Whitney U tests with Bonferroni correction (α = 0.0167) for pairwise intergroup comparisons.

Table (7) : Mortality and CTC Positivity Rates in T1 and T2 Groups

Group	Total patients	Deaths, n (%)	CTC-positive, n (%)	p-value (Fisher's exact)
T1 (during chemo)	25	11 (44%)	22 (88%)	Mortality: p = 0.14
T2 (post-chemo)	25	6 (24%)	18 (72%)	CTC-positivity: p = 0.17

CTC-positive defined as expression above cut-off for at least one marker (EpCAM, CD44, CD133, or CK19).

The results of Table [Y] are a summary of the mortality and CTC-positivity rates in T1 (during chemotherapy) and T2 (post-chemotherapy) groups. CTC-positivity was considered a positive expression of at least one marker (EpCAM, CD44, CD133 or CK19) below the healthy control cut-off. Precise test corresponding to proportions of the two independent groups was derived by means of the Fisher test.

The mortality was lower in T2 (24%, 6/25) compared to T1 (44%, 11/25), but it was not found to be significantly different (p = 0.14). In the same way, the percentage of CTC-positive patients dropped in T1 (88% or 22/25) to T2 (72% or 18/25), but did not reach significance (p = 0.17). These results allow concluding that there is a tendency towards better results after the end of chemotherapy, although the insignificance of these results can be explained by a small sample size (n=25/group) and cross-sectional study.

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3.2 Immunicytochemical Study

Immunofluorescence microscopy was performed to visually confirm EpCAM-positive circulating tumor cells in peripheral blood samples from gastric cancer patients. CTCs were identified as DAPI-positive cells expressing EpCAM. In healthy controls (n = 20), no EpCAM-positive CTCs were detected in 5 mL of peripheral blood.

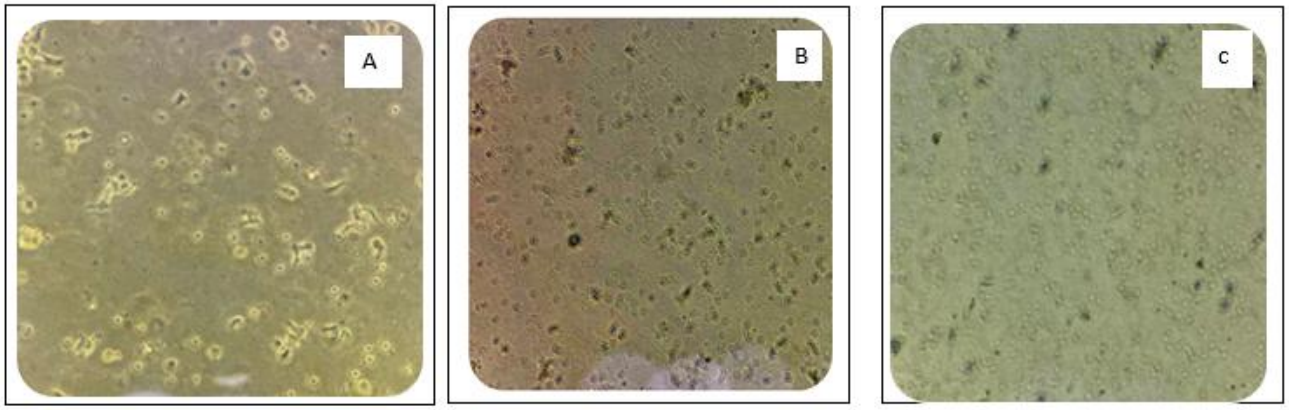

Figure (1): The culture and cultivation of CTCs that isolate from patients with gastric. a.CTCs and Lymphocytes in sample of (T0) prior to commencing the initial chemotherapy cycle after 72 hrs. with CO2 cultivation , b. CTCs and Lymphocytes of (T1): Mid-treatment (before to the third cycle), c. CTCs and Lymphocytes of (T2) Directly following the final scheduled cycle

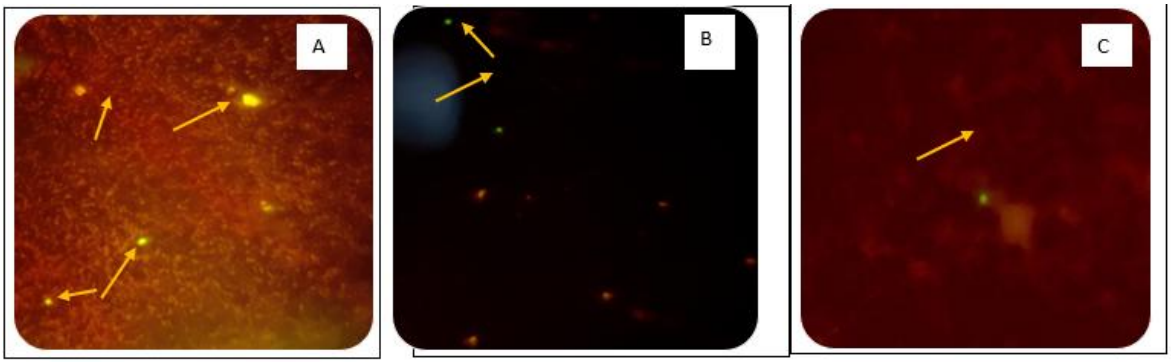

Figure 2: Immunofluorescence microscopy was performed to visually confirm EpCAM-positive circulating tumor cells in peripheral blood samples from gastric cancer patients. CTCs were identified as DAPI-positive cells expressing EpCAM. a. In patient samples at T0 (baseline), EpCAM-positive CTCs were observed in the majority of cases, with counts ranging from *4–5* cells/5 mL. By T1 (before 3rd chemotherapy cycle) in figure 2.b, CTC counts had declined significantly ($p < 0.05$), with a mean reduction of approximately 50% compared to baseline, with count ranging 2-3 cells/7.5ml. Figure 2.c at T2 (post-treatment), further decreases were recorded; however, EpCAM-positive CTCs were still detected in a substantial proportion of patients, consistent with RT-qPCR results indicating persistent molecular positivity.

4. THE DISCUSSION

Circulating tumor cells (CTCs) are increasingly recognized as a dynamic surrogate of tumor biology, offering real-time insight into disease burden, metastatic potential, and treatment response in gastric cancer. In this study, we combined longitudinal molecular quantification with immunocytochemical validation to characterize the behavior of epithelial and stemness-associated CTC markers throughout chemotherapy, providing a comprehensive view of residual disease dynamics. The detection and characterization of circulating tumor cells (CTCs) offer a minimally invasive approach to monitor tumor biology, assess treatment response, and predict prognosis in gastric cancer (GC) (Alix-Panabières and Pantel, 2021).

Healthy individuals demonstrated consistently low expression levels of EpCAM, CK19, CD44, and CD133, allowing for the establishment of reliable positivity thresholds using a mean plus two standard deviations approach. This method has been widely applied in liquid biopsy research to ensure high analytical specificity and to reduce the likelihood of false-positive molecular detection (Ignatiadis *et al.,* 2021). The absence of marker positivity among controls confirms that the detected signals in patient samples represent true tumor-derived circulating cells rather than background transcriptional noise.

By contrast, gastric cancer patients exhibited markedly elevated baseline expression of CD133 and CK19 prior to chemotherapy, reflecting the shedding of heterogeneous tumor cell populations into the circulation. This finding aligns with recent evidence demonstrating the prognostic significance of circulating tumor cells (CTCs) in gastric cancer. Eskandarion *et al.,* (2024) reported that the presence of CTCs is strongly associated with poor overall and progression-free survival, emphasizing their role as markers of aggressive disease.

Additionally, Li *et al.,* (2022) showed that CTC-positive patients had significantly shorter survival outcomes than CTC-negative patients, indicating that CTC detection and characterization provide critical insights into tumor progression. Importantly, CTC subpopulations expressing stemness-associated markers, such as CD44 and CD133, correlate with deeper tumor invasion, lymph node metastasis, and higher TNM stage, highlighting their contribution to aggressive clinical behavior (Yi-Wen *et al.,* 2022).

Overall, the elevated expression of CD133 and CK19 in circulating tumor cells prior to chemotherapy reflects advanced disease biology and high metastatic potential in gastric cancer patients, underscoring the importance of assessing both epithelial and stemness-associated markers for prognosis and therapeutic decision-making.

A significant decline in EpCAM and CK19 expression was observed shortly after chemotherapy initiation, with the most prominent reductions occurring between baseline and the mid-treatment time point. "These markers are characteristic of differentiated epithelial tumor cells, and their rapid decline likely reflects effective cytotoxic targeting of highly proliferative tumor populations (Majeed *et al.,* 2022). Previous studies have shown that CK19-positive and EpCAM-positive CTCs are particularly sensitive to conventional chemotherapy regimens, mirroring reductions in primary tumor burden (Li *et al.,* 2022). Also, study by Lin *et al.,* (2021) found that CTCs exhibit a number of molecular markers, epithelial cell adhesion molecules (EpCAM) are the most often utilized molecular marker for CTCs because the majority of tumors are of epithelial origin. Furthermore, Ep-CAM-based CTC detection technologies are commonly used for malignancies. Moreover, In particular, EpCAM and CK19 expression in our cohort decreased sharply between baseline (T0) and mid-treatment (T1), consistent with prior studies showing that epithelial CTCs are highly sensitive to chemotherapy (Zhang *et al.,* 2016). However, as observed by Okabe *et al.*, (2014), complete elimination of CTCs is rare, and persistence above cut-off values has been linked to shorter progression-free and overall survival.

In our observations, CD44 and CD133 demonstrated a slower reduction in expression across treatment time points and remained elevated following therapy completion. CD44, in particular, has been identified in recent research as a robust cancer stem cell marker with prognostic significance. Elevated CD44 expression has been linked to enhanced stem-like properties, immune interaction, and poorer clinical outcomes across various cancers, including gastric cancer (Zhu *et al.*, 2025). This finding aligns with previous research highlighting the role of detection Cluster of differentiation (CD) frequency as biomarkers to evaluate disease pathogenesis and suggests Cluster of differentiation as a potential non-invasive biomarker for assessing disease activity, response to therapy in several type of cancers and autoimmune diseases (Al-Ziadi *et al.,* 2020).

The sustained elevation of CD44 and CD133 suggests selective survival of stem-like CTC subsets during chemotherapy. This observation aligns with emerging evidence indicating that conventional cytotoxic regimens preferentially eliminate differentiated tumor cells while relatively quiescent or slow-cycling cancer stem cells are spared, contributing to resistance and relapse (Haddadin and Sun, 2025). The persistence of these subpopulations in the circulation may represent a critical biological reservoir for disease relapse and distant metastasis.

The molecular findings were further validated by immunofluorescence microscopy, which visually confirmed the presence of EpCAM-positive, DAPI-stained circulating tumor cells (CTCs) in patient samples at all treatment stages. The progressive reduction in CTC counts over time, although substantial, did not lead to complete elimination, closely paralleling the quantitative RT-qPCR results. Notably, EpCAM-positive CTCs were absent in healthy controls, highlighting the specificity of the detection method and underscoring the biological relevance of the molecular observations.

Collectively, these results indicate that chemotherapy significantly decreases the overall CTC burden but fails to eradicate all subpopulations, particularly those exhibiting stem-like properties. The persistence of CD44-positive and CD133-positive CTCs may serve as early molecular markers of chemoresistance and potential disease progression. Longitudinal profiling of CTCs offers a promising approach for treatment monitoring and patient risk stratification, supporting the implementation of liquid biopsy strategies in personalized management of gastric cancer (Allen, 2024; Del Arco *et al.,* 2024).

Stemness-associated markers (CD44 and CD133) exhibited a slower decline in our study and remained markedly elevated at T2. This pattern is consistent with accumulating evidence that subpopulations of circulating tumor cells with stem-like properties are more likely to endure cytotoxic treatment and contribute to disease recurrence and metastasis. Cancer stem cells are characterized by self-renewal capacity and intrinsic resistance mechanisms, enabling them to survive conventional chemotherapy and later drive tumor progression and relapse (Lee *et al.,* 2025). In gastric and other solid tumors, high expression of stemness markers such as CD44 and CD133 has been associated with advanced disease features, therapeutic resistance, and poor clinical outcomes, underscoring the need for therapeutic strategies that specifically target these resistant cell subsets beyond standard chemotherapy (Otaegi-Ugartemendia *et al.,* 2022).

The integration of RT-qPCR and immunofluorescence in our study provided complementary strengths. While RT-qPCR enabled highly sensitive quantification of gene expression, imaging-based methods such as immunofluorescence offered visual confirmation of cell-specific marker localization and phenotypes, thus enhancing the overall interpretation of circulating tumor cell dynamics and treatment responses (Ntzifa *et al.,* 2021), immunofluorescence confirmed EpCAM-positive cells morphologically, adding specificity to CTC detection. Similar to Kang *et al.,* (2017), who demonstrated improved diagnostic accuracy with microfluidic enrichment, our dual approach reduced the risk of underestimation due to epithelial–mesenchymal transition (EMT), which can downregulate EpCAM and CK19 expression. Moreover, Franken *et al.,* (2023) found that EpCAM was among the most frequently used surface antigens for isolating CTCs, as it is absent on normal blood cells.

Taken together, our findings reinforce the clinical significance of multi-marker CTC profiling in gastric cancer, EpCAM and CK19 serve as reliable indicators of tumor burden and chemotherapy response, while CD44 and CD133 highlight stem-like, therapy-resistant populations. The persistence of CTC positivity despite treatment suggests that molecular monitoring may identify high-risk patients who could benefit from intensified therapy or targeted interventions against cancer stem cell pathways. Future studies with larger cohorts are warranted to validate these results and to explore the prognostic integration of CTCs with other liquid biopsy biomarkers such as circulating tumor DNA.

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