Detection of circulating tumor cells by nested RT-PCR targeting EGFR/CEA/CK20mRNAs in colorectal carcinoma patients

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Abstract  Background: EGFR is involved in the epidermal growth factors pathway that regulates cellular processes and is associated with the development of many types of cancer including colorectal cancer. Molecular methods with high sensitivity such as nested polymerase chain reaction (PCR) based assays have been used to search for tumor cell specific markers. This study aimed to detect the circulating EGFRmRNA expressing tumor cells and its diagnostic value in colorectal cancer compared with that of known markers of circulating cancer cells CEA and CK20.

Subjects and methods: This study included 36 patients diagnosed as having colon cancer of different stages and 18 matched healthy controls. The staging was carried out according to the TNM classification. We used nested RT-PCR-based reverse transcription PCR assay for the detection of circulating cancer cells in the peripheral blood.

Results: The blood samples from the colon cancer patients showed detection of EGFR in 15/36 patients (41.7%); CEAmRNA in 22/36 patients (61.1%) and CK20mRNA in 24/36 patients (66.7%). No evidence of EGFR mRNA expression in any of the samples used as controls. 3/18 (16.7%) and 4/18 (22.2%) of healthy controls gave a positive result of CEA/CK20mRNAs. There was a statistically significant difference in the prevalence of EGFR/CEA and CK20mRNAs expression between the early disease group (stage I and II) and the advanced disease group (stage III and IV) ($P < 0.01$). Colon cancer patients with a high level of serum CEA exhibited detectable...
concentrations of EGFR and CEA and CK20mRNAs more often than those with a low serum CEA level, there is significant difference ($P < 0.01$).

**Conclusion:** EGFR assay might represent a suitable marker for detection of circulating tumor cells in colon cancer patients. CEA and CK20mRNAs are significantly more frequently detected in colon cancer patients than in healthy controls supports the hypothesis that they are promising complementary markers for CRC diagnosis. The assessment of multiple molecular tumor markers improved the sensitivity in detecting circulating tumor cells but due to limited specificity; identification and validation of genes and proteins implicated in metastatic processes need to be further investigated.

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### 1. Introduction

Colorectal cancer is currently the third most common type of cancer in both men and women and the third leading cause of cancer death. The incidence of colorectal cancer begins to rise at age 40 and peaks between ages 60 and 75 [1]. A recent report published by Egypt’s National Cancer Institute revealed that colon cancer is the third most dangerous type of cancer that causes death in the country. The report said that it found that infection rates range from 10 to 12 percent of the total cancer cases annually reported in Egypt.

Metastatic spreading through blood vessels is the most important factor affecting the prognosis of patients with primary carcinomas [2]. The detection of circulating tumor cells has crucial prognostic and therapeutic implications in all cancer patients [3]. The question is whether circulating tumor cells represent metastatic dissemination or are merely cancer cells without metastatic potential that have detached from the primary tumor.

The most widely used screening technique for colorectal cancer is the fecal occult blood test. However, this simple, inexpensive, and noninvasive test is heavily prone to produce not only false positive results but also false negative results because colorectal tumors bleed intermittently. On the other hand, colonoscopy, which has very high diagnostic accuracy in terms of both sensitivity and specificity, is characterized by a moderate compliance because it is invasive and not without potentially adverse events. Its use is limited to second-level diagnostic tests within screening programs [4]. Numerous serum markers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 19–9, and lipid-associated sialic acid, have been investigated in colorectal cancer, but their low sensitivity has induced the American Society of Clinical Oncology to state that none can be recommended for screening and diagnosis and that their use should be limited to postsurgery surveillance [5,6]. Cytokeratines (CKs); CK18, CK19, and CK20 are generally used for the detection of most epithelial cell type tumors. Recently, the epidermal growth factor receptor (EGFR) is widely used.

Epidermal growth factor receptor (EGFR) is over expressed in 50–70% of human primary breast, lung and colon carcinoma, whereas it is not usually expressed in hematopoietic cells. It is a 170-kDa transmembrane glycoprotein/cell surface receptor composed of an extracellular ligand binding domain, a transmembrane lipophilic segment and an intracellular tyrosine kinase [7,8]. Epidermal growth factor receptor belongs to the ErbB tyrosine kinase receptor family which includes four proteins encoded by the erb B proto-oncogene, namely ErbB1 (EGFR), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). Ligand binding produces dimerization of the receptor and activation of intrinsic protein tyrosine kinase activity leading to the transduction of signaling pathways involved in proliferation, cell division and differentiation. The MAP kinase and AKT signaling pathways have been found to mediate intracellular EGFR signaling. The biologic responses to MAP kinase induction result in increased expression of proteins governing cell-cycle regulation. AKT, an anti-apoptotic kinase, is implicated in cell survival and promotion of angiogenesis and has also been linked to activation of matrix metalloproteinase protein facilitating tumor growth and promotion [9].

Carcinoembryonic antigen (CEA), the gene for CEA is one of the most widely expressed genes in cancer cells. It is a glycoprotein involved in cell adhesion. It is normally produced during fetal development, but the production of CEA stops before birth. Therefore, it is not usually present in the blood of healthy adults. The gene for CEA is one of the most widely expressed genes in cancer cells. It is expressed in 95% of colorectal, gastric, and pancreatic cancers; in the majority of non-small cell lung cancers and other carcinoma types, such as squamous cell cancer of the head and neck; and in 50% of breast cancers [10].

Cytokeratines (CKs) belong to the intermediate filament system and can be divided into five classes according to their biochemically distinct subunits. Cytokeratins are almost exclusively expressed in epithelial tissues and have therefore been used to detect disseminated tumor cells of epithelial origin [11–13].

Cytokeratin 20 (CK20), tumor marker is expressed in pancreatic, gastric and colorectal cancers. Further, CK20 expression has been shown in normal colonic epithelial cells as well as in cells of patients with colorectal and stomach cancers. Recently, a CK20 expression pattern characterized 100% of primary and 88% of metastatic colorectal adenocarcinoma [14–16]. In other reports, the expression of CK20 has been shown to be almost entirely restricted to gastric and intestinal epithelium, urothelium and Merkel cells [17,18].

The current study aimed both to evaluate the feasibility of detection of cancerous cells in the peripheral blood of colon cancer patients based on the nested RT-PCR amplification of EGFR mRNA with the simultaneous identification of CEA/CK20mRNAs and to examine the clinical significance of our findings.
2. Subjects and methods

2.1. Subjects

This study included 54 subjects classified into two main groups:

I. Patients group: Included 36 patients diagnosed as having colon cancer and confirmed by pathological examination of the biopsies. Clinical and histological staging was as follows: stage I, 6 (16.7); stage II, 9 (25%); stage III, 10 (27.8%); stage IV, 11 (30.5%).

II. Control group: Included 18 apparently healthy people of matched age and sex as the malignant group.

All the patients involved in the study were chosen from Internal Medicine Department; Ain Shams Hospital from December 2009 to May 2010. All subjects provided their informed consent to participate in the study. Each patient was subjected to full history taking, complete general and abdominal examination; laboratory evaluation; imaging investigation (abdominal ultrasonography and C.T).

2.2. Methods

2.2.1. Blood samples

Blood samples from the peripheral veins from each patient and controls were collected into two tubes; EDTA tube blood for RNA preparation and another plain tube blood for separation of serum to detect CEA. To reduce the false positive risk from needle cored epithelial cell entering the venesection needle lumen, an intravenous cannula was inserted and 5 ml was aspirated before sample collection.

For all the specimens collected of all groups, the following procedures were carried out:

2.2.1.1. RNA preparation. Aliquots of peripheral blood were processed within 1-h of being obtained from the patients. The blood samples were mixed with 1 ml of 5%dextran-saline solution and left to set for 30 min. at room temperature to yield erythrocyte sediment. Supernatant was collected and centrifuged at 500 g for 10 min. at 4 °C. The cells were then suspended in 1 ml of nucleic acid extraction buffer and frozen at −70 °C until RNA extraction was performed.

2.2.1.2. Extraction of total RNA. Total RNA of nuclear cells was extracted using RNA extraction kit (Qiagen) according to the manufacturer’s instruction. All RNA preparation and handling steps took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was resuspended in RNase-free water and stored at −80 °C until assay. The RNA concentration was assessed by absorbance reading at 260 nm with UV spectrophotometry (Beckman; Du series 650, INC, USA).

2.2.1.3. Amplification by nested reverse transcriptase polymerase chain reaction (RT-PCR). Three different PCR reactions were performed with each sample in order to amplify fragments of EGFR/CEA and CK20mRNAs.

Reverse Transcription (Complementary cDNA synthesis): Reverse transcription was carried out in 20 μL reaction mixture using 2 μg of RNA by using first strand cDNA synthesis kit (Promega; USA) according to manufacturer’s instruction.

Nested PCR for EGFR/CEA/CK20mRNAs: A total amount of 50 μL reaction solution contained 5 μL 10× PCR buffer, 0.2 mmol/L of each dNTP, 30 pmol of each primer, 2.5U Taq DNA polymerase (Promega; USA) and cDNA. Thermocycling in either an MJ Research PTC 200 (MJ Research, Inc., Boston, Mass.) or Perkin–Elmer 9600 (Perkin–Elmer, Cambridge, United Kingdom) was done according to the following cycle profile. For sequences of oligonucleotide primers used for PCR reaction see (Table 1).

Nested PCR for CEA mRNA: Thirty cycles consisting of 5 cycles of 30 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C and 25 cycles of 30 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C; the samples were heated for 10 min. at 94 °C before the first cycle, and the extension was lengthened to 10 min. during the last cycle.

After completion of the first PCR reaction, 1 μl of the first PCR amplification product was added to the second PCR solution. The PCR conditions for the nested PCR reaction were similar to those for the first round PCR with the following exception that the PCR number of cycles was 35 [2].

Nested PCR for CEA and CK20mRNAs were performed as described by Lagoudianakis et al. 2009 [1].

To verify the successful preparation of mRNA, samples were detected for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Analysis of each sample was repeated at least two times.

2.2.1.4. Gel electrophoresis. The final amplification product was electrophoresed on 2% agarose gel and stained with ethidium bromide for the specific band of bp.

2.2.1.5. Assay for CEA. For the analysis of CEA; we used commercially available kit (Roche, Mannheim, Germany).

Table 1: Sequences of oligonucleotide primers used for PCR reaction.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence of primers (5’–3’)</th>
<th>(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR primer 2</td>
<td>Outer-sense</td>
<td>TCTCAGCAACATGTCGATGG</td>
</tr>
<tr>
<td></td>
<td>Outer-antisense</td>
<td>TCCGACCTTCTACATTGCG</td>
</tr>
<tr>
<td></td>
<td>Inner-sense</td>
<td>TCTCAGCAACATGTCGATGG</td>
</tr>
<tr>
<td></td>
<td>Inner-antisense</td>
<td>TCACATTCATCTGTACGTTG</td>
</tr>
<tr>
<td>CEA primer 1</td>
<td>Outer-sense</td>
<td>CCAATGGGTCTTCCTCG</td>
</tr>
<tr>
<td></td>
<td>Outer-antisense</td>
<td>GTAGTTGTGTTGTCATTTC</td>
</tr>
<tr>
<td></td>
<td>Inner-sense</td>
<td>AGGTGGCTTCTACATTGTCACCAA</td>
</tr>
<tr>
<td></td>
<td>Inner-antisense</td>
<td>GCCAGTGTCTTCTCTCATTCA</td>
</tr>
<tr>
<td>CK20 primer 1</td>
<td>Outer-sense</td>
<td>CAGACACACGAGTGGACTATG</td>
</tr>
<tr>
<td></td>
<td>Outer-antisense</td>
<td>GTACAGCTTCTCAGTTAGAGACG</td>
</tr>
<tr>
<td></td>
<td>Inner-sense</td>
<td>GTGGGCAATGAGAAATGTC</td>
</tr>
<tr>
<td></td>
<td>Inner-antisense</td>
<td>GCATCTTATCTGACTTCGACA</td>
</tr>
<tr>
<td>GAPDH primer</td>
<td>Sense</td>
<td>CTACTGCGGC CTG CCA AGG CGT T</td>
</tr>
<tr>
<td></td>
<td>Anti sense</td>
<td>GCCATG AGG TTC ACC ACCTG T</td>
</tr>
</tbody>
</table>
The assay was performed according to manufacturer’s recommendation. Protein levels were given in ng/ml.

2.3. Statistical analysis

The results were analyzed using the Statistical Package of Social Sciences (SPSS) computer software program, version 16.0 (Chicago, IL, USA). Data are presented as numbers and percentages. Association between categorical groups was evaluated using Chi-square or Fisher’s exact test when appropriate. A $P$ value less than 0.05 was considered statistically significant.

3. Result

3.1. Interpretation of result

According to the design of primer pairs, the PCR products of (322); (260) (203) base pairs were amplified from EGFR/CEA and CK20 cDNA respectively; while GAPDH band amplification was visualized corresponding to 390 bp. The sizes of PCR products were estimated by comparison with DNA molecular mass markers (Bio-Rad; EZ Load) (Figs. 1–3).

3.2. Patient characteristics

A total of 36 patients were enrolled in the study (20 male; 16 female). Demographic data are represented in (Table 2). Neither age nor sex was correlated with the presence of EGFR/CEA and CK20mRNAs expression ($P > 0.05$).

3.2.1. Expression of EGFR/CEA/CK20mRNAs in peripheral blood from healthy volunteers

A total of 18 RNA samples extracted from healthy volunteers were subjected to RT-PCR. EGFR was not detected in any sample. However, CEA and CK20mRNAs were detected in 3/18 (16.7%) and 4/18 (22.2%) respectively.

3.2.2. Expression of EGFR/CEA/CK20mRNAs in peripheral blood from colon cancer patients

The blood samples from the 36 colon cancer patients showed detection of EGFR in 15 patients (41.7%); CEA mRNA in 22 patients (61.1%) and CK20 mRNA in 24 patients (66.7%) and analysis of these results showed that EGFR/CEA/CK20mRNAs expression showed a statistically significant difference between healthy controls and cancer patients (Table 3, Fig. 4).

The frequency of positive cases in TNM stage III and IV was significantly higher than that in stage I and II ($P < 0.05$). Colon cancer patients with a high level of serum CEA exhibited detectable concentrations of EGFR and CEA and CK20mRNAs more often than those with a low serum CEA level, thus there is significant difference. These results indicate that the incidence of the expression of cancer specific molecular markers EGFR/CEA and CK20mRNAs increased in advanced stage of disease and correlated with clinical severity (Table 4).
The detection of EGFRmRNA in peripheral blood is correlated with that of CK20mRNA and no correlation was found between EGFRmRNA and CEAmRNA (Table 5). The result indicates that 25/36 (69.4%) were positive for at least one marker and 13/36 (36.1%) positive for the three molecular markers which strongly indicate hematogenous spread.

### Table 2 Patients characteristics.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Normal</th>
<th>Colon cancer patients</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 52.8 ± 10.1</td>
<td>18</td>
<td>36</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sex Male 11 (61.1%)</td>
<td>20 (55.6%)</td>
<td>16 (44.4%)</td>
<td></td>
</tr>
<tr>
<td>Stage of disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I (Duke A)</td>
<td>6 (16.7%)</td>
<td>12 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>Stage II (Duke B)</td>
<td>9 (25%)</td>
<td>10 (27.8%)</td>
<td></td>
</tr>
<tr>
<td>Stage III (Duke C)</td>
<td>10 (27.8%)</td>
<td>11 (30.6%)</td>
<td></td>
</tr>
</tbody>
</table>

4. Discussion

Colon cancer is one of the most common malignancies in the world. With progress in diagnostic and therapeutic techniques, its prognosis has been improving.

The spread or dissemination of cancer cells from the primary tumor is the most important factor affecting prognosis in carcinoma patients. Once distant metastasis has been formed, cancer disease is generally no longer curable and medical intervention is restricted to palliative treatment. Hematogenous spread of solid cancers represents a major clinical challenge in oncology and has a fundamental influence on the outcome of the disease. Metastasis of solid malignancies like breast and colorectal cancers has been referred to as a cascade; in the beginning, cancer cells carrying multiple genetic abnormalities grow unregulated and lose their ability to adhere to each other. This, together with their ability to stimulate angiogenesis, provides a means for entry to the blood and lymphatic circulation. In the case of the blood circulation, these cells can circulate in the body until adhering to the vascular endothelium when they can leave the circulation (extravasation) [19].

### Table 3 Expression of EGFR, CEA and CK20mRNAs in peripheral blood of colon cancer patients and healthy controls.

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>Colon cancer patients (n = 36)</th>
<th>Healthy controls (n = 18)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR 15 (41.7%)</td>
<td>0 (0%)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>CEA 22 (61.1%)</td>
<td>3 (16.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK20 24 (66.7%)</td>
<td>4 (22.2%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference between cancer patients and the healthy controls.

### Table 4 Expression of EGFR, CEA and CK20mRNAs in relation to gender, stage and serum CEA.

<table>
<thead>
<tr>
<th>Gender</th>
<th>n</th>
<th>EGFR</th>
<th>P*</th>
<th>CEA</th>
<th>P*</th>
<th>CK20</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>20</td>
<td>8 (40%)</td>
<td>0.05</td>
<td>12 (60%)</td>
<td>2.33</td>
<td>12 (60%)</td>
<td>0.91</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>7 (43.8%)</td>
<td>0.82</td>
<td>10 (62.5%)</td>
<td>0.17</td>
<td>12 (75%)</td>
<td>0.27</td>
</tr>
<tr>
<td>Stage of disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>6</td>
<td>0 (0%)</td>
<td>1 (16.7%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>9</td>
<td>1 (11.1%)</td>
<td>12.32</td>
<td>5 (55.6%)</td>
<td>9.21</td>
<td>4 (44.4%)</td>
<td>14.95</td>
</tr>
<tr>
<td>Stage III</td>
<td>10</td>
<td>5 (50%)</td>
<td>0.001**</td>
<td>6 (60%)</td>
<td>0.01**</td>
<td>9 (90%)</td>
<td>0.001**</td>
</tr>
<tr>
<td>Stage IV</td>
<td>11</td>
<td>9 (81.8%)</td>
<td>10 (90.9%)</td>
<td>11 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum CEA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5</td>
<td>14</td>
<td>1 (7.1%)</td>
<td>11.23</td>
<td>5 (35.7%)</td>
<td>6.21</td>
<td>4 (28.6%)</td>
<td>14.96</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>22</td>
<td>14 (63.6%)</td>
<td>0.003</td>
<td>17 (77.2%)</td>
<td>0.01</td>
<td>20 (90.9%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Chi square test or Fisher’s exact test for small sample size.
** Significant difference between stage (I and II) and (III and IV).

### Table 5 Comparison of expression of marker genes in blood samples of colon cancer patients.

<table>
<thead>
<tr>
<th>n</th>
<th>CK20</th>
<th>EGFR</th>
<th>CEA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>21</td>
<td>12</td>
<td>9</td>
<td>12.9</td>
</tr>
<tr>
<td>Positive</td>
<td>15</td>
<td>0</td>
<td>15</td>
<td>0.001</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>12</td>
<td>24</td>
<td>14</td>
</tr>
</tbody>
</table>

* Chi square test or Fisher’s exact test for small sample size.
In recent years, protocols based on RT-PCR allowed the detection of cancer cells in the peripheral blood, bone marrow, and lymph nodes of patients with several cancer types such as breast, colon cancer, melanoma, neuroblastoma, prostate cancer, and lymphoma.

Data from the current study are as follows: The blood samples from the 36 colon cancer patients showed detection of EGFR mRNA in 15 patients (41.7%); CEA mRNA in 22 patients (61.1%); CK20 mRNA in 24 patients (66.7%) and our data result is in agreement with many previous results [1,2]. All blood samples were scored positive for mRNA expression only after performing an internal PCR.

4.1. EGFR

In our result, EGFR mRNA showed significantly strong statistical difference between healthy controls and cancer patients. EGFR was not detected in any sample of healthy volunteer. It can be concluded that in the majority of the papers reviewed, EGFR expression had been reported in 25–82% of CRCs [20–23]. RT-PCR for EGFR can detect circulating micrometastasis in a proportion of patients with advanced-stage cancers of epithelial origin, including lung, colon, and pancreas.

It is well documented that EGFR expression may be associated with an advanced disease stage [24–26]. Furthermore, our data showed EGFR correlated to TNM stage. However, these results remain controversial because an association between EGFR expression and Dukes stage or length of survival in CRC has not been detected in other studies [27].

Finally, novel therapeutic approaches based on EGFR blocking antibodies, EGFR tyrosine kinase inhibitors, or antisense oligonucleotides directed against either EGFR or its ligands have been developed. EGFR assay could be used to monitor the response of the tumor to these novel agents by monitoring the level of EGFR positive cells shed by the carcinoma during therapy.

4.2. CEA

We found that; there was a significant high positive ratio of CEA in the peripheral blood of CRC patients compared with normal. CEA is in fact widely accepted as a useful tumor marker for surveillance of gastrointestinal patients, especially with colorectal neoplasm and several studies have used CEA mRNA as a target marker to detect circulating tumor cells and high expression levels have been correlated with poor survival. Using nested RT-PCR for CEA, a large study was published that reported detection of CEA mRNA in whole blood in 69% (n = 51) of colorectal carcinoma patients, whereas only 3% (n = 60) of apparently healthy donors were positive for CEA mRNA [9].

Expression of CEA mRNA has been described recently in 76–80% of peripheral blood samples from patients with metastatic carcinomas using nested PCR. This is in contrast to other reports of detection of CEA mRNA in a significant number of blood samples of healthy donors or in patients with inflammatory bowel disease [28–31]. Abnormally high levels of CEA protein in 30% of smokers should be taken into account [24].

4.3. CK20

We found that CK20 mRNA were significantly more frequently detected in the colon cancer patients than in healthy controls and could serve as markers. CK20 appears to be a sensitive marker for detection of disseminated tumor cells in blood samples of intestinal cancer patients. Several studies showed that CK20 is a reliable marker and not detectable in the blood of non cancer patients. In contrast; RT-PCR amplification of CK20 mRNA, which is considered as a promising candidate in the method for the detection of circulating epithelial cells, seems to lack specificity because its expression is not limited to epithelial cells and an increasing number of false-positives were observed in patients without cancer. Both an illegitimate transcription and the presence of pseudogene have been described to explain the possibility of false-positive results. The reported expression of CK20 mRNA in the epidermal Merkel cells and granulocytes outlines the importance of blood sampling technique and perfect elimination of contaminating granulocytes [27].

The relatively high positive ratio of CEA and CK20 mRNAs in the peripheral blood of CRC patients compared with normal controls in our study indicates the existence of malignant tumor cells in their peripheral blood, and further supports the hypothesis that CEA and
CK20mRNAs are promising complementary markers for CRC staging and prediction of cancer progression and metastasis but false positive for CEA/CK20mRNAs seen in groups of healthy donors limit its specificity. In particular CK20 which show lower specificity.

There is reported evidence to a significant association of disseminated cancer cell detection to an advanced stage [28,29] which is in agreement with our result. On the other hand, numerous studies reported that no correlation can be detected between CEA and CK20mRNAs expression and the clinicopathological characteristics of the disease [30].

The mRNA originated from circulating cancer cells is commonly detected in peripheral blood and RT-PCR shows higher sensitivity than routine immunocytochemistry but the significance of such detection is still to be clarified. The advantage of the RNA identification is that it implies that the cell is viable since only viable cells produce mRNA and extracellular RNA is rapidly degraded [1]. However, limitations may arise from deficient expression in circulating tumor cells or low level illegitimate expression in hematopoietic cells, particularly if a nested PCR approach is used.

Possible reasons contributing to a failure to detect circulating tumor cells include intertumoral variation in mRNA expression of the tumor-associated protein used for RT-PCR detection, resulting in varying detection thresholds between tumors from different patients. In addition, experimental metastasis studies suggest that circulating tumor cells are aggregated in clumps of varying size, and this may result in a sample-to-sample variation in the number of tumor cells contained within different blood samples from the same patient. Thus, while patient positivity for circulating cancer cells is based on identification of one mRNA marker within a single blood sample, the detection sensitivity might be limited by both the amount of mRNA marker in circulating tumor cells and the number of tumor cells in the blood sample [31]. Also, since cancer cells are usually very heterogeneous, different cancers express different markers and even cells from the same tumor may not be identical. Thus, single-marker RT-PCR has its limitations in sensitivity and specificity. It has therefore been proposed that the assessment of multiple tumor markers in one blood sample would enhance the sensitivity of tumor cell detection. But due to limited specificity as reported by Gradilone et al. [32], further investigation is needed.

Recently Lagoudianakis et al. 2009; reported CEA/CK20mRNAs were not detected in any sample of healthy controls using OncoQuick Density Gradient Centrifugation. By using tumor cell enrichment technique; specific cancer marker detection could be improved [1,33,34].

The reason for discrepancy among various studies may be multifactorial. The limited number of tested patients and in the method of isolating circulating carcinoma cells may be contributory factors; amount of cDNA; increasing amount of DNA used increased sensitivity of the assay as reported by [2] and the specificity of a RT-PCR assay is largely influenced by several factors, among which the most important are: (a) carryover contamination, (b) illegitimate transcription, and (c) marker expression by non tumor cells in the sample. The best-defined problem in RT-PCR assays is carry-over contamination. The exponential amplification method efficiently amplifies a few DNA copies one-million-fold to amounts of DNA that are easily detectable on agarose gels. In addition to illegitimate transcription, samples may be false-positive for a particular marker because a small subset of the non tumor cells might express the transcript and the antigen; false positivity [35].

Although only 0.01% of circulating tumor cells survives the passive transport in the blood stream and actually forms metastasis at distant site, the quantification of cancer cells released into the blood stream is a helpful piece of information to allow precise diagnosis of the metastatic potential of the primary tumor in each individual case.

A standardization of blood sampling; multiple blood sample, RNA Preparation, cDNA preparation, gene-specific primer pairs, optimal RT-PCR conditions and the determination of possible threshold caused by cells of non-tumor origin is mandatory to obtain reliable and comparable results.

5. Conclusion

EGFR assay might represent a suitable marker for detection of circulating tumor cells in colon cancer patients. That CEA and CK20mRNAs are significantly more frequently detected in colon cancer patients than in healthy controls supports the hypothesis that CEA and CK20mRNAs are promising complementary markers for CRC staging and prediction of cancer progression and metastasis. RT-PCR assays with multiple tumor markers were shown to be superior in comparison to the assessment of single markers but due to their limited specificity; further data; investigation and clarification of the prognostic significance of genes and proteins implicated in metastatic process in colon cancer needs to be further investigated.

Conflict of interest

The authors declare that there is no conflict of interest.

References


