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Comparison of three distinct methods for the detection of circulating tumor cells in colorectal cancer patients

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Abstract. The detection of circulating tumor cells (CTCs) has considerable utility in the clinical management of patients with solid cancers. However, the phenotypic heterogeneity of CTCs and their low numbers in the bloodstream of patients means that no standardized detection method currently exists for these cells. This, together with differences in pre-analytical sample processing, has led to the collection and accumulation of inconsistent data among independent studies. Here, we compare the ability of three methods to detect CTCs in the blood of colorectal cancer patients. Specifically, different aliquots of the same blood sample were screened for the presence of CTCs by a multimarker RT-PCR assay, the standardized CellSearch assay and dHPLC-based gene mutation analysis. In the population tested, none of the blood samples analysed appeared to be positive by all three methods. Of the samples, 75% were positive for the presence of CTCs by the RT-PCR method. Only 20% were positive by the CellSearch assay, while 14.3% of samples displayed gene mutations consistent with the presence of CTCs when the dHPLC method was applied. The samples which were positive for CTCs by the CellSearch assay did not overlap with those that were positive by dHPLC. Interestingly, however, all of these samples were positive when assessed by RT-PCR. Conversely, of the samples that resulted negative by RT-PCR analysis, none appeared to be positive by either of the other methods. These data, therefore, indicate that of the three methods tested, the multimarker RT-PCR assay provides maximal probability of CTC detection. Here, we present the preliminary results of an ongoing clinical study. Future follow-up involving detection of CTCs in the blood of colorectal cancer patients using these three distinct methods will allow us to verify whether either a single method, or a combination of different assays, is necessary to uncover further prognostic significance of circulating tumor cells.

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Introduction

Epidemiological studies indicate that colorectal cancer is the second most prevalent malignant tumor in terms of incidence and mortality in the general population of western countries (1). The incidence in Italy is about 200,000 cases per year (FISMAD Congress 2009, Italian Federation of Digestive System Diseases) and despite curative resection as the major treatment option, about half of all patients develop distant metastases. The detection of circulating tumor cells (CTCs) in the peripheral blood of patients with different neoplastic diseases has been used as an early potential marker of the metastatic spread of malignant cells in the bloodstream (2-6), and is, therefore, considered a sign of poor prognosis (7-11). In fact, the malignant nature of CTCs has been established in previous studies by the identification of tumor-specific chromosomal aberrations (2) and other genetic changes (12,13), as well as by their *in vitro* growth with cancer cell-like behaviour (14). In addition, in several studies the number of CTCs detected seems to correlate with the aggressiveness of different advanced malignancies, such as breast (9,15,16), prostate (17,18) and colorectal cancer (19). Moreover, detectable CTCs at the end of chemotherapy was found to be associated with an increased risk of relapse and death (Rack BK, J Clin Oncol, 2008 ASCO Proc; 20,21). Independent studies have also reported a significant genetic heterogeneity among early disseminated tumor cells in breast cancer patients, demonstrating the existence of a subpopulation of CTCs with putative stem cell phenotypes in patients with metastatic breast cancer which are either associated (22) or not (23) with worse patient outcome, partially

supporting the hypothesis that tumor progression and resistance to chemotherapy is dependent on a group of cells that share stem cell features (24).

Several techniques have been used to detect CTCs, some analysing tissue- or tumor-specific gene expression (25-28) or genome mutation analysis (29), detecting CTCs as intact cells, such as by the CellSearch System. This last method is the only system approved for clinical use by the American Food and Drug Administration (9,30). Interestingly, it was observed that the ability of early changes in CTC numbers to predict the outcome in metastatic breast cancer patients undergoing a new therapy was at least as good as that of traditional radiologic evaluation (31) and also that the CTC assay was more reproducible and more informative than radiographic interpretation. In another study, the prognostic value of CTC detection was also superior to FDG-PET imaging in metastatic breast cancer patients (32).

However, the lack of a standardized method to detect CTCs, that is, differences in preanalytical sample processing and differences in analytical methods, produced inconsistent results among different studies (26,27). The discrepancies were mainly observed in the proportion of CTC-positive patients and in the number of detectable cells.

In order to identify the most effective way to detect CTCs, a few studies compared different enrichment techniques and methods for detection of CTCs in the blood of breast cancer (33,34), prostate cancer (35) and other metastatic carcinoma patients (36,37), sometimes showing concordance in results between methods of analysis or variation in CTC detection rates due to the higher sensitivity of the molecular methodology utilized in the analysis (33,34).

In this study we compared three distinct techniques to detect CTCs in the peripheral blood of patients undergoing surgery for colorectal cancer, in order to investigate whether a single method or a combination of different methods may offer a more sensitive and specific approach to the detection and enumeration of CTCs in patient samples. Specifically, different aliquots of the same blood sample were screened for CTCs by: a highly sensitive multimarker RT-PCR assay, previously described by Gervasoni *et al* (38), the standardized and regulatory-approved CellSearch assay, and a dHPLC-based gene mutation analysis. Secondly, we evaluated the association between the presence of CTCs and clinical-pathological variables.

Materials and methods

Patient characteristics. The study was conducted in 20 patients undergoing elective surgery for colorectal adenocarcinoma between November 2008 and December 2009. Written informed consent was obtained from each patient enrolled in the study according to the guidelines of the Ethics Committee of the Catholic Hospital (CEIOC).

Exclusion criteria were the following: urgent or emergency surgical procedure, synchronous or previous colorectal cancer, co-existence of adenomatous polyposis, ulcerative colitis or Crohn's disease.

The following patient and tumor variables were prospectively recorded: age and gender, tumor site (right colon, left colon and rectum), stage (as defined according to TNM definitions and AJCC groupings), grade of cellular differentiation, mucinous or signet ring cell variants, circumferential margin involvement, presence of lymphatic or vascular invasion and tumor budding. The following treatment variables were prospectively recorded: intent of surgery (palliative or curative, depending on the presence or absence of macroscopic residual disease at the end of surgical treatment) and chemoradiation prior or after surgery (performed or not). The characteristics of the study cohort according to these variables are summarized in Table I.

Table I. Patient, tumor and treatment variables (20 subjects, 19 tumor specimens available).

Variable	Values	No. of patients
Patient age (years)		
Median	70.5	
Range	53-84	
Patient gender		
Male	15	
Female	5	
Tumor site		
Right colon		7
Left colon		6
Rectum		7
Tumor stage		
I		5
II		8
III		6
IV		1
Grade of cellular differentiation		
Grade 1		1
Grade 2		15
Grade 3		4
Mucinous variant		3
Signet ring cell		0
Circumferential margin involvement		0
Lymphatic invasion		8
Vascular invasion		1
Tumor budding		14
Palliative surgery		2
Chemoradiation		13

Sampling of biological material. Before surgery, peripheral blood samples were obtained from each patient, drawn into distinct collection tubes and processed independently with the three distinct CTC detection methodologies as described in the following sections. Five 5-ml EDTA-containing tubes were processed for multimarker molecular expression, one 5-ml serum tube and one 5-ml EDTA-containing tube were analysed for gene mutation detection, and one 7.5-ml CellSave tube (CellSave Preservative Tubes, Veridex

Table II. Marker panel with forward and reverse primers, annealing temperatures, product sizes and references/GeneBank sequence accession numbers.

A. Gene	Primer sequence (5'→3')	Temperature (°C)	Expected size (bp)	Refs./GeneBank accession nos.
CK20	F: GCGACTACAGTGCATATTACAGACAA R: GCAGGACACACCGAGCATTT	58	87	(42)
CEA	F: GCCAAATAATAACGGGACCTA R: CCAGCTGAGAGACCAGGAGAA	60	120	M17303
GCC	F: GAAGATGCGGTGAATGAGGGGCT R: ATGAGGACACAGCCCATCCGTTGTG	62	176	S57551
CK19	F: CCCGCGACTACAGCCACTA R: GCTCATGCGCAGAGCCTGTT	58	164	NM_002276

B. Gene	Primer sequence (5'→3')	Temperature (°C)	Expected size (bp)	Refs./GeneBank accession nos.
APC	Exon15.8F: GTCAAATGAAACCCTCGATTGA Exon15.8R: TTTGCCTTCCAGAGTTCAACT	50	195	M74088
	Exon15.13F: CAAAGCAGTAAAACCGAACAT Exon15.13R: CTTCCTGTGTCGTCTGATTA	56	277	
	Exon 15.15F: CGAAGTTCCAGCAGTGTCAC Exon 15.15R: TGGCAATGCAACGACTCTC	56	245	
p53	Exon5F: TGTGCCCTGACTTTCAACTC Exon5R: ACCAGCCCTGCTGTCTCTCC	60	262	NM_OOO546
	Exon6F: TCCTCACTGATTGCTCTT Exon6R: CACATCTCATGGGGTTAT	55	259	
	Exon7F: TCATCTTGGGCCTGTGTTAT Exon7R: AGGTGGATGGGTAGTAGTAT	58	251	
	Exon8F: TGCTTCTCTTTTCCTATCCT Exon8R: TTGTCCTGCTTGCTTACCTC	56	252	
BRAF	Exon 15F: TCATAATGCTTGCTCTGATAGGA Exon15R: CTTTCTAGTAACTCAGCAGC	58	251	NM_004333
K-ras	Exon2F: GTGTGACATGTTCTAATATAG Exon2R: TACCTCTATTGTTGGATCATATTC	56	173	BC013572
	Exon3F: CCTTCTCAGGATTCCTACAG Exon3R: TTATTTATGGCAAATACACA	54	154	

LLC, Warren, NJ, USA) was processed for CTC count by CellSearch immunomagnetic selection. To prevent contamination with epithelial cells, the first 5 ml of blood from each patient were discarded.

Biopsies of the tumor and normal mucosa (at least 5 cm distant from tumor margin) were collected from each fresh surgical specimen within 20 min of detachment from the vascular supply. In one patient (Stage I, T1N0M0) no fresh tumor tissue was obtained due to the small size of the lesion (<0.7 cm in diameter).

Multimarker RT-PCR assay. The molecular multimarker assay that we have developed and described in detail in a previous study (38), enables the immunomagnetic enrichment of tumor cells from the peripheral blood of cancer patients and the subsequent RT-PCR expression analysis of 4 genes, CEA, CK20, CK19 and GCC, selected for detection of colorectal tumor cells.

Twenty-five millilitres of peripheral blood were obtained from each patient and processed within 2 h, as previously described in detail (38). Briefly, peripheral blood mononuclear cells (PBMCs) were collected by centrifugation through a Ficoll density gradient (Lymphoprep; Axis-Shield, Oslo, Norway). Cells were then subjected to white blood cell depletion. Negative immunomagnetic selection using anti-CD45 specific antibodies (Dynabeads M-450 CD45 Pan Leukocyte, Dynal Biotech ASA, Oslo, Norway) was performed to enrich for tumor cells, according to the manufacturer's instructions. All non-bound cells, the CD45 negative ($CD45^-$) fraction (enriched in tumor cells) were collected and lysed to release RNA in 750 μl QIAzol reagent (Qiagen Inc., Valencia, CA,

USA). Subsequently, RNA was isolated from whole blood specimens and from enriched cell fractions, reverse transcribed and analysed for the expression of CEA, GCC, CK20 and CK19 genes. Primer sequences, predicted product sizes and annealing temperatures are indicated in Table IIA. Amplification conditions were: 10 min at 95˚C for 1 cycle; 30 sec at 95˚C, 40 sec at the annealing temperature for each set of primers (Table IIA) and 30 sec extension at 70˚C for 45 cycles, with a final extension cycle of 7 min at 70˚C. For each sample and gene, 3 independent RT-PCR assays were performed. The spiked tumor cell recovery experiments performed as controls for the assay method and the sensitivity of the method are described in a previous paper (38).

CellSearch System™. Blood samples were kept at room temperature and processed within 96 h after collection. The CellSearch System was used to detect the presence of CTCs. The method has been described in detail elsewhere (30). Briefly, ferrofluid particles conjugated to anti-EpCAM antibodies are used for the isolation of EpCAM positive cells, using a magnetic field. After removing the supernatant, the cells are fluorescently stained using 4',6-diamidino-2-phenylindole (DAPI) for nucleic acid, anti-cytokeratin-phycoerythrin for epithelial cells, and allophycocyanin-conjugated anti-CD45 antibody to detect and exclude leucocytes. Stained cells are then analyzed on a fluorescence microscope that automatically scans the surface of the reaction cartridge, and CTCs are defined as nucleated cells (DAPI positive) lacking CD45 but expressing cytokeratins (CKs). The presence of 1 or more cells per 7.5 ml of blood was considered a positive result.

Mutation detection. Specific genomic mutations were first assessed in patient tumor tissues. The gene mutations identified were then followed in whole blood, serum samples and cDNAs from PBMCs of the same patient. Genomic DNA was extracted from 200 μl of EDTA-anticoagulated blood by use of the QIAamp DNA Blood Mini Kit (Qiagen spa, Milan) according to the manufacturer's instructions (Qiagen) from normal and tumor tissue samples by a desalting NaCl extraction method. Primers for PCR amplifications of specific gene fragments were obtained from MWG Biotech (M.Medical srl, Cornaredo, Mi). The specific primer sequences for amplifying the different gene mutations are reported in Table IIB.

PCR reactions were carried out in a 50-μl volume with 100 ng of template genomic DNA, 200 μM each of the deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1.5 U of AmpliTaq Gold (Applera Italia, Monza) and 25 pmol of each primer.

DHPLC mutation analysis. Heteroduplexes were obtained by denaturing the PCR products at 94˚C for 10 min and cooling at 56˚C for 60 min. Mutation analysis was performed according to a previously described method (39,40) on a Transgenomic WAVE System (Transgenomic, NE, USA) equipped with a preheated C18 reversed-phase column based on non-porous polystyrene/divinylbenzene particles (DNASep™; Transgenomics). For UV detection, 8 μl of the PCR mixture was injected into the column, and the heteroduplexes and homoduplexes were eluted with a linear gradient formed by mixing buffer A (0.1 mol/l triethylamine acetate, pH 7.0) and buffer B (0.1 mol/l triethylamine acetate, pH 7.0, containing 250 ml/l acetonitrile) at a constant flow rate of 0.9 ml/min. DNA was detected by monitoring the absorbance at 260 nm. For each fragment, the initial and final concentrations of buffer B were adjusted to obtain a retention time between 3 and 5 min. The column was then washed with 100% buffer B for 30 sec and equilibrated at starting conditions for 1 min. The melting characteristics of the DNA fragments were predicted by use of the Wavemaker™ software (Transgenomic Ltd., Glasgow). Following UV detection, a DNA-intercalating dye (WAVE Optimized HS Staining Solution I; Transgenomic) was mixed with the eluted sample to increase the sensitivity and measured by fluorescence with excitation at 490 nm and emission at 520 nm, according to the instructions of the manufacturer. A 100-fold higher sensitivity was obtained compared to UV reading. Gene variants with altered elution profiles in dHPLC were sequenced directly in both directions with the appropriate primers.

Sensitivity of mutation detection using DNA from MYH mutant patients. The sensitivity of heteroduplex detection on the WAVE HS system was tested by means of dilution curves. An MYH fragment containing the heterozygous mutation IVS6+35A was diluted with white blood cells, which did not express the MYH mutation as they were obtained from a healthy donor. Genomic DNA was isolated from cell mixtures containing 100, 10, 1, 0.1 or 0% mutant cells.

To confirm that the mutant allele could be identified, these samples were fractionated using partially denaturating high-performance liquid chromatography (dHPLC). Under these conditions, heteroduplexes were eluted first. To enrich for heteroduplexes, the first fractions from the chromatogram were collected and sequenced. dHPLC analysis of the fractions isolated from 100, 10, 1, 0.1 or 0% dilutions of the MYH (IVS6+35A) could detect the mutation (by UV) only in the samples containing 100 and 10% of mutant DNA (not shown). Using fractionation, re-amplification and sequencing we were able to enrich for the mutant-containing heteroduplex PCR product by at least 4-fold.

The DNA fragment MYH (IVS6+35A) was amplified and fractionated on the WAVE HS system using the high-sensitivity fluorescence detector, with WAVE Optimized Staining Solution I. With this method the mutant allele is easily detected in the 100% mutant DNA (double peak) and remains visible in genomic DNA mixtures containing as little as 1-0.1% of mutant DNA.

Statistical analysis. We evaluated the association between each of the three methods (positive for CTCs vs. negative) with all the clinicopathologic characteristics using the Fisher's exact test. We decided not to report any measure of agreement between tests because of the evident variability in the detection rates of positive samples. For that reason we have reported all the individual results and a descriptive analysis of the agreement.

Results

CTC detection by single methods. The data obtained from three independent RT-PCR assays for CTC marker expression are summarized in Table III. Using this first method of molecular analysis, a total of 15 (75%) out of 20 patient samples

Table III. Comparative data of CTC detection using the three methods: CellSearch assay, molecular expression and oncogene mutation detection in blood, serum and cDNA samples.

Patient ID	Mutation detection					cDNA[a]	Cells[b]
	Tumoral tissue	Normal tissue	Blood	Serum	cDNA		
1	APC EX15.30 A>T nt5465 D1822VD1822V	APC EX15.30 A>T nt5465 D1822V	APC EX15.30 A>T nt5465 D1822V	APC EX15.30 A>T nt5465 D1822V	APC EX15.30 A>T nt5465	+	+
2	APC EX15.15 C>G nt 4031 S1344STOP	No mutation	No mutation	No mutation	No mutation	-	-
3	APC EX15.32 del T nt5799 1969STOP	No mutation	No mutation	No mutation	No mutation	+	-
4	APC EX 15.15 C>T nt 4099 Q1367STOP	No mutation	No mutation	No mutation	No mutation	+	-
5	p53 EX7 G>T nt 734 G245V p53 EX8 C>T nt 916 R306STOP	No mutation	No mutation	No mutation	No mutation	+	-
6	No mutation	/	/	/	/	+	-
7	No mutation	/	/	/	/	+	+
8	APC EX 15.9 delAA nt3186-87 E1064STOP	No mutation	No mutation	No mutation	No mutation	+	-
9	APC EX15.30 A>T nt5465 D1822V omo	APC EX15.30 A>T nt5465 D1822V omo	APC EX15.30 A>T nt5465 D1822V omo	APC EX15.30 A>T nt5465 D1822V omo	APC EX15.30 A>T nt5465 D1822V omo	+	+
10	p53 EX 4:5' C>G nt466 P72R	C>G nt466 P72R	C>G nt466 P72R	C>G nt466 P72R	/	-	-
11	p53 EX7 C>A nt705 N235K	No mutation	p53 EX7 C>A nt705 N235K	p53 EX7 C>A nt705 N235K	/	+	-
12	BRAF T>A nt1799 V600E p53 EX7 C>A nt705 N235K	No mutation	p53 EX7 C>A nt705 N235K	p53 EX7 C>A nt705 N235K	/	+	-
13	APC EX15.15 delGT nt4160-61 1394X	No mutation	No mutation	No mutation	/	+	-
14	APC EX15.13 delTT nt3783-84 1275X	No mutation	No mutation	APC EX15.13 delTT nt3783-84 1275X	/	+	-
15	p53 EX 8 C>T nt817 R273C T>G nt4128 Y1376X	No mutation	No mutation	No mutation		-	-
16	No mutation	/	/	/	/	-	-
17	p53 EX 4:5' del C nt518 128X	No mutation	No mutation	No mutation	/	-	-
18	K-ras EX2 G>T nt35 G12V	No mutation	No mutation	No mutation	/	+	-
19	No tissue	/	/	/	/	+	-
20	p53 EX4:5' C>G nt466 P72R omo	C>G nt466 P72R omo	C>G nt466 P72R omo	C>G nt466 P72R omo	/	+	-

[a]CDNA presence (+) or absence (-) as detected by molecular expression analysis; [b]Presence (+) or absence (-) of CTCs as detected by the CellSearch System.

were observed to be positive for CTC marker expression. In particular, when considering the CD45-negative fraction, 9 out of these were positive for CEA, 10 patients were positive for CK19, 4 patients for CK20 and 4 patients for the GCC marker. We also observed that 2 patients were double positive for CEA and CK19 transcript expression, 1 patient for CK19 and CK20, 2 patients were positive for both CEA and CK20, 1 patient for CEA and GCC, 2 patients were triple positive for CEA, CK19 and GCC, and 1 for CEA, CK20 and GCC. Five patients (25%) were CTC negative with this method.

The data obtained by dHPLC screening of hotspot regions of the APC, p53, K-ras and BRAF genes (see Material and methods) in tissue samples, whole blood and serum of patients are shown in Table III. Mutant DNA was identified in 71% of the tumor tissue samples analysed. The mutations observed in patient tumor samples were then identified in whole blood and serum to follow the presence of tumor cells by detection of mutant DNA or mutation-free DNA. A total of 15 patients were positive for mutations in one of the 4 gene fragments analysed from tumor tissues. Of these, 2 patient samples (P11, P12) were found to be positive in both whole PBMCs and serum and one (P14) only in the serum sample. From patient 12 we found two different mutations (BRAF V600E and p53 N235K) in the tumor tissue, but only one in whole blood and serum (p53 N235K). Four patients (P1, P9, P10, P20) showed positive in all samples indicating a germline mutation. Thus, these are not suitable to assess disease recurrence and follow-up. Using the CellSearch System, CTCs were detected in 3 patients (20%): 2 patients (P1, P7) presented 1 CTC/7.5 ml of whole blood, while 1 patient (P9) presented 2 CTCs/7.5 ml of whole blood.

CTC detection by combined methods. We observed variability in the detection rates of positive samples using the three different techniques: 14.3% (3/20) of patient samples with colon cancer were positive for CTCs by the CellSearch System, 20% (3/15) by the gene mutation detection method and 75% (15/20) using multimarker RT-PCR.

The three patient samples that tested positive with the CellSearch System and the three patient samples in which the dHPLC-based assay showed the presence of the same CTC-associated oncogene mutations in blood, serum or cDNA from PBMCs as that detected in tumor tissue, were also positive by the RT-PCR analysis (Table III). No blood sample analysed tested positive by all three methods.

Correlation between CTC detection and clinicopathologic factors. None of the considered clinicopathologic parameters of the study cohort (Table I) showed a significant correlation with one or more methods applied to detect the CTCs.

Discussion

The detection and enumeration of CTCs have considerable potential in the clinical management of patients with solid cancers. However, CTCs are phenotypically heterogeneous cells, that are rare in the blood of cancer patients. Therefore, different techniques have been developed and have been used in combination in efforts to maximize the ability to detect and identify CTCs.

In this study, for the first time to our knowledge, we compared three CTC detection methods, two based on a molecular approach (one based on tumor marker transcript analysis, and one based on the detection of gene mutations) and one based on a cytofluorometric technique, the CellSearch System. We found that the detection rate was higher for the RT-PCR-based method for 4 tumor associated markers (75% of patient samples were positive by this method) compared with the other two approaches, which presented a much lower detection rate (14.3 or 20% of the samples were positive).

Our first goal was to compare the methods of CTC detection and then to possibly identify and maximize the possibility of CTC detection by combination of one or more of the methods.

Few studies have compared the performance of different methods for CTC detection in the same population and even fewer included patients with colon cancer (36,37). It is well known that the PCR assays show a higher sensitivity compared to the cytofluorometric approach. In fact, a recent paper by Van der Auwera *et al* (33) showed that in patients with metastatic breast cancer, the CTC positive rate was higher when a qRT-PCR approach for the detection of CK19 and mammoglobin was used compared to other systems like CellSearch or Adnagen, approved CTC detection method. In our population sample, the highest rate of positive samples was observed using the RT-PCR approach (75% positive samples). The data suggest that this method might be the most sensitive method for detecting CTCs among the three methods under study. Interestingly, all 3 blood samples positive for tumor cells using the CellSearch System and the 3 samples displaying gene mutations were also positive by the RT-PCR analysis assay. None of the 5 CTC-marker negative samples were positive by either CellSearch or dHPLC. On the other hand, we observed that in blood samples of patients with detectable tumor cells by the CellSearch System, no mutations were identified in circulating DNA by the dHPLC analysis, since no pathogenetic sporadic mutations were found in these tumors. The mutations identified in the blood of three patients were not confirmed by cellular detection using CellSearch. The variability of CTC detection among the three methods may be explained by the fact that the tumor cells disseminated in blood might be heterogeneous and therefore, certain types might not be identified by the CellSearch method either because it is based on anti-EpCAM antibody enrichment (41), or because with this system only intact cells are counted. This might certainly be considered a positive characteristic, as damaged cells will likely not be able to enter into the tissues and metastasize. It is also noteworthy, that the low detection rate with dHPLC may be explained by the fact that specific dHPLC assays can be applied to patient blood samples only if a gene mutation is previously identified in the patient tumor tissue. Actually, in 4 (P1, P9, P10 and P20) out of 20 patients, no mutations could be identified in the analysed genes beyond the reported germinal mutations.

Another possible explanation of these discrepancies in the data observed using different CTC detection methodologies is probably due to the different volumes of blood required for the distinct investigation methodologies. Indeed, CTCs are rarely found in peripheral blood of cancer patients, therefore, it is likely that larger volumes of blood have to be processed to increase the probability of tumor cell identification and consequently to improve the sensitivity of the assay.

Our comparative data also revealed that there is a partial overlap in positive results regarding the detection rate of tumor cells obtained using the multimarker RT-PCR assay and either the CellSearch assay or the gene mutation analysis. The data show that the multimarker RT-PCR assay is likely to be the method that provides the highest chance of CTC detection in the peripheral blood of colorectal patients. In particular, we also observed that this molecular method allowed identification of tumor cells in patients with more advanced stages (TNM 3 and 4), with respect to TNM 1 or 2 patients. However, none of the three CTC detection methods showed a significant correlation with any of the clinicopathologic parameters analysed for the study cohort and recorded in Table I.

Herein we have presented the preliminary comparative data of a clinical study that is still ongoing. During further clinical follow-up, the detection of CTCs in the blood of colorectal cancer patients by the three distinct techniques will allow us to verify whether either a single method or a combination of different methods exhibits better prognostic significance for CTC detection.

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