Molecular signature detection of circulating tumor cells using a panel of selected genes

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Abstract

Circulating tumor cell (CTC) detection in peripheral blood of colon and other epithelial cancer patients is becoming a scientifically recognised indicator for the presence of primary tumors and/or metastasis. The resulting need to further develop CTC detection-based systems for improved diagnosis, prognosis and assessment of therapy efficacy in tumour patients has prompted the application of different approaches, including expression analysis of tissue-specific and epithelial genes. In this context, lack of specificity of the analysed genes remains a fundamental problem for reliable CTC detection. In this study, we have selected a panel of highly specific epithelial genes: cytokeratin 20 (CK20), cytokeratin 19 (CK19), carcinoembryonic antigen (CEA) and guanylyl cyclase C (GCC), and performed RT-PCR analysis to assess their expression in total blood and in different cell fractions of peripheral blood (PBMC and CD45-negative population) of cancer patients and healthy controls. Our results demonstrate that analysis of a single gene in a CTC-enriched population (CD45−/CD211− peripheral blood cells) of cancer patients allows detection of a CTC molecular signature in at most 63.3% of cases, while analysis of all four genes performed in all three sample types increases the detection of positive patient samples to 87.7%. Healthy controls did not show positivity for any combination of these genes, although positivity was observed for the CEA marker alone, which was detected in 3 (6.6%) out of 45 donors, and only in the CD45− fraction.

Here, we demonstrate that combined analysis of the genes above, in multiple blood fractions, results in a highly specific and sensitive CTC detection system in patients with metastatic solid tumors. Therefore, we believe that validation on a large scale of this approach, which demonstrates higher specificity in patients compared to controls, could become a relevant CTC screening test in patients with established metastatic disease, and furthermore, may also be useful for evaluating the possible presence of CTCs before the onset of clinically manifested metastatic spreading.

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1. Introduction

Epidemiological studies indicate that, despite modest improvements in colon and breast cancer patient survival rates in the last few decades, the
This fact strongly suggests that the dissemination of metastatic cells from the primary solid tumor to distant sites probably occurs early in the disease process [2–4]. Extensive insights have been gained from minimal residual disease (MRD) detection in the peripheral blood of cancer patients for therapeutic management of haematological malignancies [5]. In the case of solid tumors, the biological significance of circulating tumor cell (CTC) presence in the blood, which is necessary but not sufficient for their metastatic spreading [6,7], is still object of discussion [8]. In the 19th century, T.R. Ashworth first described CTCs in the peripheral blood compartment and S. Paget hypothesized the “seed and soil” theory, which relates to the preferential homing to particular target organs (the “soil”) by selected metastatic tumor cells (the “seed”) which can then proliferate. This hypothesis still holds true today as a model which takes into account the fact that solid tumors are composed of genetically heterogeneous cell subpopulations with different molecular profiles and metastatic potentials [9,10]. Through previous studies, the malignant nature of CTCs has been established by the identification of tumor-specific chromosomal aberrations [11] and other genetic changes [12,13], as well as their in vitro growth with cancer cell-like behaviour [14]. Current evidence shows that malignant cells can settle in bone marrow [8,15–19] and lymph nodes [19–22] and can also circulate in the peripheral blood of patients [11,14,23–26] resembling a “leukemic phase” of solid cancers, as suggested by Mocellin et al. [7]. Following this assumption, CTC detection in the peripheral blood of carcinoma patients could potentially have important clinical [27,28], prognostic [29–37] and therapeutic implications [38–42]. This emphasizes the need to develop feasible methods with sufficient sensitivity and specificity to detect these cells. It is generally accepted that CTCs are present in the bloodstream in very low numbers which are difficult to detect by conventional methods. Indeed, the reliability of using CTCs for predicting disease progression has also been debated from a methodological perspective, because it has been reported that tumor cells are only shed intermittently and often circulate in clumps, characteristics that can make accurate detection problematic [43]. Therefore, a wide range of different techniques have been used to try to detect these cells in peripheral blood of cancer patients, including gene expression [44–47], quantification of cancer-associated serological proteins [48–52], detection of free nucleic acids in peripheral blood [53–55] and more recently, enumeration of single CTCs in blood samples using the CellSearch System [34,56], the Isolation by Size of Epithelial Tumor cells (ISET, Metagenex) [57–59] or a novel in vivo method for quantification of rare circulating tumor cells by multiphoton intravital flow cytometry, which has been validated in mice [60]. The reverse transcriptase-polymerase chain reaction (RT-PCR) has been demonstrated to be able to detect a single specific messenger RNA in a mixed cell population, achieving a detection sensitivity of one tumor cell in $1 \times 10^6–10^7$ normal cells [61]; this method has therefore been used as a sensitive means of identifying CTCs in the peripheral blood of solid tumor patients [46], with a prognostic value reported [62–64]. Although extremely powerful and reliable, this technique has the disadvantages of being unable to enumerate circulating tumor cells, as well as being potentially oversensitive, in that it can detect minimal amounts of mRNA expression resulting from non tissue-specific transcription [65]. The expression of single or multiple mRNAs of organ-specific and epithelial genes has been used as a marker of CTC presence [1,30,66–69] in different types of solid malignancies, but questions remain regarding the clinical value of the detection of these cells and the best strategy to be used for CTC detection. Some of the most frequently accepted genetic markers used to detect CTCs in peripheral blood of cancer patients include the cytokeratins (CKs), which are a group of markers expressed at various levels in epithelial cells [70,71] that under normal circumstances should not circulate unless they become metastatic. In particular, CK18 has been found to be expressed in gastric [72] and prostate tumors [73], but it is used less frequently than CK19, which has been used for CTC detection in blood samples of patients with breast cancer [74] and other types of solid tumors [21,75], including colorectal cancer [27,32]. The CK20 gene is mainly expressed in gastric and intestinal epithelium, urothelium and Merkel cells [76]. Its expression has also been observed in breast cancer cells [77], and colorectal [29,30,78], pancreatic [37] and oral tumors [79]. Another marker often used for CTC detection is the carcinoembryonic antigen (CEA) which is known to have a role in several biological functions, including cell–cell adhesion in embryonic cells and also tumor cells...
It is expressed in breast cancers [82] as well as in colorectal [23], gastric [24], pancreatic and other carcinomas [26,44]. In colorectal tumors, the high expression levels of both mRNA and serological CEA have been correlated with poor survival [31,83]. Guanylyl cyclase C (GCC), the receptor for a bacterial heat-stable enterotoxin, is expressed selectively by cells derived from intestinal mucosa, including normal intestinal cells and colorectal tumor cells [19,22,84], but not by extra-gastrointestinal tissues [85]. The expression of this marker in other solid tumors is yet to be completely proven, even though one study has reported its expression in ovarian, prostate, lung and hepatic tumors [86].

A series of studies have used human telomerase (hTERT) mRNA expression as a molecular tumor marker to detect circulating breast [13], colon [65], cervical [87] and hepatic [88] tumor cells. The Epithelial Cell–cell Adhesion Molecule (EpCAM) is ubiquitously expressed on the surface of epithelial cells and its mRNA has been used for CTC detection in several types of carcinoma, namely: colorectal [65], prostate [89] and breast cancer [90]. Several studies have reported the expression of mucin-1 (MUC-1) in colorectal [27], breast [91], ovarian [92] and prostate [89] cancers, where it has been shown to decrease cell adhesion and favour dissemination [93]. Various other tumor markers have been used to detect CTCs in peripheral blood of patients with several different types of tumor: these markers include the ephrin receptor (EPHB4) and E74-like factor (ELF3) in breast cancers [90], gastrin (GAS) and villin-1 (VIL 1) in colorectal cancers [65], and claudin-7 (CLDN7) [94,95] and matrix metalloproteinase-9 (MMP9) [96] in breast cancers. Several studies have demonstrated that some of these organ-specific markers, which have been used to detect CTCs, are also expressed in the haematopoietic cell lineage [97,98], thus confounding the accurate identification of tumor cells. A solution to this problem is offered by the possibility of combining multi-marker expression analysis with cell selection methods, as has been previously reported in a few studies [27,30,99–101].

Here, we investigate whether the detection of circulating tumor cell-associated markers, performed by carrying out an immunobead multi-marker RT-PCR assay on peripheral blood samples obtained from colon and breast cancer patients, may help in identifying a minimal set of genes, from the panel outlined above, with characteristics of (a) being sensitive enough to allow detection of CTC presence even in the face of down-regulation or heterogeneous expression within solid tumors, and (b) having the highest specificity for CTC detection in patients compared to controls. We also set out to define which blood fraction should be used for CTC detection studies. Here, we propose a methodological strategy for demonstrating that the expression of a combination of four genes (CK20, CK19, CEA, GCC) allows specific detection of CTCs in both un-fractionated and CTC-enriched cell populations of peripheral blood of colon and breast cancer patients.

2. Materials and methods

2.1. Patients and healthy controls

Written informed consent was obtained from each subject. Sample acquisition and subsequent use were performed according to the guidelines of the Ethical Committee of the Catholic Hospital (CEIOC). Thirty million peripheral blood were obtained from each patient and processed within 2 h. Blood samples were drawn into six 5-ml EDTA-containing vacutainer tubes and pooled. To prevent contamination with epithelial cells, the first 5 ml of blood were discarded. Forty stages III–IV solid cancer patients, classified according to TNM (Tumor, Nodes and Metastases) staging system, with clinically manifested metastases, were admitted to the Oncology Department of the Istituto Ospedaliero – Fondazione Poliambulanza, enrolled in the study and analysed at least once. Fifteen patients, 12 affected with colorectal cancer and 3 with breast cancer, were regularly followed up for 6 months during chemotherapy treatment. Fourteen out of these 15 patients were at stage IV, with the remaining patient being at stage III. At each clinical follow-up time point, before chemotherapy treatment, 30 ml of peripheral blood were obtained from each patient for CTC determination. As negative controls for assessing the CTC specificity of the candidate markers, 30 ml of peripheral blood samples were taken from 45 healthy adult individuals and processed. Physical examination, routine blood analysis, serum CEA and CA 19.9 (Carbohydrate Antigen 19.9) measurement, liver function tests and computed tomography (CT) studies of the patients were conducted periodically. The combination of these clinical data allowed classification of the patients as being in Progressive Disease (PD), Stable Disease (SD) or Response (R) according to the Response Evaluation Criteria in Solid Tumors (RECIST). From each blood sample, peripheral blood mononuclear cells (PBMC) were collected by centrifugation through a Ficoll density gradient (Lymphoprep; Axis-Shipol, Oslo, Norway). Cells at the interface were harvested and then washed twice with 1X phosphate-buffered saline (PBS; Sigma, St. Louis Aldrich, MO, USA). Cells were then subjected to white blood cell depletion.
2.2. Cell enrichment

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. The PBMC fraction was added to 35 μl of beads (4 × 10^6/ml) and incubated for 30 min at 4 °C. The bead-cell pellet was then isolated using a magnetic field. The CD45-negative (CD45−) fraction (enriched in tumor cells) was carefully removed by pipetting and centrifuged for 15 min at 1200 rpm to collect all non-bound cells. The pellet was lysed to release RNA in 750 μl Qiazol reagent (Qiagen Inc., Valencia, CA, USA) and stored at −80 °C until RNA extraction.

2.3. Assay validation

Cell mixing experiments were used to test the sensitivity of the techniques applied for the detection of cancer cells in peripheral blood. MCF-7 human breast cancer cells (kindly provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna, Brescia, Italy), expressing CEA and CK19, were cultured in MEM (Cambrex, Verviers, Belgium) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1% Na-pyruvate (Cambrex) and 10% heat-inactivated foetal bovine serum (FBS; Cambrex), in plastic flasks at 37 °C in a 5% CO₂ environment. Known numbers of MCF-7 cells were serially diluted in 1X PBS (Sigma) and each dilution was added to 10 ml of normal whole blood samples from a healthy volunteer to obtain final MCF-7 cell concentrations of 1000 cells/ml; 100 cells/ml; 50 cells/ml; 10 cells/ml; 5 cells/ml and 1 cell/ml. Negative immunomagnetic enrichment, total RNA extraction and RT-PCR were performed as described in following sections. A blood sample from the same healthy subject, without the addition of tumor cells, was used as negative control and processed using the same procedure.

2.4. RNA isolation

RNA was isolated from whole blood specimens using the Versagene Total RNA Purification Kit (Gentra Systems, Minneapolis USA) and from enriched cell fractions using the EZ1 RNA Universal Tissue Kit for the automated Bio Robot EZ1 (Qiagen) according to the manufacturers’ instructions.

2.5. First-strand cDNA synthesis and polymerase chain reaction

One fifth of the total RNA extracted from either 300 μl of total blood, 1 million PBMC or the CD45− cell fraction were reverse transcribed to cDNA using the Improm II Reverse Transcription System (Promega, Madison, USA), according to the manufacturer’s instructions. To check the integrity of the synthesized cDNA, 2 μl of the mixture were subjected to polymerase chain reaction (PCR) for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). Reactions in which either RNA or nuclease free water replaced cDNA were used as RT-PCR negative controls. Primer sequences, predicted product sizes and annealing temperatures (Ta) are indicated in Table 1. Amplification conditions were: 10 min at 95 °C for 1 cycle; 30 s at 95 °C, 40 s at the annealing temperature for each set of primers (Table 1) and 30 s extension at 70 °C for 45 cycles, with a final extension cycle of 7 min at 70 °C. Primers were either selected from previous studies or were newly designed in order to avoid unspecific sequence amplification. PCR experiments were performed in a final volume of 25 μl, containing 2 μl RT mix, 0.3 U GoTaq DNA Polymerase (Promega), 0.2 mM of each dNTP, 1.5 mM MgCl₂, 1X Green Reaction Buffer (Promega) and 10 pmol/μl of each primer, using an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). For each sample and gene, three independent RT-PCR assays were performed. The volume of a single RT reaction was not sufficient to test all of the molecular markers, so at least three different cDNA syntheses were performed from total RNA. PCR products were separated by electrophoresis on a 2.5% agarose gel analysed by direct visualization on a trans-illuminator, and photographed after etidium bromide staining (0.5 μg/ml).

3. Results

3.1. Selection of epithelial- and tissue-specific genes detectable in peripheral blood of patients with metastatic solid tumors

In a primary screening, we analysed the expression of a panel of 14 genes, which are currently accepted as CTC-associated markers, in peripheral blood samples of patients with different types of solid tumors. We performed NCBI BLAST searches to assess the specificity of primers which had been used and reported by others for amplification of the selected genes. As reported in Table 1, for the EpCAM, CEA, GCC and CK19 genes we designed new oligonucleotide sequences to increase the target specificity. The multi-marker RT-PCR assay was first performed on total PBMC and on the CTC-enriched fraction (CD45− fraction). Analysis of samples from 10 healthy subjects showed that all samples were positive for the majority of the genes analysed, both in total PBMC and in CD45− blood fractions, as detailed in Table 2. Out of the 14 markers analysed, only four (CK20, CK19, CEA and GCC) were negative in both fractions in all control samples. In this first phase of our study, we also included 10 patients with different types of solid metastatic tumors: 5 with colon carcinoma, 2 with pancreatic cancer, 1 with breast cancer, 1 with ovarian cancer and 1 with gastric cancer. Our data highlighted the fact...
that the patients were all positive for the 10 markers which had also been positive in the majority of the healthy controls, while the pattern of expression of the four markers, which were negative in the controls, varied among patients, although no patient was ever negative for all these four markers. Specifically, 1 patient was positive for all markers, 1 patient was positive for three markers, 1 patient was positive for two markers and 7 patients were positive for one marker. Because our detection system does not allow quantification of the expression levels of the markers, we focused our attention on the analysis of the four genes that were expressed only in patients and not in the control group. In the second phase of our study, we enrolled 35 new controls. Out of a total of 45 healthy controls analysed, only three (6.6%) of these showed positivity for the CEA gene, which in all cases was detected in only one out of three PCRs performed on the CD45^-/C0^+ CTC-enriched population. None of the control samples demonstrated positivity for CK20, GCC and CK19 (Table 3).

### 3.2. Sensitivity of the CTC detection method

To establish the limit of sensitivity of the immunobead RT-PCR assay using the most frequently expressed CEA and CK19 markers, serial dilutions of MCF-7 human
breast cancer cells in peripheral blood samples were subjected to the RNA extraction and RT-PCR procedure. Representative results of the immunobead RT-PCR assay performed for each sample are shown in Fig. 1. In the PBMC fraction, the highest detection sensitivity observed was 5 MCF-7 cells in 1 ml of whole blood. When the RT-PCR was performed on the CD45-depleted cell population, as expected, a higher sensitivity (1 cell/ml) was observed.

3.3. Expression of CK20, CK19, GCC and CEA genes in metastatic cancer patients at study entry and over time

We studied the expression of the four selected markers in 30 metastatic cancer patients (25 with colorectal carcinoma and 5 with breast cancer), 15 of whom were subsequently monitored over a 6-month-time period with multiple peripheral blood withdrawals. RT-PCR assays were performed for each gene on total peripheral blood, total PBMC and CD45− cell fractions from each patient. The data collected from three independent RT-PCR assays are reported in Table 3. At study entry, we observed positive expression for CEA and CK19 in the PBMC fraction of 8 (26.6%) and 10 (33.3%) out of 30 patients, respectively. In the CD45− fraction, 6 patients (20%) showed positivity for CK20, 19 patients (63.3%) for CEA, 1 patient (3.3%) for the GCC marker and 18 patients (60%) were CK19 positive. We observed that from the combined analysis of all three sample types, the percentage of patients who tested positive for CK20, CEA, GCC and CK19 were 6.7%, 35.5%, 1.1% and 44.4%, respectively. In aggregate, all four genes in all three sample types allowed us to detect a CTC molecular signature in 87.7% of the patients analysed (Table 3). The markers that were most frequently detected in any of the three fractions analysed, and which therefore appeared to allow the best detection of a CTC molecular signature, were CK19 (in 23, or 76.6%, of 30 patient samples) and CEA (in 21, or 70%, of 30 patient samples) (data not shown). The expression of GCC and CK20, whenever present, were always associated with the expression of at least one other marker. Fifteen patients were studied multiple times over a 6-month-period and followed clinically for 12 months. Table 4 shows the molecular data obtained from the RT-PCR analysis of the four genes in the peripheral blood of these patients as well as clinical data, where available, reported according to RECIST. In all patients, we detected marker positivity over the time period during which samples were drawn. At single time points during follow-up, patients 2, 5 and 7 showed positive RT-PCR signals for two or three markers together with clinical signs of disease progression. In particular, in samples from patient 5, who showed disease progression, we detected

Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Controls (n = 45)</th>
<th>Patients (n = 30)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>% of positive sample types</td>
<td>% of total positive samples</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>CD45−</td>
</tr>
<tr>
<td>----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK20</td>
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<td>0</td>
</tr>
<tr>
<td>CEA</td>
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<tr>
<td>GCC</td>
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<td>0</td>
</tr>
<tr>
<td>CK19</td>
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<td>0</td>
</tr>
<tr>
<td>Overall</td>
<td>2.2</td>
<td>87.7</td>
</tr>
</tbody>
</table>

“ND” indicates that no analysis were performed.

Fig. 1. Different numbers of MCF-7 human breast cancer cells were added to 10 ml whole blood samples. The number of tumor cells added was 10,000–1000–500–100–50–10, in lanes 1–6, respectively. RT-PCR experiments were performed in CD45-depleted fractions for CK19 and CEA, and in total PBMC for CK19 (NC, negative control: PBMC from a healthy subject; PC, positive control: MCF-7 breast cancer cell line).
constant positivity for three markers. Interestingly, in patients 1 and 6 we observed stabilization of disease together with a decrease in the number of positive markers. Over the duration of the follow-up period, we noticed that patient 4 displayed an increase in the number of positive markers as well as a clinical worsening. In patients 10 and 11, we saw an increase in the number of positive markers during the first six months of follow up with no clinical worsening during this period, however, these patients underwent subsequent clinical decline. For the remaining 3 patients (3, 8 and 9) out of the 11 studied, we did not observe a clear relationship between marker positivity and clinical outcome.

4. Discussion

In this study, we have investigated whether the RT-PCR-based detection of CTC-associated markers in peripheral blood samples from patients with solid cancers may help to identify a panel of genes indicative of CTC presence. Our goal was to maximize the possibility of CTC detection while minimizing the possibility of obtaining false positive results in healthy controls. In addition, we set out to overcome the limitations associated with the wide variation of gene expression observed in solid tumor patients, by selecting genes which are identified prevalently in metastatic patients, and analysing different blood fractions for the expression of these genes, in order to increase the reliability of the detection system. The data thus far reported in literature often show expression of some tumor-associated markers not only in cancer patients but also in healthy control samples, which confounds the interpretation of the results [74,102–106]. We have therefore selected a set of markers and conditions to try to maximise specificity for detection of a CTC molecular signature in peripheral blood of carcinoma patients. To this end, we started out with a panel of 14 genes, which had been reported by others to be expressed at elevated levels in cancer patients with respect to controls. From the initial marker panel, we then selected four genes that, according to our data, were expressed only in cancer patients. We then performed RT-PCR experiments

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Table 4
Molecular marker analysis and clinical outcome in cancer patients

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<td>Time (months)</td>
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<td>SD</td>
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<td>Dead</td>
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<tr>
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<td>up</td>
<td>(January 2007)</td>
<td>(February 2007)</td>
<td>up</td>
<td>(April 2007)</td>
<td>up</td>
<td>(June 2007)</td>
<td>Clinical outcome</td>
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</table>

The molecular markers CEA, CK19, CK20 and GCC were analysed for metastatic cancer patients by RT-PCR over multiple withdrawals. The patients were clinically followed for a total of 12 months following study admission. Following RECIST, the patients were classified as being in Progressive Disease (PD), Stable Disease (SD), Response (R).
for the four genes selected on total blood, PBMC and CD45\(^{-}\) cells, obtained after PBMC isolation and/or immunomagnetic tumor cell enrichment of each blood sample, with the aim of gaining results that would confirm and complement each other. We decided to perform an immunomagnetic isolation step before the RT-PCR assay, because this method has been demonstrated to increase CTC gene transcript detection [90,99]. Specifically, we decided to employ an immunobead-based negative selection method to deplete the peripheral blood cells from leukocytes, rather than using positive enrichment with immunobeads specific for the epithelial molecule EpCAM, in order to avoid potential loss of non-haematopoietic, EpCAM negative CTCs. In our study, we combined epithelial-specific genes (CK20 and CK19) and CEA, the latter of which is also related to invasiveness and metastasis development [64,107], together with the tissue-specific tumor marker GCC. From our data, the expression of GCC was always associated with the expression of the CK20 marker. Furthermore, we observed that the genes that were most frequently expressed in cancer patients, and therefore indirectly allowed easiest detection of CTCs, were CK19 and CEA (Table 3). This is in keeping with another study that showed that CEA expression alone increased with the progression of metastatic disease [108]. Even though blood samples were not analysed for the expression of CK20 mRNA, due to the fact that this marker is expressed in granulocytes and is therefore detectable in total blood specimens, as reported by Jung and colleagues [109], we have observed that CK20 is useful for CTC detection in the CD45\(^{-}\) fraction.

In both mixing experiments and marker analysis of patient peripheral blood, we observed that the CD45-depleted fraction, which is expected to be enriched in tumor cells, allowed the most sensitive detection of CTC-associated markers. Consistent with this, expression analysis of each of the four selected genes performed in the CD45-depleted fraction of each patient allowed us to reach a percentage of detection of 63.3\% (Table 3), which was the highest observed for any sample type. In addition, when the combination of all four genes was analysed in all three blood fractions, the detection of positive patient samples increased to 87.7\%. In contrast, only 6.6\% of the controls were positive for a single marker in any single sample type (Table 3), and when all the sample types were considered, the percentage of positivity for the controls decreased to 2.2\%. Furthermore, we noticed that the healthy controls were never positive for two markers, even when considering all three blood fractions. For this reason, our results support that the use of a combination of several molecular markers is important for obtaining a specific and reliable positive result. This is in agreement with other studies which have confirmed that the more molecular markers used for CTC detection, the higher the sensitivity of the method becomes [110,111].

We obtained molecular data from multiple blood withdrawals for a series of metastatic cancer patients and verified that CTC detection was consistently determined in almost all cases. Although our data are too preliminary to establish a clear correlation between CTC detection using this method and clinical outcome, we observed a general association between the detection of two or more markers and negative clinical outcome. These observations warrant further studies in a larger and statistically significant patient cohort, and are in keeping with the notion that there might be a correlation between CTC detection and disease progression, as recently discussed by Zieglschmid et al. [45]. If validated, therefore, the CTC detection method proposed here could become an important tool with predictive and prognostic value that may complement traditional measures of treatment efficacy during clinical follow up.

In conclusion, we have proposed a qualitative method that shows high specificity for detection of CTCs in peripheral blood samples of metastatic cancer patients. In particular, by combining the assessment of four selected specific genes, with expression analysis in more than one sample type, we achieve detection of CTCs in 87.7\% of patient samples tested, compared to 2.2\% in samples from healthy controls. Future clinical studies should allow validation of the method described here as a reliable test for CTC detection, and should also allow definition of the utility of CTC detection as a prognostic tool in cancer patients.

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