The Egyptian Journal of Medical Human Genetics (2010) **11**, 173–180

Ain Shams University

The Egyptian Journal of Medical Human Genetics

www.ejmhg.eg.net
www.sciencedirect.com

ORIGINAL ARTICLE

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

Salwa H. Teama *, Sara H.A. Agwa

Medical Research Center, Molecular Biology Unit, Ain Shams University, Egypt

Received 15 July 2010; accepted 11 October 2010

KEYWORDS

Colorectal carcinoma;
EGFR;
CEA;
CK20;
mRNA

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

* Corresponding author.
E-mail address: salwateama2004@yahoo.com (S.H. Teama).

Peer review under responsibility of Ain Shams University.
doi:10.1016/j.ejmhg.2009.10.001

Production and hosting by Elsevier

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.

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 c-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 canula 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 reaction 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 EGFR 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].

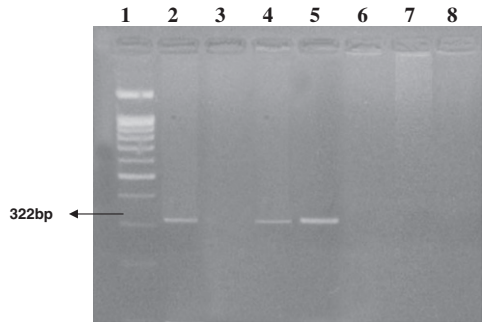
To verify the successful preparation of mRNA, samples were detected for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Analysis of each sample was repeated at least two times.

2.2.1.4. Gel electrophoresis. The final amplification product was electrophoresed on 2% agrose 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.

Primers	Sequence of primers (5′–3′)	(bp)
EGFR primer 2		
Outer-sense	TCTCAGCAACATGTCGATGG	
Outer-antisense	TCGCACTTCTTACACTTGCG	473
Inner-sense	TCTCAGCAACATGTCGATGG	
Inner-antisense	TCACATCCATCTGGTACGTG	322
CEA primer 1		
Outer-sense	CCATGGAGTCTCCCTCG	
Outer-antisense	GTAGCTTGCTGTGTCATTTC	641
Inner-sense	AGGTGCTTCTACTTGTCCACAA	
Inner-antisense	GCCAGTTGCTTCTTCATTCA	260
CK20 primer 1		
Outer-sense	CAGACACACGGTGAACTATGG	
Outer-antisense	GATCAGCTTCCACTGTTAGAGACG	371
Inner-sense	GTTGGCAATGAGAAAATGGC	
Inner-antisense	GCATCCTTAATCTGACTTCGCA	203
GAPDH primer		
Sense	CTACTGGCG CTG CCA AGG CTG T	
Anti sense	GCCATG AGG TCC ACC ACCCTG T	390



Lane 1: DNA molecular size marker
Lane 2: Positive control
Lane 3: Negative control
Lane 4,5: Positive cases of EGFR gene transcript
Lane 6, 7: Negative cases

Figure 1 Shows the result of PCR product. Electrophoretic separation of PCR amplified product; Detection of gene transcripts in blood specimens from Colon cancer patients.

The assay was performed according to manufacturer's recommendation. Protein levels were given an ng/ml.

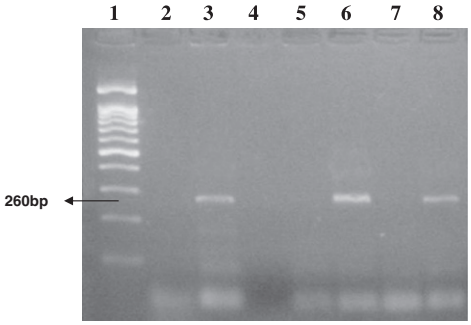
2.3. Statistical analysis

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

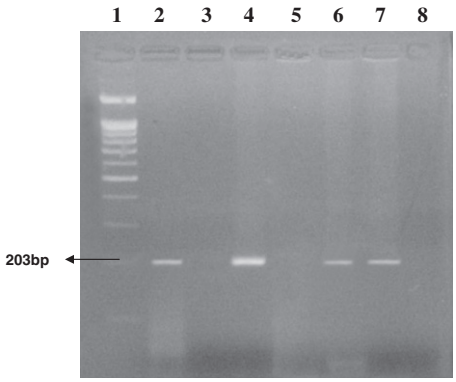
3.1. Interpretation of result

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).



Lane 1: DNA molecular size marker
Lane 2,4,5,7: Negative Case
Lane 3,6,8: Cases showing CEA positive gene transcript

Figure 2 Shows the result of PCR product. Electrophoretic separation of PCR amplified product; Detection of gene transcripts in blood specimens from Colon cancer patients.



Lane 1: DNA molecular size marker
Lane 2: Positive control
Lane 3: Negative control
Lane 4,6,7: Positive cases of CK20 positive gene
Lane 5,8: Negative cases

Figure 3 Shows the result of PCR product. Electrophoretic separation of PCR amplified product; Detection of gene transcripts in blood specimens from Colon cancer patients.

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%).

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%); CEAmRNA in 22 patients (61.1%) and CK20mRNA 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).

Table 2 Patients characteristics.

	Normal	Colon cancer patients	*P*-value
No. of patients	18	36	
Age	52.8 ± 10.1	55.8 ± 9.9	>0.05
Sex			
Male	11 (61.1%)	20 (55.6%)	
Female	7 (38.9%)	16 (44.4%)	
Stage of disease			
Stage I (Duke A)		6 (16.7%)	
Stage II (Duke B)		9 (25%)	
Stage III (Duke C)		10 (27.8%)	
Stage IV (Duke D)		11 (30.5%)	

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.

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.

mRNA expression	Colon cancer patients (n = 36)	Healthy controls (n = 18)	*P*-value
EGFR	15 (41.7%)	0 (0%)	<0.05*
CEA	22 (61.1%)	3 (16.7%)	
CK20	24 (66.7%)	4 (22.2%)	

* Significant difference between cancer patients and the healthy controls.

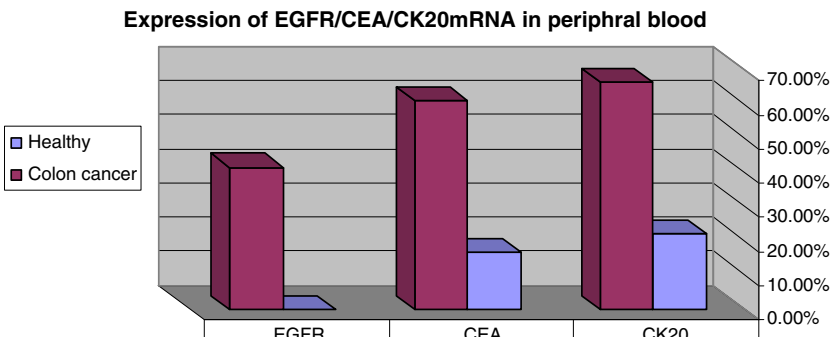
Table 4 Expression of EGFR/CEA and CK20mRNAs in relation to gender, stage and serum CEA.

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

* 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.

	n	CK20		P^*	CEA		P^*
		Negative	Positive		Negative	Positive	
EGFR							
Negative	21	12	9	12.9	10	11	1.61
Positive	15	0	15	0.001	4	11	0.17
Total	36	12	24		14	22	



	EGFR	CEA	CK20
Healthy	0%	16.70%	22.20%
Colon cancer	41.70%	61.10%	66.70%

Figure 4 Expression of EGFR/CEA/CK20mRNAs in peripheral blood.

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%); CEAmRNA in 22 patients (61.1%); CK20mRNA 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 CEAmRNA 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 CEAmRNA in whole blood in 69% (n = 51) of colorectal carcinoma patients, whereas only 3% (n = 60) of apparently healthy donors were positive for CEAmRNA [9].

Expression of CEAmRNA 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 CEAmRNA 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 CK20mRNA 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 CK20mRNA, 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 CK20mRNA in the epidermal Merkel cells and granulocytes outlines the importance of blood sampling technique and perfect elimination of contaminating granuloytes [27].

The relatively high positive ratio of CEA and CK20mRNAs 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) carry-over 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

[1] Lagoudianakis EE, Kataki A, et al. Detection of epithelial cells by RT-PCR targeting CEA, CK20and TEM-8 in colorectal carcinoma patients using oncoquick density gradient centrifugation system. J Surg Res 2009;155:183–90.
[2] Antonella De Luca, Sandro Pignata, Amelia Casamassimi, et al. Detection of circulating tumor cells in carcinoma patients by a novel epidermal growth factor receptor reverse transcription-PCR assay. Clin Cancer Res 2000;6:1439–44.
[3] Ghossein RA, Bhattacharya S, Rosai J. Molecular detection of micrometastases and circulating tumor cells in solid tumors. Clin Cancer Res 1999;5:1950–60.
[4] Funaki NO, Tanaka J, Itami A, et al. Detection of colorectal carcinoma cells in circulating peripheral blood by reverse transcription-polymerase chain reaction targeting cytokeratin 20mRNA. Life Sci 1997;60:643.
[5] Bromer MQ, Weinsberg DS. Screening for colorectal cancer: now and the near future. Semin Oncol 2005;32:3–10.
[6] Bast RC, Ravdin P, Hayes DF, et al. Update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. J Clin Oncol 2001;19:1865–78.
[7] Zhang H, Berezov A, Wang Q, Zhang G, Drebin J, Murali R, Greene MI. ErbB receptors: from oncogenes to targeted cancer therapies. J Clin Invest 2007;117(8):2051–8.
[8] Herbst RS. Review of epidermal growth factor receptor biology. Int J Radiat Oncol Biol Phys 2004;59(2 Suppl):21–6.
[9] Arkom Cheirsilpa, Preecha Ruangvejvorachai, Anant Karalak, Soisuda Sangprakarn, Sangduan Pummai, Suleeporn Sangrajrang.

Determination of epidermal growth factor receptor (EGFR) in patients with colorectal cancer (Institutional series). Cancer Ther 2007;5:137–42.

[10] Fiorella Guadagni, Judith Kantor, Simona Aloe, et al. Detection of blood borne Cells in colorectal cancer patients by nested reverse transcription polymerase chain reaction for carcinoembryonic antigen messenger RNA: longitudinal analyses and demonstration of its potential importance as an adjunct to multiple serum markers. Cancer Res 2001;6:2523–32.

[11] Gaforio JJ, Serrano MJ, Sanchez-Rovira P, Sirvent A, Delgado-Rodriguez M, Campos M, de la Torre N, et al. Detection of disseminated tumor cells in peripheral blood positively correlated with estrogen-receptor status and predicts for poor prognosis. Int J Cancer 2003;107:984–90.

[12] Pierga JY, Bonneton C, Vincent-Salomon A, de Cremoux P, Nos C, Blin N, Pouillart P, et al. Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients. Clin Cancer Res 2004;10:1392–400.

[13] Taubert H, Blumke K, Bilkenroth U, Meye A, Kutz A, Bartel F, Lautenschlager C, et al. Detection of disseminated tumor cells in peripheral blood of patients with breast cancer: correlation to nodal status and occurrence of metastases. Gynecol Oncol 2004;92:256–61.

[14] Ukyanchuk VV, Friess H, Kleeff J, Osinsky SP, Ayuni E, Candinas D, Roggo A. Detection of circulating tumor cells by cytokeratin 20 and prostate stem cell antigen RT-PCR in blood of patients with gastrointestinal cancers. Anticancer Res 2003;23:2711–6.

[15] Ascoli V, Taccogna S, Scalzo CC, Nardi F. Utility of cytokeratin 20 in identifying the origin of metastatic carcinomas in effusions. Diagn Cytopathol 1995;12:303–8.

[16] Moll R, Lowe A, Laufer J, Franke WW. Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. Am J Pathol 1992;140:427–47.

[17] Kummar S, Fogarasi M, Canova A, Mota A, Ciesielski T. Cytokeratin 7 and 20 staining for the diagnosis of lung and colorectal adenocarcinoma. Br J Cancer 2002;86:1884–7.

[18] Moll R, Zimbelmann R, Goldschmidt MD, Keith M, Laufer J, Kasper M, Koch PJ, et al. The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. Differentiation 1993;53:75–93.

[19] Zieglschmid V, Hollmann C, Böcher O. Detection of disseminated tumor cells in peripheral blood. Crit Rev Clin Lab Sci 2005;42(02):155–96.

[20] Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127–37.

[21] Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004;351:337–45.

[22] Spano JP, Fagard R, Soria JC, Rixe O, Khayat D, Milano G. Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives. Ann Oncol 2005;16:189–94.

[23] Prewett MC, Hooper AT, Bassi R, Ellis LM, Waksal HW, Hicklin DJ. Enhanced antitumour activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumour xenografts. Clin Cancer Res 2002;8:994–1003.

[24] Gross ME, Zorbas MA, Danels YJ, Garcia R, Gallick GE, Olive M, Brattain MG, et al. Cellular growth response to epidermal growth factor in colon carcinoma cells with an amplified epidermal growth factor receptor derived from a familial adenomatous polyposis patient. Cancer Res 1991;51:1452–9.

[25] Radinsky R. Modulation of tumor cell gene expression and phenotype by the organ-specific metastatic environment. Cancer Metastasis Rev 1995;14:323–38.

[26] Radinsky R, Risin S, Fan D, Dong Z, Bielenberg D, Bucana CD, Fidler IJ. Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. Clin Cancer Res 1995;1:19–31.

[27] McKay JA, Murray LJ, Curran S, Ross VG, Clark C, Murray GI, Cassidy J, et al. Evaluation of the epidermal growth factor receptor (EGFR) in colorectal tumours and lymph node metastases. Eur J Cancer 2002;38:2258–64.

[28] Silva JM, Rodriguez R, Garcia JM, et al. Detection of epithelial tumor RNA in plasma of colon cancer patients associated with advanced stages, circulating tumor cells. Gut 2002;50:530.

[29] Sadahiro S, Suzuki T, Tokunaga N, et al. Detection of tumor cells in the portal, peripheral blood of patients with colorectal carcinoma using competitive reverse transcriptase polymerase chain reaction. Cancer 2001;92:1251.

[30] Schuster R, Max N, Mann B, et al. Quantitative real time RT-PCR for detection of disseminated tumor cells in peripheral blood of patients with colorectal cancer using mRNA markers. Int J Cancer 2004;108:219.

[31] Richard Q. Wharton, Sonja K. Jonas, Clare Glover, et al. Increased detection of circulating tumor cells in the blood of colorectal carcinoma patients using two reversetranscription-PCR assays and multiple blood samples. Clin Cancer Res 1999;5:4158–63.

[32] Gradilone A, Gazzaniga P, Silvestri I, Gandini O, Trasatti L, Lauro S, Frati S, et al. Detection of CK19, CK20 and EGFR mRNAs in peripheral blood of carcinoma patients: correlation with clinical stage of disease. Oncol Rep 2003;10(1):217–22.

[33] Michael Raynor, Sally-Anne Stephenson, David CA Walsh, Kenneth B Pittmanand, Alexander Dobrovic. Optimisation of the RT-PCR detection of immunomagnetically enriched carcinoma. BMC cancer. J Hematol Oncol 2009;2:24.

[34] Vlems FA, Diepstra JH, Cornelissen IM, et al. Investigations for a multi-marker RT-PCR to improve sensitivity of disseminated tumor cell detection. Anticancer Res 2003;23(1A):179–86.

[35] Hampton R, Walker M, Marshall J, Juhl H. Differential expression of carcinoembryonic antigen CEA) splice variants in whole blood of colon cancer patients and healthy volunteers: implication for the detection of circulating colon cancer cells. Oncogene 2002;21:7817–23.