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Prognostic significance of circulating tumor cells (CTCs) in Egyptian non-metastatic colorectal cancer patients: A comparative study for four different techniques of detection (Flowcytometry, CellSearch, Quantitative Real-time PCR and Cytomorphology)

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ABSTRACT

Purpose: We assessed CTCs counts in NMCRC patients using four different techniques.
Methods: CTCs were detected in 63 NMCRC patients, 40 benign bowel diseases (BBD) and 40 normal controls (NC) using, flow-cytometry (FCM), CellSearch (CS), cytomorphology and quantitative real time (qPCR) for *CK19*, *MUC1*, *CD44*, *CD133*, *ALDH1* expression. Results were correlated to progression free (PFS) and overall (OS).
Results: Positive CTCs (≥4 cells /7.5 mL blood) were detected in 50.8% (32/63) NMCRC by FCM and 7.5% (3/40) BBD ($p < .001$). CTCs were detected in 34/63 (54%) NMCRC, 4/40 (10%) BBD ($p < .001$) by CS. *CK19*, *MUC1*, *CD44*, *CD133* and *ALDH1* were expressed in 35 (55.6%), 29 (46.0%), 28 (44.4%), 26 (41.3%) and 25 (41.3%) cases of NMCRC. In BBD 4/40 (10%) cases expressed *CK19*, *MUC1* and *CD44*, while 2/40 (5%) expressed *CD133*. Cytomorphology showed the lowest sensitivity (47.6%) and specificity (90%) for CTCs detection. The combined use of FCM or CS with CTCs-mRNA markers improved the sensitivity and specificity to 68.3%, and 95.0%; respectively. Positive CTCs and mRNA markers expression were significantly associated with shorter 5-yr PFS and OS. In multivariate analysis, CTCs mRNA markers were independent prognostic factors for PFS and OS.
Conclusions: Enumeration of CTCs by FCM and RNA expression for specific colon cancer markers are comparable to CS regarding sensitivity and specificity. CTCs also represent novel therapeutic targets for NMCRC cases.

1. Introduction

Colorectal cancer (CRC) is one of most common malignancies, and the third leading cause of cancer related death worldwide (Siegel et al., 2017). In Egypt, CRC has been detected in about 13% of the patients undergoing colonoscopy, and the incidence is increasing annually (Gado et al., 2014). Surgical resection is still the treatment of choice for early stage CRC, while adjuvant chemo- or radiotherapy are introduced in advanced cases (Bayraktar et al., 2010). However, the current tumor staging histology or radio-imaging techniques are still lacking the sensitivity required to detect early dissemination. Moreover, 20% of the cases diagnosed with early stage CRC and up to 30% of stage III patients, subsequently relapse after 5 years of curative surgery (Roder et al., 2015; Siegel et al., 2017), suggesting the occurrence of undetected micro metastatic event(s), or shedding of viable tumor cells in the circulation (Hardingham et al., 2015).

Circulating tumor cells (CTCs) are specific tumor cells that disseminated into the peripheral circulation from the primary or the metastatic tumors. They have gained increasing significance as non-invasive liquid biopsy markers for cancer diagnosis and treatment

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monitoring (Han et al., 2017). Several studies on CTCs in metastatic tumors produced evidence that the number of CTCs associated significantly with poor prognosis, reduced response to treatment and reduced survival rates (Cohen et al., 2008; Resel Folkersma, 2012). Also CTCs could be found in patients with NMCRC which correlated significantly with the type of tissue differentiation of colorectal tumors (Yang et al., 2018), and benign tumors (Tsai et al., 2016). In addition, the detection of CTCs in patients with early breast cancer represent an independent prognostic factor associated with an unfavorable clinical outcome (Economopoulou et al., 2017).

Till now, the most widely used detection method for separation of CTCs involves utilization of monoclonal antibodies against the epithelial cell adhesion molecule (EpCAM), with the FDA approved CellSearch system (CS). However, the use of EpCAM-based technique failed to identify CTCs that have undergone epithelial to mesenchymal transition (Gorges et al., 2012). Other techniques including imaging cytometry and size-based separation approaches, however they showed less sensitivity and specificity (Kowalik et al., 2017). Therefore, the limited ability of these techniques to detect intact CTCs produces an additional challenge for highly sensitive technique(s) that could detect CTCs in early stage of NMCRC patients. This will help in identifying those at risk of developing metastasis, and thus could benefit from early treatment. In a previous study by our group using FCM (Bahnassy et al., 2014), we were able to enumerate CTCs in hepatocellular carcinoma (HCC) patients, with high sensitivity and specificity, especially when combined with RT-qPCR. To the best of our knowledge, very limited studies (Yang et al., 2018; Tsai et al., 2016) assessed CTCs in non-metastatic CRC (NMCRC) patients for long follow-up periods especially in the Egyptian population.

In this study, we aimed to assess the prognostic impact of CTCs in a cohort of NMCRC patients, compared to those with BBDs and normal subjects using four detection methods, and those patients were followed up for a period of 5 years. We compared the sensitivity and specificity of flow cytometry (FCM), RT-qPCR, CS and cytomorphology for enumeration of CTCs in NMCRC and BDD to determine the optimal methodology for analyzing CTCs count impact.

2. Methods

This prospective cohort study included 63 patients with pathologically confirmed non-metastatic colorectal cancer (NMCRC), and 40 patients with benign bowel disease (BBD), Compared to 40 age and sex matched normal control (NC). Patients were admitted to the National Cancer Institute (NCI) during the period 2012 to 2013, where diagnosis and staging done according to the guidelines of the seventh edition of the International Union against Cancer (UICC) (Sobin et al., 2010). Patients were treated according to the NCI, Cairo guideline. Detailed patients' characteristics were illustrated in Table 1. The study was approved by the institutional Review Board of the NCI, Cairo University which was in accordance with declaration of Helsinki and an informed consent was obtained from each subject prior to enrollment in the study. None of the patients received therapy prior to blood acquisition.

2.1. Blood samples acquisition

Two Peripheral blood samples (7.5 mL each) were collected from each individual into CellSave blood collection tubes (Immunicon Inc., USA) containing EDTA and cellular preservative and processed within 72 h after collection to recover the peripheral blood mononuclear cells (PBMCs). The PBMCs was isolated by density gradient centrifugation using Ficoll-Hypaque 1077 (Sigma).

Analytical samples were prepared for validation to simulate the tested samples depending on knowing the starting concentration of target cells. Prostate cancer (PC3) and breast cancer (MDA-MB-231and SKBR3) cell lines were used, as they have known (EpCAM) expression range. Cells suspended in a stock solution of Dulbecco's modification of Eagle medium (MDEM; HAM-F12, volume per volume) were counted on a hemocytometer and spiked into 7 mL whole blood in varying concentrations.

Table 1
Clinicopathological features of colorectal cancer patients.

Characteristics	Colorectal cancer patients (n = 63) N (%)
Age	
Mean ± SD (Range)	44.8 ± 12.7 (21–72)
≤ 40	25 (39.7)
41–60	38 (60.3)
Gender	
Male	32 (50.8)
Female	31 (49.2)
Family history	
Positive	11 (21.2)
Negative	52 (78.8)
PS	
I	55 (87.3)
II&III	8 (12.70)
Tumor site	
Colon	40 (63.5)
Rectum	23 (32.1)
Tumor type	
Adenocarcinoma	49 (77.8)
Mucinous	14 (22.2)
Grade	
1	1(1.6)
2	53 (84.1)
3	9 (14.31)
Lymph node status	
Negative	35 (55.6)
Positive	28 (44.4)
T Stage	
T1	2 (3.2)
T2	8 (12.7)
T3	38 (60.3)
T4	15 (23.8)
Stage	
IA	2 (3.2)
IB	6 (9.5)
IIA	16 (25.4)
IIB	9 (14.3)
IIIA	3 (4.8)
IIIB	13 (20.6)
IIIC	14 (22.2)
Neo-adjuvant	
Yes	18
Adjuvant CTH treatment	
Yes	33
Relapse	
Free	40 (63.5)
Relapsed	14 (22.2)
Missing	9 (14.3)
Progression	
1	41 (75.9)
2	13 (24.1)
missing	9
Survival	
Alive	36 (66.7)
Dead	18 (33.3)
Missing	9

PS: performance status, RTH: Radiotherapy, CTH: Chemotherapy.

2.2. CTCs enrichment

CD45 Human MicroBeads (130-045-801; Miltenyi Biotec, Germany) were used for enrichment of epithelial tumor cells from PBMCs via CD45+ cells depletion and cell number determination. The recovered PBMCs were resuspended in binding buffer and the cell suspension was loaded onto a separator column placed in a magnet (MiniMACS Starting kit; Miltenyi Biotec). CD45+ cells were retained in the column, while the targeted CD45- fraction enriched in epithelial tumor cells were collected. The cells were further enriched by using magnetic beads

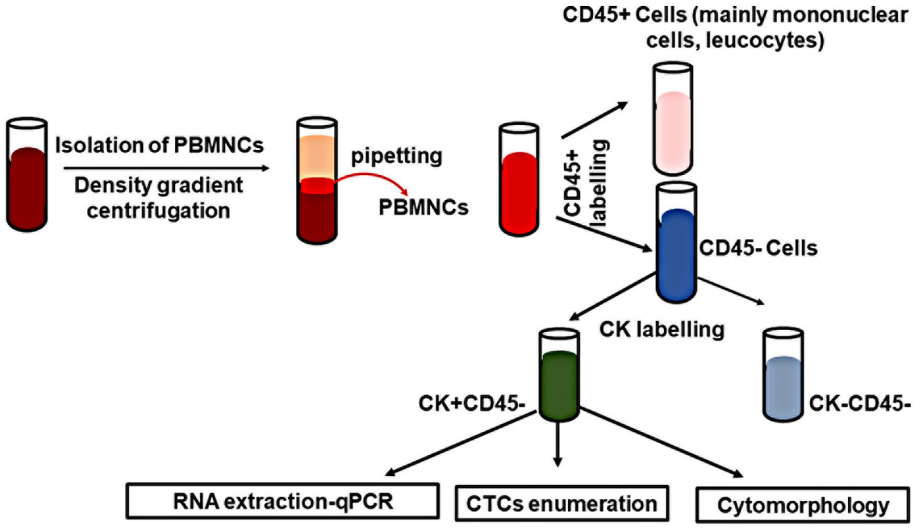

Fig. 1. Isolation and enrichment for circulating tumor cells.

Table 2
The primer sequences.

	Forward	Reverse
Ck19	5′TCGACAACGCCCGTCTG3′	5′GACGCGTACTCGCACC3′
MUC1	5′GTGCCCCCTAGCAGTACCG3′	5′GGTTGAACATCCCCGTGCAG3′
CD44	5′GACACATATTGCTTCAATGCTTCAGC3′	5′TACTAGGAGTTGCCTGGATGGTAG3′
CD133	5′AGCAGCAGTCTGACCAGCGTGA3′	5′CCACGGGTGGAAGCTGCCTCAG3′
ALDH1	5′GCTGGCGACAATGGAGTCAA3′	5′ACGGCCCTGGATCTTGTCAG3′
B-actin	5′GTGAAGGTGACAGCAGTCGGTT3′	5′GAAGTGGGGTGGCTTTTAGGAT3′
K-ras 12	5′ACTGAATATAAACTTGTGGTAGTTGGACCT3′	5′TAATATGTCGACAAAACAAGATTTACCTC3′
K-ras 13	5′GTACTGGTGGAGTATTTGATAGTGTATTAA3′	5′GTATCGTCAAGGCACTCTTGCCTAGG3′

CK19, cytokeratin 19; MUC1, mucin1; *CD*, cluster of differentiation; *ALDH1*, aldhyde-dehydrogenase-1.

labeled with cytokeratin-specific antibody (CK3-11D5, Miltenyi Biotec, Germany). The separated CD45-CK+ cells were used for a) enumeration of CTCs by FCM and CellSearch (CS) b) RNA extraction & RT-qPCR of tested genes, c) cytomorphology and d) DNA extraction for detection of *K-ras* mutations (Fig. 1).

For comparison of CTCs enumeration, 20 cases were further enriched by addition of anti-EpCAM pre-conjugated immunomagnetic beads (Miltenyi Biotec, Germany) to the separated cells.

2.3. Enumeration of CTCs by FCM

The spiked in and the human samples (with/without EpCAM enrichment) were stained with anti CK-FITC and anti-CD45-TRITC monoclonal antibodies (Thermo Scientific) according to manufacturers' protocols. Normal lymphocytes were used as a negative control.

2.4. CellSearch system (CS)

Cells from spiked in samples were recovered using the CellSearch Profile Kit after magnetic separation. Recovered cells were applied to adhesive slides (Fischer, Germany) and then fixed with 2% paraformaldehyde, permeabilized with cold methanol, washed and 10% goat serum were added for 20 min to block nonspecific binding sites. Slides were incubated at 37 °C with monoclonal anti-pan cytokeratin (Sigma, MO), conjugated CD45 antibody (Alexa 647 antibodies Serotec), and the secondary antibody (Alexa555 Invitrogen) was added for 20 min after washing, and counterstained with DAPI. Enumeration of CTCs (CK+/CD45-/DAPI +) was done using fluorescent microscope (Nikon, Japan). For human samples (with/without EpCAM enrichment), separated cells were fluorescently stained by using anti-cytokeratin (CK), anti-CD45 and DAPI. Cells were considered as CTCs if they were 1) CK+/CD45−, 2) nucleated, and 3) morphologically intact. The total cell number was determined by counting all intact nuclei in the smear. Imaging was performed using Inverted epifluorescence microscope (AxioObserver Z1; Carl Zeiss, Germany) and the Cell Tracks System imaging software (Veridex).

2.5. Confirmation of CTCs results by RT-qPCR

RNA was extracted from the separated CK+/CD45 − CTCs of human samples (NMCRC, BBD and controls) using RNeasy kit (QIAGEN) according to manufacturers' instructions. The expression levels of *CK19, MUC1, CD44, CD133 and ALDH1* markers were assessed in a final volume of 25 μL SYBR Green Master mix, 1 μL cDNA and 400 nM of primers of the tested genes in stratagene MAX3000P (Applied Biosystems, USA) according to manufacturer's protocols. Standard c-DNA for the used markers (obtained either from previously tested cases or cell lines with a known RNA level for each gene) were used for adjustment (compensation) of the PCR especially if there was a difference in the dilutions of the tested samples. Assays were done in triplicates for each sample using β-actin for normalization. The primer sequences are illustrated in Table 2. Correlative Ct values were recorded, and the data was expressed as relative expression units.

2.6. Cytomorphology

Aliquots of separated CK+/CD45− CTCs in PBS were applied to adhesive slides (Fischer, Germany) and incubated at 37 °C for 40 min. Cells were then fixed with 2% paraformaldehyde, permeabilized with cold methanol and washed. Slides were stained with Papanicolaou stain, cover-slipped and cells were examined by a specialized pathologist using bright-field microscope to confirm their origin as epithelial

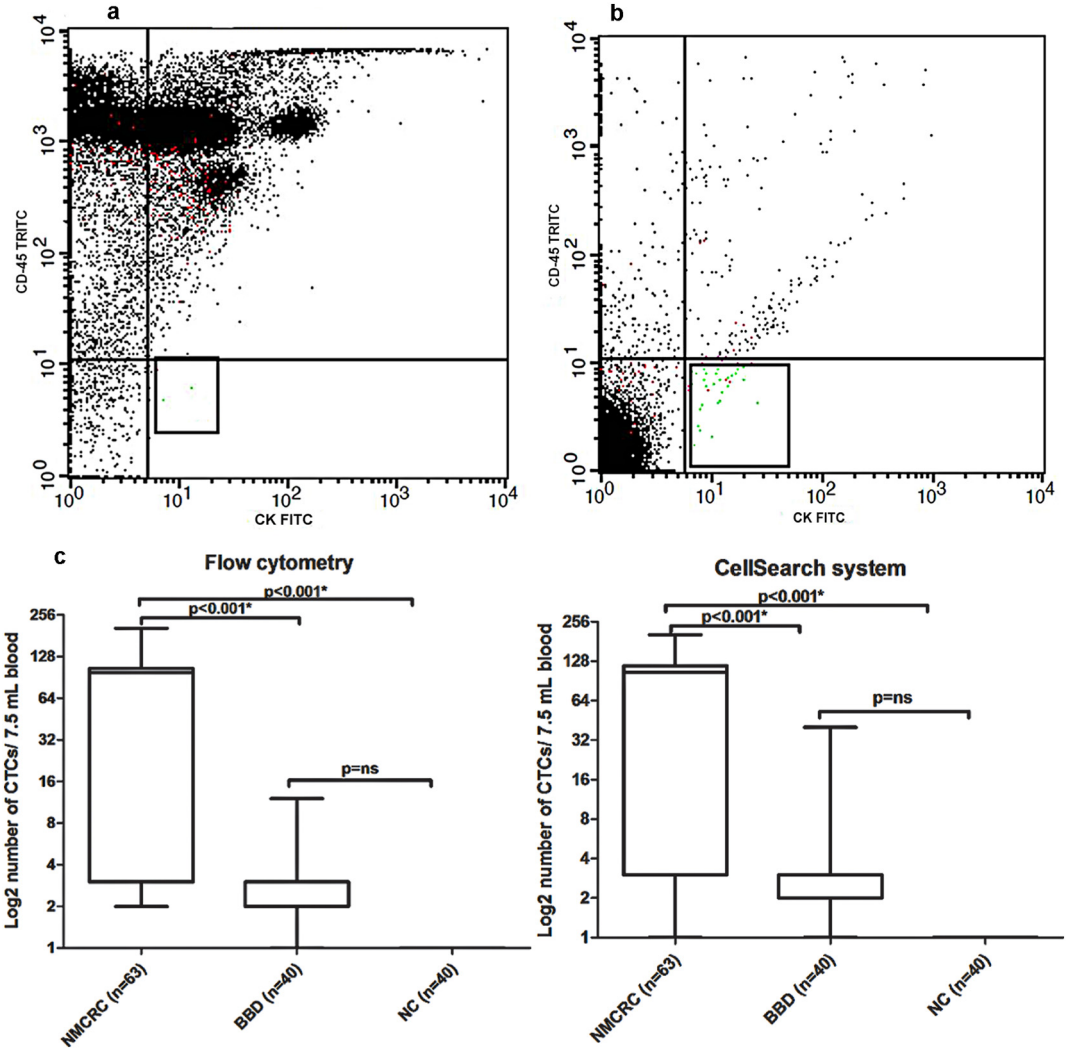

Fig. 2. Flow cytometric analysis of circulating tumor cells in a) benign bowel disease and b) non-metastatic colorectal cancer patients. The gated areas are as follows: 1) the lower right quadrant represents the CTCs (cell positive for CK and negative for CD45), 2) the upper left quadrant represents CD45 positive cells and CK negative (mononuclear cells and lymphocytes). The upper right quadrant represents cells positive for CD45 and CK). The lower left quadrant represents debris. c, d Box plot of circulating tumor cells count among different groups using c) flow cytometry and d) CellSearch. The CTCs count in NMCRC was compared to benign and NC using F-test of ANOVA. *Significance at $p < .001$

Table 3
Combined sensitivity and specificity with accuracy for the investigated parameters in non-metastatic colorectal cancer patients.

Parameters	Sensitivity	Specificity	PPV	NPV	Accuracy
FCM	50.8%	96.3%	91.4%	71.3%	76.2%
CS	54.0%	95.0%	89.5%	72.4%	76.9%
Cytomorphology	47.6%	90.0%	78.9%	68.8%	71.3%
CK19	55.6%	95.0%	89.7%	73.1%	77.6%
MUC1	46.0%	95.0%	87.9%	69.1%	73.4%
CD44	44.4%	95.0%	87.5%	68.5%	72.7%
CD133	41.3%	97.5%	92.9%	67.8%	72.7%
ALDH1	41.3%	100.0%	100.0%	68.4%	74.1%
CK19 + MUC1 + CD44 + CD133 + ALDH1	63.5%	95.0%	90.9%	76.8%	81.1%
FCM + CS + *CK19 + MUC1 + CD44 + CD133 + ALD1*	68.3%	95.0%	91.5%	79.2%	83.2%

FCM, flowcytometry; CS, CellSearch; *CK*19, cytokeratin 19; *MUC1*, mucin1; *CD*, cluster of differentiation; *ALDH1*, aldehyde dehydrogenase1.

cells.

2.7. K-ras mutation detection

Analysis of *K-ras* mutations (codons 12 and 13) was detected in the tissues and CTCs of the NMCRC and BBD patients using PCR-RFLP and line strip assay (ViennaLab® Diagnostics GmbH, Vienna, Austria). The DNA was isolated from FFPE tissues and CTCs. The primer sequences and PCR conditions were done according to Popovic Hadzija et al. (Popovic Hadzija et al., 2007), which include two rounds of PCR amplification and restriction enzymes cut as follow; after the 1st PCR, the products were digested overnight at 60 °C with 5 units *Bst*NI (for assessment of mutation at codon 12), and at 37 °C with 5 units of *Hae*III for revealing mutation at codon 13. Then, the first digest was used as a

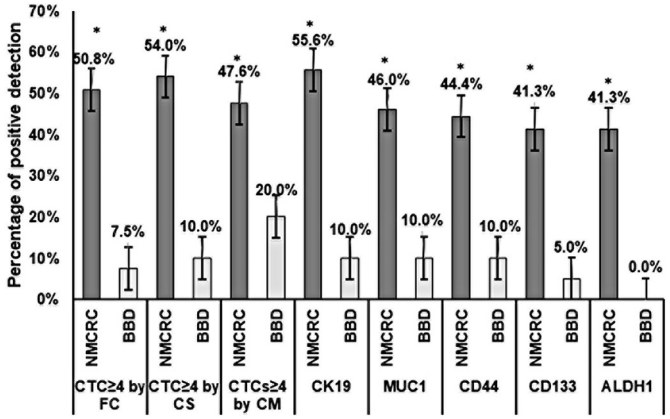

Fig. 3. Positive circulating tumor cells detection rate and expression of studied markers in patients with non-metastatic colorectal cancer (NMCRC) and benign bowel disease (BBD). Positive detection of CTCs was compared between NMCRC and BBD using $\chi 2$ test. *Significance at $p < .001$. FC; flowcytometry, CS; CellSearch, CM; Cytomorphology.

template for the 2nd PCR. Products of the second PCR were digested with either *Bst*NI or HaeIII. The final digestion products were electrophoresed in 4% ethidium bromide stained agarose gel and analyzed under UV light. (Table 2).

2.8. Statistical analysis

data was analyzed using the SPSS package (version 20 for Windows; SPSS Inc., Chicago, IL, USA). Pearson's $\chi 2$ was used to determine the significance of associations between categorical variables. Spearman's test was used to detect the strength of concordance between two variables. Kaplan–Meier was used for comparing survival rates using log-rank test. Progression-free survival (PFS) was defined as time from date of primary treatment till date of relapse/progressive disease, while overall survival (OS) is time from date of diagnosis till date of death. The Cox proportional-hazards model was used to determine the independent significant risk of individual factors. *P*-values ≤.05 were considered as statistically significant.

3. Results

3.1. Flowcytometry and CellSearch

The EpCAM expression level was evaluated in three tumor cell lines spiked into the healthy donor blood to simulate the clinical conditions. It ranged from low (MDA-MB-231 and PC3 at 2.3× and 6× background fluorescence; respectively) to high (SKBR3 at 25× background fluorescence). Recovery linearity was measured as previously described by Harb et al. (Harb et al., 2013) using PC3 cell line spiked in at levels ranging from 20 to 300 target cells/7.5 mL blood in triplicates. According to EpCAM expression, the recovery percentages using FCM was 74%, 75%, and 85% for MDAMB-231(n = 17), PC3(n = 28), and SKBR3(n = 11), respectively compared to 12% for MDA-MB-231, 48% for PC3 and 88% for SKBR3 cells using the CellSearch.

As for human samples, the median CTCs count by FCM was 98(range, 2-198/7.5 mL blood) in NMCRC compared to 3(range, 0-3/7.5 mL blood) in BBD ($p \leq .001$). While by using CS, the median CTCs count was 105(range, 1-214/7.5 mL blood) in NMCRC compared to 3(range, 1-3/7.5 mL blood, $p \leq .001$, Fig. 2). By using FCM, there was no statistically significant difference in CTCs count with or without enrichment (median no. with EpCAM-enrichment was 98 versus 85

Table 4
The correlation between the number of circulating tumor cells (CTCs) by flow cytometry or CellSearch and the expression levels of the studied markers in NMCRC patients.

	CTCs Flow cytometry		Concord[b]	p-value[a]	CTCs CellSearch		Concord[b]	p-value[a]
	-ve (n = 31) N (%)	+ve (n = 32) N (%)			-ve (n = 29) N (%)	+ve n = 34) N (%)		
CK19								
Negative	26 (83.9)	2 (6.3)	77.70%	$\chi 2$ = 38.4	23(79.3%)	5(14.7)	64.8%	$\chi 2$ = 26.5
Positive	5 (6.1)	30 (93.7)		p < .001*	6(20.7%)	29(85.3%)		p < .001*
MUC1								
Negative	28 (90.3)	6 (18.8)	71.5%	$\chi 2$ = 32.5	24(82.8%)	10(29.4)	53.0%	$\chi 2$ = 17.9
Positive	3 (9.7)	26 (81.2)		p < .001*	5(17.2%)	24(70.6)		p < .001*
CD44								
Negative	30 (96.8)	5 (7.9)	81.0%	$\chi 2$ = 41.9	27(93.1)	8(23.5)	68.5%	$\chi 2$ = 30.7
Positive	1 (3.2)	27 (84.4)		p < .001*	2(6.9)	26(76.5)		p < .001*
CD133								
Negative	29 (93.5)	8 (25.0)	68.3%	$\chi 2$ = 30.5	27(93.1)	10(29.4)	62.4%	$\chi 2$ = 26.2
Positive	2 (6.5)	24 (75.0)		p < .001*	2 (6.9)	24 (70.6)		< 0.001*
ALDH1								
Negative	30 (96.8)	7 (21.9)	74.7%	$\chi 2$ = 36.4	27(93.1)	10(29.4)	62.4%	$\chi 2$ = 26.2
Positive	1 (3.2)	25 (78.1)		p < .001*	2(6.9)	24 (70.6)		p < .001*
K-ras mutation								
WT	24 (77.4)	11(34.4)	41.0%	$\chi 2$ = 11.8	21(72.4)	11(41.2)	33.1%	$\chi 2$ = 6.2
Mutant	7 (22.6)	21 (65.6)		p < .001*	8(27.6)	23(58.8)		p = .01*
CTCs by PAP								
-ve	28(90.3)	5(15.6)	74.8%	$\chi 2$ = 35.2	24(82.8)	9(26.5)	56.2%	$\chi 2$ = 19.9
+ve	3(9.7)	27(84.4)		p < .001*	5(17.2)	25(73.5)		p < .001*
CTCs by CS								
-ve	27(81.1)	2(6.2)	81.1%	$\chi 2$ = 41.4				
+ve	4(112.9)	30(93.8)		p < .001*				

CK19, cytokeratin19; MUC1, mucin1; *CD*, cluster of differentiation; *ALDH1*, aldhyde-dehydrogenase-1; CTCs, circulating tumor cells; PAP, Papanicolaou staining (cytomorphology); Concord., concordance.
+ve CTCs, CTCs ≥ 4cells/7.5 mL blood, −ve CTCs, CTCs < 4 cells/7.5 mL blood.
[a] Different variables and occurrence of CTCs was tested by chi square test. [b]Concordance was detected using Cohen's kappa. *Significance at $p < .05$.

Table 5
The concordance between the epithelial cell markers expression and *K-ras* mutation in NMCRC.

	CK19	*MUC-1*	*CD44*	*CD133*	*ALDH1*	*K-ras mutation*
CK19		$r = 0.698_{**}$	$r = 0.607_{**}$	$r = 0.685^{**}$	$r = 0.62_{**}$	$r = 0.31^{**}$
	1	$p < .001$	$p < .001$	$p < .001$	$p < .001$	$p = .012$
MUC1	$r = 0.698_{**}$		$r = 584_{**}$	$r = 0.52^{**}$	$r = 0.46_{**}$	$r = 0.36_{**}$
	$p < .001$	1	$p < .001$	$p < .001$	$p < .001$	$p = .004$
CD44	$r = 0.607_{**}$	$r = 584_{**}$		$r = 678^{**}$	$r = 743^{**}$	$r = 0.46_{**}$
	$p < .001$	$p < .001$	1	$p < .001$	$p < .001$	$p < .001$
CD133	$r = 0.685^{**}$	$r = 0.52^{**}$	$r = 678^{**}$		$r = 0.607^{**}$	$r = 0.33^{**}$
	$p < .001$	$p < .001$	$p < .001$	1	$p < .001$	$p < .01$
ALDH1	$r = 0.62_{**}$	$r = 0.46_{**}$	$r = 743^{**}$	$r = 0.607^{**}$		$r = 0.46^{**}$
	$p < .001$	$p < .001$	$p < .00$	$p < .001$	1	$p < .009$
K-ras mutation	$r = 0.35^{**}$	$r = 0.39^{**}$	$r = 42^{**}$	$r = 0.30^{**}$	$r = 0.42^{**}$	1
	$p = .005$	$p = .002$	$p = .001$	$p = .01$	$p < .001$	

** significant at $p < .01$; r, spearman correlation coefficient; *CK 19*: cytokeratin 19; *MUC1*, mucin1; *CD*, cluster of differentiation; *ALDH1*, aldehyde dehydrogenase1.

without EpCAM-enrichment, $p = .64$) whereas, the count of CTCs differed significantly with enrichment by CS (median no. with EpCAM-enrichment was 105 versus 62 without EpCAM-enrichment, $p = .031$).

The sensitivity and specificity for detection of CTCs (≥4 cells /7.5 mL blood) were 50.8% and 96.3%; respectively by FCM, and by CS it was 54% and 95%; respectively (Table 3). The positive detection rate of CTCs (defined as nucleated cells CK+/CD45-) was 50.8% (32/63) in NMCRC patients compared to 7.5% (3/40) in patients with BBD by using FCM ($p < .001$). Whereas by CS system, CTCs (≥4 cells /7.5 mL blood) were detected in 34/63(54%) of the NMCRC group compared to 4/40 (10%) of patients with BBD ($p < .001$, Fig. 3). All cases of BBD with CTCs≥4/7.5 mL blood were histopathologically diagnosed as colonic adenomas with atypia. On the other hand, none of the NC samples showed CTCs by both techniques. The concordance between the two techniques was 81.1% ($p < .001$, Table 4).

3.2. Markers expression in CTCs by RT-qPCR

Positive expression was detected in The separated CD45-CK+ cells for *CK19:* 35/63(55.6%), *MUC1:* 29/63(46.0%), *CD44:* 28/63(44.4%), *CD133:* 26/63(41.3%) and *ALDH1:* 25/63(41.3%) of the NMCRC cases. In the BBD patients there were 4/40(10%) cases only showed positive expression of *CK19*, *MUC1* and *CD44,* and 2/40(5%) cases showed positive expression of *CD133* (Fig. 3). None of the NC samples showed positive expression of any of the studied epithelial or stem cell markers. There was a statistically significant concordance in NMCRC between the expressions of the assessed markers by RT-qPCR ($r \geq 0.52$, *p-values* $< .001$, Table 5). A significant correlation was also found between the expression of studied markers at the RNA level and the CTCs detected by FCM (*p-values* $< .001$) and CS (*p-values* $< .001$, Table 4).

3.3. Cytomorphological evaluation of CTCs

In the NMCRC patients, we were able to detect; 1) highly pleomorphic population of CTCs, mostly large cells with high nucleo-cytoplasmic (N/C) ratio, hyperchromasia and large nuclear lobation. 2) Smaller cells with irregular N/C condensation and/or fragmentation (most probably apoptotic cells), 3) cells with eccentric nuclei, slightly irregular contour and a rim of CK+ cytoplasm. Cells having plasmacytoid appearance with Papanicolaou stain were considered adenocarcinoma cells. The median CTCs count was 45(range, 1-78) in NMCRC, 4(range, 0-6) in BBD compared to zero in NC. The positive detection rate of CTCs (≥4 cells) was 30/63(47.6%) in NMCRC compared to 8/40(20%) in BBD and zero in NC ($p = .005$, Fig. 3). The sensitivity and specificity of cytomorphology for detection of CTCs in NMCRC was 47.6% and 90%, respectively (Table 3). The concordance between PAP-staining and FCM was 74.8% and between PAP-staining and CS was 56.2% (*p values* $< .001$, Table 4).

3.4. Combination of different techniques

The combined application of CTCs detection by FCM, CS system and RT-qPCR of *CK19*, *MUC1, CD44, CD133* and *ALDH* improved the sensitivity, specificity and positive prediction value of NMCRC to 68.3%, 95.0% and 91.5%, respectively (Table 3).

3.5. Assessment of K-ras mutation

We also assessed the K-ras status in CTCs. By conventional PCR, K12-ras mutation were detected in 34.9% of CTCs and 31.7% of the tissues obtained from NMCRC patients (*concordance; 0.929,* $p < .001$). Whereas *K13-ras* mutation were detected in 42.9% and 34.9% of NMCRC patients' CTCs and tissues, respectively (*concordance; 0.834,* $p < .001$). By using another technique for the assessment of K-ras mutation, which is the line strip assay (LSA), we found that *K12-ras* mutations were detected in 34.9% and 28.6% of NMCRC patients' CTCs and tissues, respectively (*concordance; 0.24,* $p = .03$), whereas *K*13-*ras* mutations were detected in 33.3% and 30.2% of the NMCRC patients' CTCs and tissues, respectively (*concordance; 0.67,* $p < .001$). Using either methods, 11 out of the 40 BBD (27.5%) cases showed *K-ras* mutation (6 with *K-ras* 12, 5 with *K-ras*13 and 3 with *K-ras*12&13). The positive detection rates of CTCs count by FCM and CS was significantly correlated to *K-ras* mutation ($p < .001$ *and* $p = .01$; respectively, Table 4). A statistically significant correlation was also found between positive RNA expression of the studied markers and the presence of *K-ras* mutation in CTCs of the NMCRC patients ($r \geq 0.31$, *p-values* $\leq .02$, Table 5).

3.6. Correlations with the clinic-pathological features of the patients

Patients with positive CTCs showed a significantly higher T stage, positive LNs and TNM stage III ($p = .034$, $p = .005$ *and* $p = .004$; respectively) compared to those with negative CTCs. No correlation was found between CTCs and the rest of the clinic-pathological factors assessed (Table 6).

The expression levels of *CK19*, *MUC1*, *CD44*, *CD133* and *ALDH1* associated significantly with lymph nodes (LN) metastasis ($p = .001$, $p = .012$, $p = .026$, $p = .007$ & $p = .007$; respectively) and TNM stage III ($p < .001$, $p = .002$, $p = .02$, $p = .004$& $p = .004$; respectively). In addition, positive expression of *CK*, *MUC1*, *CD44* and *CD133* was significantly higher in patients with mucinous adenocarcinoma compared to the conventional adenocarcinoma ($p = .001$, $p = .006$, $p = .02$& $p = .009$; respectively, Table 6).

3.7. Survival analysis

The median follow-up period was 5.3 yrs.(range, 1-5.5 yrs). Out of

Table 6
Clinical-pathological characteristics of the non-metastatic colorectal cancer patients, positive CTCs and expression of *CK19*, *MUC1*, *CD44*, *CD133* and *ALDH 1*.

Parameters	+ve CTCs	+ve *CK19*	+ve *MUC1*	+ve *CD44*	+ve *CD133*	+ve *ALDH1*
	(*n* = 32)	(*n* = 35)	(n = 29)	(n = 28)	(*n* = 26)	(n = 26)
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Age	44.3 ± 13.8	43.3 ± 12.7	42.7 ± 12.4	42.6 ± 13.0	42.9 ± 14.5	42.6 ± 13.1
Age						
≤40 (n = 26)	17 (65.4)	17 (65.4)	14 (53.8)	15 (57.7)	13 (50.0)	14 (53.8)
> 40 (*n* = 37)	15 (40.5)	18 (48.6)	15 (40.5)	13 (35.1)	13 (35.1)	12 (32.4)
χ2	χ2 = 3.7	1.7	1.1	3.1	1.4	2.9
p-value[a]	0.06	0.18	0.30	0.08	0.24	0.09
Gender						
Male (n = 32)	13(40.6)	14 (43.8)	12 (37.5)	11(34.4)	11 (34.4)	10 (31.2)
Female (n = 31)	19(61.3)	21 (67.7)	17 (54.8)	17 (54.8)	15 (48.4)	16 (51.6)
χ2	χ2 = 2.7	3.7	1.9	2.7	1.3	2.7
p-value[a]	0.1	0.06	0.17	0.10	0.26	0.10
FH						
+ve (n = 11)	4(36.4)	4 (36.4)	5 (45.5)	4 (36.4)	4 (36.4)	3 (27.3)
-ve (*n* = 52)	28(53.8)	31 (59.6)	24 (46.2)	24 (46.2)	22 (42.3)	23 (44.2)
χ2	1.1	2.0	0.002	0.35	0.13	1.1
p-value[a]	0.29	0.16	0.97	0.55	0.72	0.30
PS						
I (*n* = 55)	29(52.7)	31 (56.4)	26 (47.3)	26 (47.3)	24 (43.6)	24 (43.6)
II–III (*n* = 8)	3(37.5)	4 (50.0)	3 (37.5)	2 (25.0)	2 (25.0)	2 (25.0)
χ2	0.65	0.12	0.27	1.4	1.0	1.0
p-value[a]	0.42	0.74	0.60	0.24	0.32	0.32
Neoadjuvant						
Yes (*n* = 18)	10(31.3)	10 (55.6)	7 (38.9)	6 (33.3)	6 (33.3)	6 (33.3)
No (*n* = 45)	22(48.9)	25 (55.6)	22 (48.9)	22 (48.9)	20 (44.4)	20 (44.4)
χ2	0.23	0.00	0.52	1.3	0.66	0.66
p-value[a]	0.63	1.00	0.47	0.26	0.42	0.42
Pathology						
A (*n* = 49)	22(44.9)	22 (44.9)	18 (36.7)	18 (36.7)	16 (32.7)	18 (36.7)
M (*n* = 14)	10(71.4)	13 (92.6)	11 (78.6)	10 (71.4)	10 (71.4)	8 (57.1)
χ2	3.1	10.1	7.7	5.4	6.8	1.9
p-value[a]	0.08	0.001**	0.006**	0.02*	0.009**	0.17
LN						
-ve (n = 35)	12 (34.3)	13 (37.1)	11 (31.4)	11 (31.4)	9 (25.7)	9 (25.7)
+ve (n = 28)	20 (71.4)	22 (78.6)	18 (64.3)	17 (60.7)	17 (60.7)	17 (60.7)
χ2	8.3	10.2	6.3	5.0	7.4	7.4
p-value[a]	0.005*	0.001**	0.012*	0.026*	0.007**	0.007**
T-stage						
T1-T2 (*n* = 10)	2(20)	3 (30.0)	1 (10.0)	3 (30.0)	1 (10.0)	2 (20.0)
T3-T4 (*n* = 53)	30(56.6)	32 (60.4)	28 (52.8)	25 (47.2)	25 (47.2)	24 (45.3)
χ2	4.5	3.1	6.2	1.00	4.8	2.2
p-value[a]	0.034*	0.07	0.01*	0.32	0.03*	0.14
TNM Stage						
I-II (*n* = 33)	11(33.3)	11 (33.3)	9 (27.3)	10 (30.3)	8 (24.2)	8 (24.2)
III (*n* = 30)	21(70.0)	24 (80.0)	20 (66.7)	18 (60.0)	18 (60.0)	18 (60.0)
χ2	8.45	13.9	9.8	5.6	8.3	8.3
p-value[a]	0.004*	< 0.001**	0.002**	0.02*	0.004**	0.004**
Grade						
1-2 (*n* = 54)	26(48.1)	28 (51.9)	22 (40.7)	22 (40.7)	20 (37.0)	20 (37.0)
3 (*n* = 9)	6(66.7)	7 (77.8)	7 (77.8)	6 (66.7)	6 (66.7)	6 (66.7)
χ2	1.06	2.1	4.3	2.1	2.8	2.8
p-value[a]	0.30	0.15	0.04	0.15	0.1	0.1
CTH						
Yes (n = 33)	18(60.0)	18 (60.0)	13 (43.3)	11 (36.7)	12 (40.0)	11 (36.7)
No (n = 30)	14(46.7)	17 (51.5)	16 (48.5)	17 (51.5)	14 (42.4)	15 (45.5)
χ2	0.39	0.46	0.17	1.4	0.04	0.5
p-value[a]	0.53	0.49	0.68	0.24	0.85	0.48

CK 19, cytokeratin 19; *MUC1*, mucin1; *CD*, cluster of differentiation; *ALDH1*, aldehyde dehydrogenase; PS, performance status; LN, lymph node; T-stage, tumor stage; TNM, tumor-node-metastasis; CTH, chemotherapy. [a]Patients' characteristics and studied markers expression was compared by chi- square test. *Significance at p < .05.

the 54 NMCRC patients who were followed-up, 25.9% relapsed, 24.1% showed progressive disease and 33.3% died. Kaplan Meier survival analysis demonstrated that NMCRC patients with CTCs counts ≥4 cells had statistically reduced 5 yr PFS (27.5% vs 100%) and OS (17.2% vs 91.7%) than those with CTCs counts < 4 cell (Fig. 4).

In NMCRC patients, reduced PFS associated significantly with advanced disease stage ($p = .03$), positive LN metastasis ($p = .005$), positive expression of all assessed RNA markers ($p < .001$) and *K-ras*

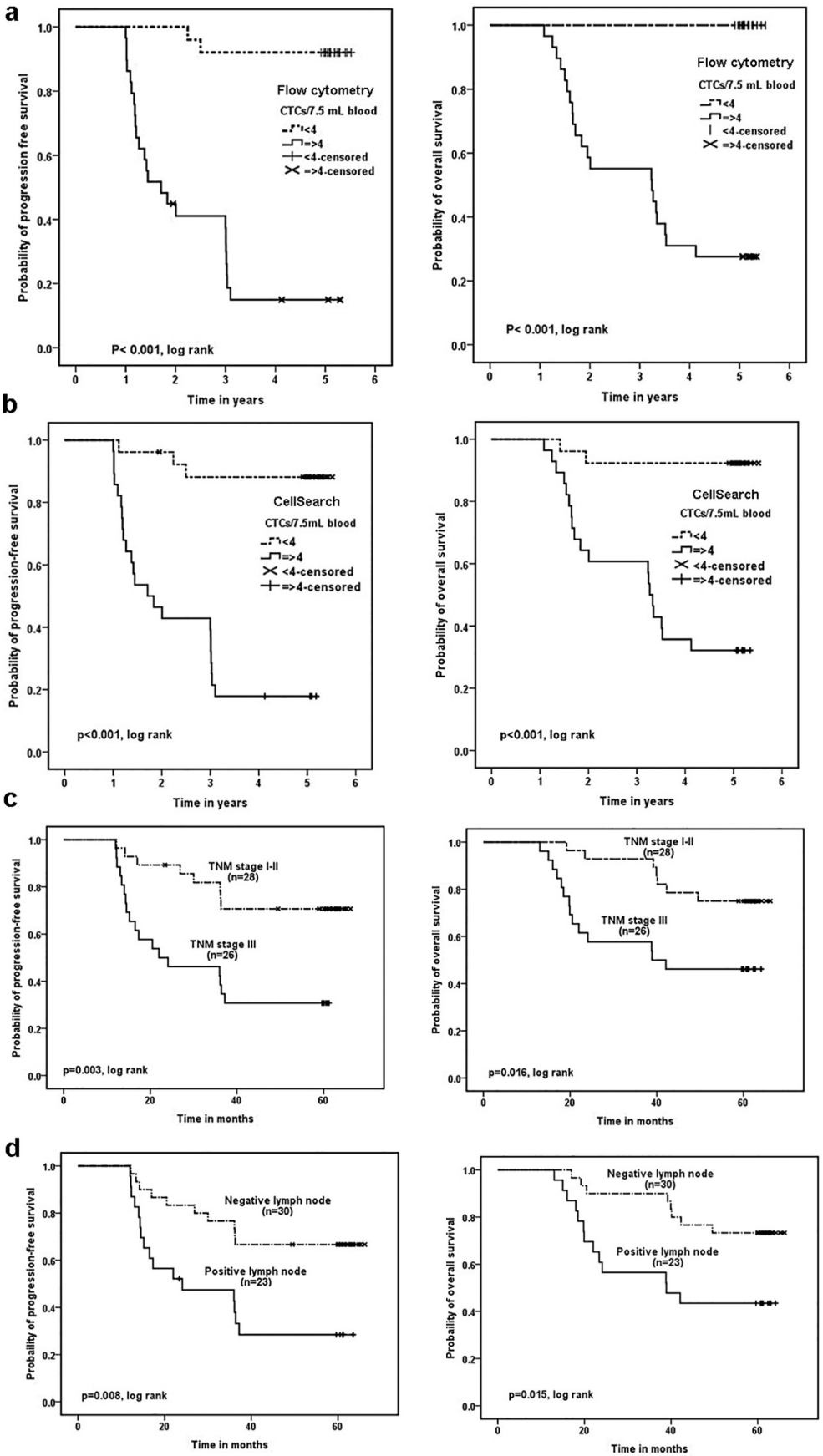

Fig. 4. Correlation between circulating tumor cells detection by a) flowcytometry or b) CellSearch and overall and progression free survival in non-metastatic colorectal cancer patients. c) Probability of survival of non-metastatic colorectal cancer patients in relation to tumor stage. d) Probability of survival of non-metastatic colorectal cancer patients in relation to lymph node status.

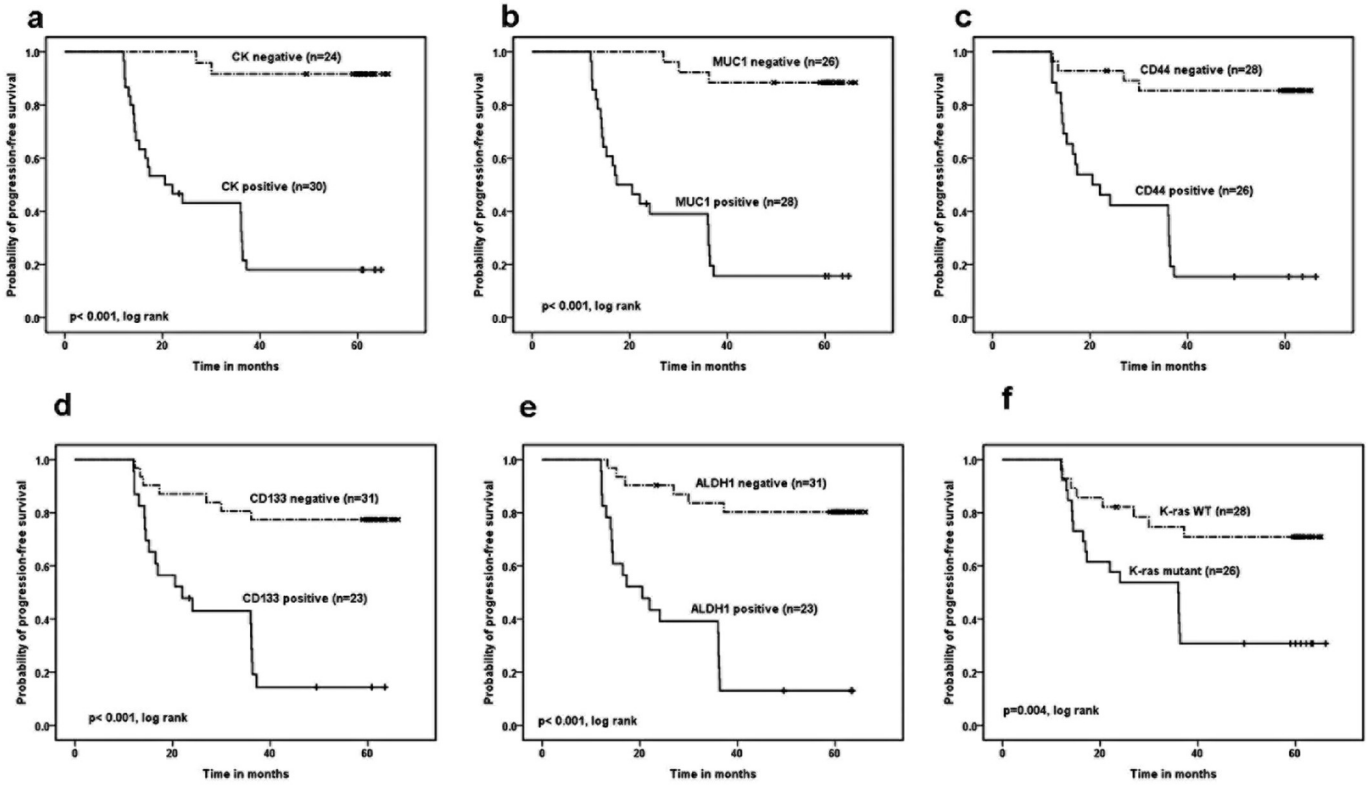

Fig. 5. The probability of progression-free survival in non-metastatic colorectal cancer patients in relation to a) *CK19*, b) *MUC1*, c) *CD44*, d) *CD133*, e) *ALDH1* expression and f) *K-ras* mutation.

mutations by either technique ($p = .003$, Figs. 4 & 5, Table 7). Also, the 5-yr OS of the NMCRC patients was significantly reduced with higher TNM stage ($p = .01$), positive LN metastasis ($p = .02$), mucinous-carcinoma type ($p = .016$), positive RNA expression of the studied epithelial and CSC markers ($p < .001$) and *K-ras* mutation ($p = .02$,Figs.4 & 6, Table 7).

Multivariate Cox regression analysis was performed for the statistically significant factors in the univariate analysis. In NMCRC patients, CTCs detected by FCM ($p < .001$), RT-qPCR expression of the CTCs markers *CK19*, *MUC1*, *CD44*, *CD133* and *ALDH1* ($p = .002$, $p = .001$, $p < .001$, $p = .006$& $p = .001$; respectively) were independent prognostic factors for PFS. However only high *CK19*, *MUC1*, *CD44*, *CD133* and *ALDH1* expressions were an independent prognostic indicator of OS ($p = .004$, $p = .002$, $p = .002$, $p = .002$& $p = .001$; respectively, Table 7).

4. Discussion

The concept of rapid detection of CTCs has always been a point in the modern medicine, however the rarity of these cells in the circulation makes it difficult to isolate (Weng et al., 2018). Compared to other diagnostic methods, CTCs detection is an easy, rapid, frequently non-invasive and relatively at lower cost. All these advantages encouraged its use to unravel mechanisms of metastases, monitor treatment outcome, help clinicians to take accurate decisions and identify new drug targets (Small et al., 2012). Earlier studies have shown that the presence of tumor cells in patient's blood before surgery reflects the invasive potential of primary tumor in the bloodstream and the aggressive behavior, since these CTCs are more likely to develop distant metastasis (Cohen et al., 2008).

The prognostic and predictive values of CTCs have been recently approved for metastatic colorectal cancer (mCRC) (Seeberg et al., 2015). However, limited clinical studies addressed the role of CTCs in the non-metastatic colorectal cancer (NMCRC) (Sotelo et al., 2015; Bork et al., 2015) and none of these studies were done on Egyptian patients. In the majority of recent studies, CTCs were enumerated using the FDA-approved CellSearch system (CS) system (Huang et al., 2016). Although the CS is highly sensitive for the quantification of CTCs in metastatic cancer patients, it is very expensive and depends on the epithelial marker EpCAM to enumerate CTCs that might preclude the identification of CTCs undergoing epithelial to mesenchymal transition (EMT). Till now there is no generally acceptable method for isolation and detection of CTCs in early CRC cases. In addition, conflicting results regarding CTCs detection rates among different techniques used for detection have been reported (Huang et al., 2016).

In the present study, we sought to assess the role of CTCs in NMCRC patients and to compare between four different techniques for detection: 1) CS, 2) flow cytometry (FCM), 3) RT-qPCR and 4) cytomorphology, regarding the sensitivity and specificity for CTCs detection in NMCRC patients compared to patients with BBD and NC subjects. We also assessed the possible prognostic value of CTCs number over 5 years follow-up.

In the current study, the presence of CTCs ≥ 4 cells/7.5 mL blood was significantly higher in the NMCRC compared to those with BBD using FCM (*50.8%* vs *7.5%*, respectively) and CS (*54%* vs *10%*, respectively). The few available studies on CTCs showed low detection rates in the non-metastatic setting using the CS technique (Thorsteinsson et al., 2011). Other studies that addressed the role of CTCs in NMCRC cases, used immunofluorescence in situ hybridization and a sensitive CTC capture platform (Yang et al., 2018; Bork et al., 2015). The difference in the results of the two previously published studies and our study could be attributed, at least in part, to differences in the clinic-pathological features of the patients assessed in each study e.g. the size of the tumor, the histopathological type, or grade, disease stage at presentation…. etc. In our study most of the cases were stage III (*47.6%*) and only 8 cases (*12.7%*) presented with stage I.

Although FCM is highly sensitive for characterization, isolation and enumeration of blood cells, the very small number of CTCs and the

Table 7
Univariate and Multivariate survival analysis for 5 yr overall survival and progression free survival.

Factors	Overall survival			Progression free survival		
	p-value[a]	95% CI	HR	p value[a]	95% CI	HR
Univariate						
Age group ≤40 *vs* > 40	0.16	0.2-1.3	0.54	0.06	0.29-1.03	0.48
Gender M *vs* F	0.4	0.6-3.5	1.5	0.24	0.73-3.5	1.6
FH (No *vs* Yes)	0.6	0.2-2.4	0.7	0.95	0.39-2.7	1.03
PS (III *vs* I-II)	0.6	0.16-3.0	0.7	0.72	0.24-2.7	0.8
Neoadjuvant No *vs* Yes	0.8	0.3-2.4	0.8	0.15	0.16-1.3	0.46
Pathology (A *vs* M)	0.02*	1.2-6.9	2.8	0.13	0.8-4.4	1.9
Grade (I-II *vs* III)	0.07	0.9-7.0	2.6	0.12	0.84-5.2	2.1
T-stage (T1-T2 *vs* T3-T4)	0.3	0.4-24.2	3.2	0.16	0.26-266	26.4
TNM Stage (I-II *vs* III)	0.016*	1.2-7.6	3.1	0.003*	1.5-7.8	3.4
LN (−ve vs + ve)	0.016*	1.2-7.1	3	0.008*	1.3-6.5	2.9
CTCs (< 4 vs ≥4)	0.01*	2.8-83.0	38	< 0.001*	4.8-87.9	20.5
CK19 (−ve vs + ve)	< 0.001*	3.5-198.0	26.5	< 0.001*	4.2-76.9	17.9
MUC1 (−ve *vs* + ve)	< 0.001*	3.5-64.9	14.9	< 0.001*	4.2-48.0	14.2
CD44 (−ve *vs* + ve)	< 0.001*	2.7-31.8	9.3	< 0.001*	3.4-29.0	9.9
CD133 (−ve *vs* + ve)	< 0.001*	2.8-25.4	8.5	< 0.001*	2.5-14.5	6
ALDH1 (−ve *vs* + ve)	< 0.001*	2.8-25.0	8.4	< 0.001*	3.2-20.9	8.2
K-ras (WT *vs* Mutant)	0.02*	1.1-6.9	2.8	0.003*	1.5-8.2	3.5
Multivariate factors						
Pathology (A vs M)	0.2	0.70-4.8	1.8			
T-stage (T1-T2 *vs* T3-T4				0.97	0.91-126	4.3
Stage (I-II vs III)	0.47	0.38-8.4	1.78	0.09	0.8-14.8	3.5
LN (−ve vs + ve)	0.61	0.33-6.9	1.5	0.69	0.2-2.9	1.09
CTCs (< 4 vs ≥4)	0.89	0.0-102.4	41.03	< 0.001*	3.7-82.6	17.6
CK19 (−ve vs + ve)	0.004*	2.7-106	20.6	0.002*	2.5-49.4	11.1
MUC1 (−ve *vs* + ve)	0.002*	2.5-50.5	11.2	0.001*	2.7-38.0	10.1
CD44 (−ve *vs* + ve)	0.002*	2.0-26.5	7.3	< 0.001*	2.7-28.4	8.7
CD133 (−ve *vs* + ve)	0.002*	2.0-19.5	6.3	0.006*	1.5-9.8	3.8
ALDH1 (−ve *vs* + ve)	0.001*	2.1-20.9	6.7	0.001*	2.3-19.7	6.7
K-ras (WT *vs* Mutant)	0.11	0.84-5.7	2.2	0.45	0.58-3.4	1.4

M, male; F, female; FH, family history; PS, performance status; T-stage, tumor stage; TNM, tumor-node-metastasis; A, adenocarcinoma; M, mucinous-carcinoma; LN, lymph node; CTCs, circulating tumor cell; CK19, cytokeratin 19; MUC1, mucin1; CD, cluster of differentiation; ALDH1, aldehyde dehydrogenase; WT, wild type; M, mutant; HR, hazard ratio; CI, confidence interval. [a]Cox regression was used for univariate and multivariate survival analysis of factors. *Significance at $p < .05$.

possibility of inducing changes in the cellular morphology during isolation and acquisition of cells might impact on the final results. In order to compensate for this, enumeration of CTCs was augmented by morphological characterizations of an isolated patch of CTCs, and by assessment of some epithelial markers characteristic for colon cancer cells. There is a growing evidence that CTCs involve a diverse pool of cells including epithelial tumor cells, cells undergoing EMT, and cancer stem cells (CSCs) that may circulate solely or in clusters (Giuliano et al., 2018).

It has been reported that CTCs have CSCs like properties that are non-proliferating and resistant to treatment (Riethdorf and Pantel, 2008). Therefore, we confirmed the FCM data by assessment of the mRNA expressions of colonic epithelial markers (*CK19*, *MUC1)*, and CSC markers (*CD44*, *CD133*, *ALDH1*) in NMCRC and BBD using RT-qPCR. There was a high expression of the studied markers in NMCRC patients compared to the BBD. We found that 55.6%, 46.0%, 44.4%, 41.3% and 41.3% of NMCRC cases had respectively positive *CK19*, *MUC1, CD44, CD133* and *ALDH1* mRNA expression in CTCs. However, only 10% BBD cases showed positive *CK19*, *MUC1* and *CD44* expression and 5% had positive *CD133* expression. These data were in accordance to the previous published studies in literature (Iinuma et al., 2011; Dalerba et al., 2007). Moreover, there was a significant correlation between these markers at RNA level ($r \geq 0.52$ *at* $p < .001$) which supported the presence of epithelial and CSCs features for CTCs. Though RT-qPCR technique does not allow for individual cell analysis, it has the potential to detect primary tumor cells that might not express EpCAM, unlike EpCAM-dependent technologies. RT-qPCR is also considered as a highly sensitive and specific technique for quantitative detection of CTCs based on expression of specific genes (Lianidou and Markou, 2011).

Using Papanicolaou staing, we found that 47.6% of NMCRC patients had positive CTCs detection rate compared to 20% in those with BBD. Though cytomorphology allows morphological characterization of stained cells, molecular assessments are generally more sensitive (Daniele et al., 2009). Then we assessed the cytomorphology of the CTCs in NMCRC compared to the cytomorphology of the neoplastic component of the tumors. We found that the cytomorphology of the isolated CTCs showed similar cytological appearance to the neoplastic component of the primary tumors. This was also reported by Marrinucci et al., in metastatic CRC patients (Marrinucci et al., 2010) who demonstrated that even with cytomorphology only, detection of CTCs in cancer patients might aid in monitoring the risk of metastasis.

We found that RT-qPCR, CS and FCM have comparable sensitivity and specificity for detecting CTCs in NMCRC patients. The combination of FCM or CS, with mRNA expression of related genes increased the sensitivity and specificity for detecting NMCRC cases to *68.3%* and *95%,* respectively. Such high specificity for the diagnosis of NMCRC patients with low positive detection rate in BBD indicates that CTCs represent a promising marker for early detection of NMCRC.

To the best of our knowledge, this is one of the few studies that compared CTCs in NMCRC to BBD (Yang et al., 2018; Tsai et al., 2016). The first study, Yang et al. (Yang et al., 2018) demonstrated significantly higher CTCs count in NMCRC patients compared to those with colorectal polyps and the second (Tsai et al., 2016) showed that the number of CTCs increases along disease severity from polyps to NMCRC to metastatic CRC. Our results also showed significant

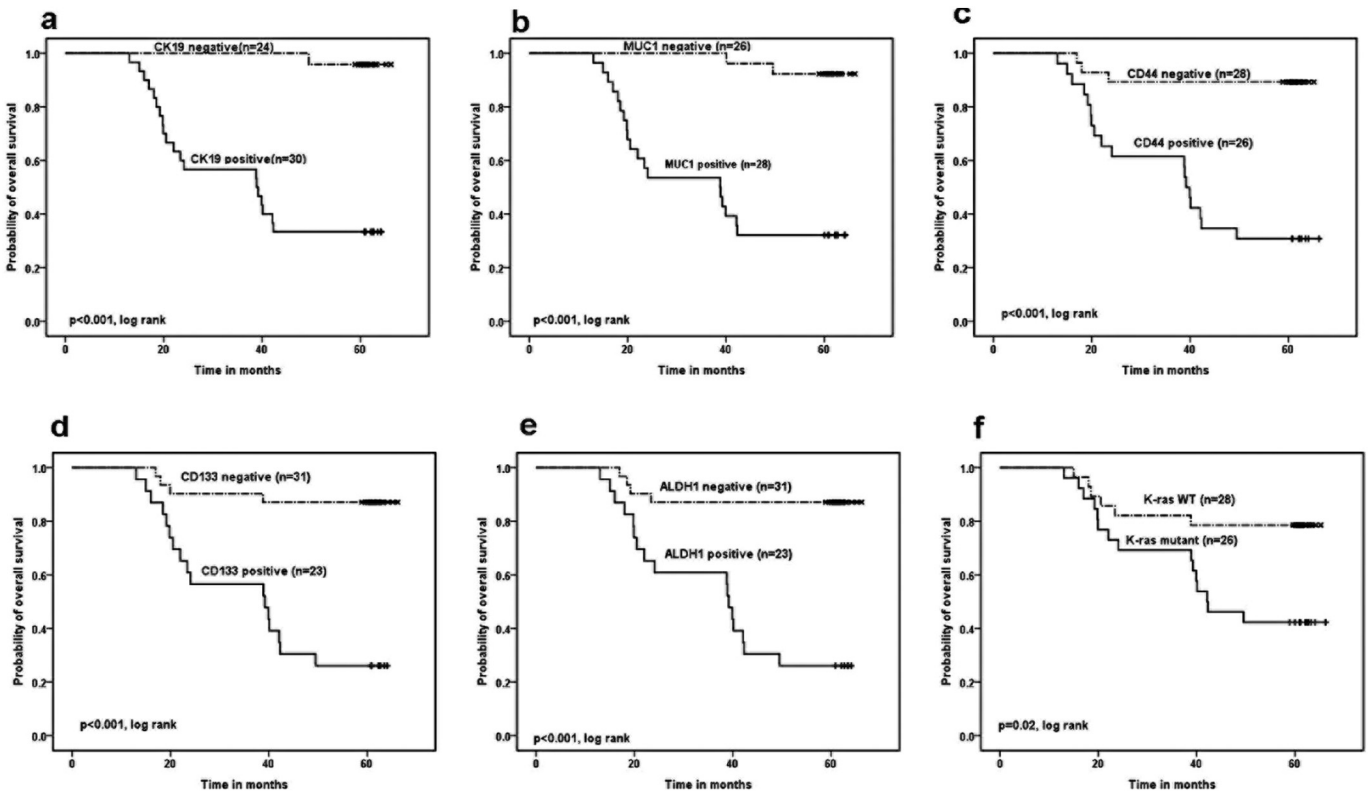

Fig. 6. The probability of overall survival in non-metastatic colorectal cancer patients in relation to a) *CK19*, b) *MUC1*, c) *CD44*, d) *CD133*, e) *ALDH1* expression and f) *K-ras* mutation.

concordance between FCM and CS (*81.1%*), FCM and cytomorphology (*74.8%*), CS and cytomorphology (*56.2%*) as well as between FCM or CS and RT-qPCR (*68.3%*&*53.0%*) for CTCs enumeration in NMCRC patients.

For further support of our data, we also assessed *K-ras* mutations of the CTCs from the different studied groups. *K-ras* mutation is one of the most common and relevant mutations in CRC patients (Kuboki et al., 2013). We found *90%* Concordance between *K-ras* mutations in CTCs (*46.0%*) and tissues *(41.3%*). Accordingly, we concluded that 1) the DNA isolated from CTCs could be used for the detection of *K-ras* mutation and 2) CTCs have genomic aberration(s) that are present in the primary tumor. Significant correlations were also found between the expression levels of tested markers and *K-ras* mutations ($r \leq 0.3$, $p \leq .01$) in CTCs of NMCRC patients.

According to literature, about 40%-60% of the early CRC cases have progressive disease in the first 3 years after surgery with the highest recurrence rate being in the second year (Aghili et al., 2010). One possible explanation for this observation is the failure for early detection of disseminated tumor cells in the blood or lymph circulation by the conventional staging methods. This support the use of such techniques, that permits early detection of secondary tumors.

We also assessed the correlation between the number of CTCs and the clinicopathological characteristics of the patients. We found that a high CTC number and/or high expression of studied markers in NMCRC associated significantly with positive LNs and higher disease stage. Similar data were previously reported by Dalerba et al. (Dalerba et al., 2007), who found significant correlation between a high *CD44* expression, tumorigenicity and metastatic potential of the CRC cells. Similarly, Iinuma et al. (Iinuma et al., 2011) reported significant correlations between CEA, CK and CD133 with poor OS and DFS in CRC patients with Dukes' stage B or C confirming its contribution to aggressiveness of CRC cases.

We correlated the number of CTCs to survival in the NMCRC patients assessed. We found that patients with unfavorable CTCs count (≥ 4 cells) as detected by FCM or CS associated significantly with poor PFS and OS rates compared to those with CTCs < 4. Accordingly, we concluded that in NMCRC patients, CTCs is a potential marker for early detection of patients with high risk to develop distant metastasis. Similarly, Bork et al. (Bork et al., 2015) demonstrated that positive detection of CTCs ≥1 CTCs/7.5 mL in NMCRC using CS correlated significantly with reduced OS. Other studies have also shown that CTC counts before and during treatment are independent predictors of PFS and OS in mCRC patients (Cohen et al., 2008; Kuboki et al., 2013). Cohen et al. (Cohen et al., 2008) reported that a change from ≥3CTCs to < 3 CTCs status during therapy improved survival rates in mCRC patients. Thus, our results demonstrated that early detection of CTCs can reflect tumor metastatic potential and help in monitoring of treatment in patients with early localized as well as advanced CRC. Thus, we recommend that clinical follow up of metastatic and non-metastatic CRC patients should include serial sampling for assessment of CTCs with therapy.

Univariate survival analysis also showed that NMCRC patients with advanced stage, LN metastasis, *K-ras* mutations and whose tumors expressed the molecular markers (*CK19*, *MUC1, CD44, CD133* and *ALDH1)* were liable to have reduced 5-yr PFS and OS. Our findings were in agreement with Lugli et al. (Lugli et al., 2010) who reported that the expression of CD44, CD133 and ALDH1 correlated to the aggressive phenotypes of CRC tumors. It was also reported by Shimada et al. (Shimada et al., 2012) that detection of *CK* and *CD133* mRNA in tumor drainage vein was as an independent prognostic marker for CRC patients with Dukes' stage B and C. In multivariate analysis, we demonstrated that the studied CTCs mRNA markers were independent prognostic markers for PFS and OS in NMCRC patients. Furthermore, we found CTCs count was an independent prognostic factor for OS. These data were in concordance with that of Bork et al. (Bork et al., 2015) who concluded that CTCs detection was a strong independent prognostic marker in NMCRC patients.

In conclusion, our findings indicate that there is more than one

technique that could be used for the detection of CTCs to suite different labs including the CS, FCM, PCR and cytomorphology. Although, the CS is the only FDA approved workstation, other techniques could also be used with comparable sensitivity and specificity. In the current study we were able to prove that the use of FCM for enumeration of CTCs together with RT-qPCR using epithelial markers specific for the tumor type and CSC markers can give results comparable to those of the CS. The selected CTCs mRNA markers can be complementary markers for CRC staging and prediction of progression. They can help to stratify the patients into groups with favorable and unfavorable survival. Moreover, these markers might be used as a novel therapeutic target for the treatment of NMCRC patients. Among the advantages of FCM is the cost-effectiveness, high accuracy, simplicity and the ability of sorting cells for subsequent use.

Future research on larger population of NMCRC patients, should draw more attention to the combining impact of these markers with FCM and CS techniques, as well as considering the use of CTCs for multiple biomarkers profiling. Studying these markers and related transcriptomics with the CTCs count, can aid in evaluating the therapeutic response, and hence choosing the best treatment option. We hope that our findings might bring forth new possibilities for the clinical application of CTCs in NMCRC patients.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

The study was approved by the institutional Review Board of the NCI, Cairo University which was in accordance to 2011 Declaration of Helsinki.

Compliance with ethical standards

All patients and healthy subjects agreed to be enrolled in this study, and informed consent was obtained from all participants.

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