# Detection of circulating carcinoma cells in peripheral blood collected from patients with ovarian cancer by using different molecular markers – a preliminary report

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## Abstract

**Introduction:** Efficient techniques enabling the ovarian cancer patients' diagnosis, prognosis and therapy remain far from perfection. Especially, the prognostic significance of circulating tumour cells' (CTC) presence remains to some extent unclear. Latest findings on this matter suggest a need to revise the modern persuasion about the meaning of single tumour cell detection in peripheral blood (PB) or bone marrow (BM) by using standard cancer diagnostic procedures, as standard histopathological, immunohisto- and immunocytochemical procedures. However, that negative state induced the investigation of mRNA transcripts present in tumour diseases, which could be easily detected by using real-time RT-PCR method. The RNA markers used in this study were: cytokeratin 19 (*CK19*), telomerase catalytic subunit (*hTERT*) and two markers thought to be tissue-specific for ovaries: steroidogenic acute regulatory protein (*StAR*) and creatine kinase B (*CKB*).
**Materials and methods:** The blood samples from 20 women were collected in two parts (blood from each 10-person group was used either to perform real-time RT-PCR, or TA PCR-ELISA [enzyme-linked immunosorbent assay]).
**Results:** Both real-time RT-PCR and TA PCR-ELISA methods showed some degree of specificity in this study, but not enough to be put in practice.
**Conclusions:** Both results of our study and the small number of articles on molecular markers suitable for detecting CTCs in PB of patients with OC show that this aim will be hard to achieve by using standard molecular biology tools, like PCR or even real-time PCR. However, these attempts should not be terminated, because if any of them succeeds, the diagnostic value of this technique will be great.

**Key words:** metastasis, telomerase (*hTERT*), steroidogenic acute regulatory protein (*StAR*), creatine kinase B (*CKB*), cytokeratin 19 (*CK-19*).

## Introduction

Despite the great improvement in primary tumour treatment, it has not been possible to significantly suppress the metastasis, which remains one of the main causes of cancer-related deaths. Our knowledge about molecular mechanisms responsible for dissemination of the tumour cells and metastasis is relatively limited. Moreover, efficient techniques enabling the patients' diagnosis, prognosis and therapy remain far from perfection. Especially, the prognostic significance of circulating tumour cells' (CTC) presence remains to

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**Table I.** Real-time RT-PCR primers and reaction conditions used for expression analysis of specified genes

| Gene primers (For/Rev) (5' → 3') | annealing temp. [°C] | detection temp. [°C] | PCR product size [bp] |
|---|---|---|---|
| *StAR*<br>ACCCCAATGTCAAGGAGATCAAGGTCCTG<br>TCGGCAGCCAGCTCGTGAGTAATG | 66 | 72 | 76 |
| *TERT*<br>AAGCTGACTCGACACCGTGTCACCTAC<br>ACTCAGCTGCGTCTGGGCTGTC | 60 | 72 | 72 |
| *KRT19*<br>AGATGAGCAGGTCCGAGGTTACTGAC<br>AGTGTGTCTTCCAAGGCAGCTTTCA | 63 | 72 | 103 |
| *CKB*<br>GGGCAACATGAAGGAGGTGTTC<br>ACTTGAAGAGAGTTTCAATCTGGGTG | 60 | 72 | 68 |

*Abbreviations: For – forward primer; Rev – reverse primer*

some extent unclear. Malignant tumours are capable of metastasizing because of the release of their cells into the vascular and lymphatic systems. Additionally, such circulating cancer cells should be capable to colonize distant and distinct tissues. The metastasis itself is considered as a cascade of events [1], all of which must be completed in order to form a secondary tumour. First, disseminated malignant cells have to survive in the blood or lymph until they are halted in a different organ [2]. Second, the cells have to survive in the secondary sites and proliferate to form a metastatic growth. Especially, these latter mechanisms are extremely inefficient. As reported in [3], approx. 2% and 0.02% of different types of tumour cells, purposely injected into mice or chicken embryos, survived and proliferated in the secondary place, respectively. These findings may suggest a need to revise the modern persuasion about the meaning of single tumour cell detection in peripheral blood (PB) or bone marrow (BM) by using standard cancer diagnostic procedures. The oldest and the most frequently used technique for identification of tumour cells is standard histopathological procedure. This involves all kinds of staining (mainly haematoxylin and eosin; H&E) in combination with cytologic assessment. However, this kind of evaluation is very arbitrary, thus it can be performed only by an experienced technician. Another disadvantage of this technique is its limitation to lymph node (LN), BM and blood analyses only. It has been proven by Ridell and Landys [4] that by using conventional histopathological methods at a time of primary diagnosis without clinical signs of metastasis to the bones, the probability of finding isolated breast cancer cells in BM is ca. 4% [4]. The development of immunohisto- and immunocytochemistry enabled a significant increase in the detection sensitivity of nodal micrometastases, but results of these assays are often affected with false-positive staining derived from expression of cytokeratins (CK), epithelial antigens or mucins (MUC) in haematopoietic cells [5]. Apart from false-positive results, immunocytochemical techniques lack the methodological standardization amongst different laboratories which is another great disadvantage and one of the major reasons why this technique has not been approved by both the International Society of Cell Therapy and the National Cancer Institute as a reliable tool for detection of disseminated tumour cells. The molecular approach to diagnosis is now routinely used only in haematological malignancies [6]. Accordingly, there were numerous attempts to evaluate distinct DNA and RNA markers for detection of CTCs in solid tumours. Among DNA markers applied in PCR-based techniques in diagnostics of various malignancies are: mutations in proto-oncogenes (for instance *k-ras*) [7, 8, 9] or tumour suppressor genes (as *p53*) [7, 10, 11]. Apart from these, approaches directed towards identification of changes in microsatellite DNA in circulating cells were also applied [12, 13]. It is thought, however, that the presence of such a kind of marker does not equal the idea that viable CTCs are found. This belief is supported by DNA relative resistance to decomposition in comparison with RNA molecules. Due to the fact that no unique DNA marker has been discovered, fast and easy development of PCR-based techniques in determination of the number of CTCs in PB as a diagnostic procedure, has been hampered. However, that negative state induced the investigation of mRNA transcripts present in tumour diseases, which could be easily detected by using RT-PCR method. This approach enables detecting of only living CTCs, but on the other hand, it does not allow quantifying CTCs in a credible way. Few techniques can be applied in order to obtain higher efficiency in isolating CTCs from the blood and to receive more specific results, mainly Histopaque®/Ficoll density gradient centrifugation and/or immunomagnetic enrichment. The first method allows for isolating of the nuclear fraction of blood cells, among which CTCs are present due to their density, similar to lymphocytes. The latter – provides a relatively easy way of separation of CTCs from normal blood cells. There are two kinds of immunomagnetic enrichment

– positive or negative, depending on the presence/absence of the unique epitopes on the CTCs. In positive selection magnetic beads are coated with monoclonal antibodies (mAbs) against surface antigens present on CTCs (CTCs are isolated with the beads); in negative selection – beads are coated with mAbs against normal blood cells surface antigens (CTCs are left in the supernatant after magnetic immobilisation of beads).

The RNA markers used in this study were: cytokeratin 19 (*CK19*), generally used for all kinds of epithelial tumours, telomerase catalytic subunit (*hTERT*) – a general tumour-associated marker and two markers thought to be tissue-specific for ovaries: steroidogenic acute regulatory protein (*StAR*) and creatine kinase B (*CKB*).

## CK19

Cytokeratins belong to the intermediate filaments, which together with the microfilaments and microtubules compose the cytoskeleton of almost all eukaryotic cells [14, 15]. In the human cells there are twenty CKs described so far [numbered from 1 to 20; 1-8 are neutral/basic = type I, whereas 9-20 CKs display more acidic character = type II [14]. This type of molecules can be used as molecular markers because different epithelial cells show contrast in combination of the CKs expressed (there is always a CK type I and a CK type II expressed), for instance most common pair found in breast cancer cells is CK8/CK19 or CK8/CK18 [16]. Although most researchers assess the expression levels of CK7 and CK20 in ovarian cancer (OC), we decided on CK19, because of its common representation in OC cells [17].

## hTERT

Telomerase is ribonucleoprotein enzyme that stabilises telomere length by adding hexameric (TTAGGG) repeats to the telomeric ends of chromosomes [18]. This way it can compensate for the loss of telomeric repeats with every cell division. Telomerase consists of three subunits: two of them are proteins (hTERT and hTP), whereas hTR is the RNA coding template element [19, 20]. The hTERT is the catalytical subunit of the human telomerase [19, 20]. The enzyme is expressed in embryonic cells and in adult male germline cells [21], but it is undetectable in most of normal somatic cells, except for proliferative cells with tissue renewing potential (e.g.: haemopoietic stem cells and activated lymphocytes [22, 23], basal cells of epidermis and intestinal crypt cells and others). It was proposed that re-expression of telomerase is a critical event responsible for continuous tumour cell growth [24, 25]. For the last ten years, using various methods, a number of cell lines and malignant tumours have been found to express telomerase activity (TA) [26-29], suggesting that telomerase activation may be a critical step in cell immortalization and carcinogenesis. One of the most promising ways to use telomerase in diagnosis of carcinomas is the

**Table II.** Results of TeloTAGGG Telomerase PCR ELISA assay

| patient's illness | FIGO stage | bodily fluid | % of Abs level |
|---|---|---|---|
| k1 | na | na | 100.00 |
| k2 | na | na | 0.00 |
| k3 | na | na | na |
| CACE | FIGO IIIc | blood | 38.08 |
| CACE | FIGO III | blood | 91.27 |
| | | perit. fluid | 76.44 |
| CACE | FIGO IV | blood | 76.21 |
| part. mucin. | | perit. fluid | 50.26 |
| metast. | na | blood | 94.15 |
| CACE | | perit. fluid | 61.29 |
| CAS | FIGO IIIc | blood | 44.35 |
| CAS | FIGO IIIb | blood | 62.21 |
| EAC | FIGO IIIa | blood | 25.13 |
| | | perit. fluid | 84.23 |
| CM | na | blood | 78.01 |
| CS | na | blood | 74.59 |

*Abbreviations: CACE – cystadenocarcinoma endometriogenes; CAS – cystadenocarcinoma serosum; CACE part. mucin. – cystadenocarcinoma endometriogenes partim mucinosum; EAC – endometrioid adenocarcinoma; metast. CACE – cystadenocarcinoma endometriogenes probably metastatic from previous intestinal tract tumour; CM – cystis mucinosa; CS – cystis simplex; k1 – positive control; k2 – negative control; perit. fluid – peritoneal fluid; na – not available*

possibility to detect circulating carcinoma cells by presence of TA. OCs are the tumour type in which TA has been also displayed [27, 30]. Unfortunately, it was found that activated lymphocytes express telomerase on the detectable level [22, 23].

## StAR

StAR is responsible for the transport of cholesterol through the outer membrane to the inner mitochondrial membrane during the biosynthesis of progesterone [31-34]. It is a 30-kDa phosphoprotein, which gene's mutations in humans lead to possibly lethal disease called congenital lipoid adrenal hyperplasia (lipoid CAH) [32]. Characteristic symptoms of this disorder are: inability to synthesise sufficient amounts of steroids, superabundant levels of cholesterol and its esters in adrenal and testicular steroidogenic cells. Patients afflicted with this disease are unable to survive unless they are treated with appropriate steroids [34]. The same symptoms were observed in StAR knockout mice [35]. Although expression of StAR has been confirmed in various human tissues (like the adrenal glands and gonads [32], discrete regions of the mouse brain [36], or human liver [37]). Recently, StAR was reported as a valuable marker in OC [38].

**Table III.** Results of real-time RT-PCR performed on 10 patients with various gynaecological ailments

| disease | patient | hTERT | | CK-19 | | CKB | | StAR | |
|---|---|---|---|---|---|---|---|---|---|
| | | PCR | gel | PCR | gel | PCR | gel | PCR | gel |
| primary ovarian tumours | 1 | + | − | + | + | + | + | − | − |
| | | + | + | − | − | + | + | − | − |
| | 2 | + | + | + | + | − | + | − | − |
| | | + | − | + | + | + | + | − | − |
| | 3 | + | + | + | + | + | + | + | − |
| | | − | − | + | + | + | + | − | − |
| | 4 | + | + | + | + | + | + | − | + |
| | | + | − | + | + | + | + | − | − |
| | 5 | + | + | + | + | − | + | − | − |
| | | + | + | + | + | + | + | − | − |
| OC CHT | 6 | − | + | + | + | + | + | + | + |
| | | − | − | + | + | + | + | − | − |
| benign cysts | 7 | − | + | + | + | + | + | + | − |
| | | − | − | + | + | + | + | − | − |
| | 8 | + | + | + | + | + | + | − | − |
| | | + | − | + | + | + | + | − | − |
| OC a o t | 9 | + | − | + | + | + | + | + | + |
| | | + | − | + | + | + | + | − | − |
| | 10 | + | − | + | + | + | + | + | − |
| | | + | − | + | + | + | + | − | − |
| NA | (+) control | + | + | + | + | + | + | + | + |
| NA | (−) control | − | − | − | − | − | − | − | − |
| NA | BrCa patient | NA | NA | + | + | − | − | − | − |

*Abbreviations: OC CHT – ovarian cancer treated with chemotherapy; OC a o t – ovarian cancer after other tumour; BrCa – breast cancer, NA – not available; (+) – (positive control – reference cDNA); (–) – negative control – H$_2$O*

## *CKB*

Creatine kinase B enzyme has been shown to be overexpressed in many tumour tissues, including OC, as far as in 1987 [39]. Although several attempts failed to assess CKB's prognostic value in OC, its levels correlation with patient age, menopausal status, clinical stage, histological grade or size of residual tumour [40, 41], this gene has been confirmed overexpressed in OC cells by using microarray technology and real-time RT-PCR in recent years [42].

## Materials and methods

### Patients

The blood samples from 20 women were collected in two parts [blood from each 10-person group was used either to perform real-time RT-PCR, or TA PCR-ELISA (enzyme-linked immunosorbent assay)]. The patients had not received any systemic chemotherapy before the blood collection.

## Blood collection

Blood samples (10 ml) were collected in tubes with sodium citrate and stored at 4°C for a maximum of 72 hours before isolation of CTCs.

### Isolation of CTCs

Peripheral blood mononuclear cells (PBMCs) were isolated by using Histopaque® (Sigma) density gradient centrifugation according to manufacturer's recommendations. After collecting mononuclear blood cells, circulating cancer cells were isolated using Dynabeads® Epithelial Enrich (Dynal Biotech) according to manufacturer's directions in order to obtain only epithelial fraction of cells isolated previously. Rosetted cells isolated with immunomagnetic beads were then frozen in liquid nitrogen and stored at -80°C until the isolation of RNAs or detection of TA. This two-step protocol assured unique specificity of markers detection, because it
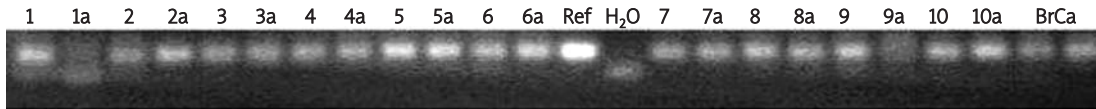
*Abbreviations: a – replica of sample; Ref – reference cDNA (positive control); H$_2$O – negative control*

**Figure 1.** Gel view of PCR products of CK-19 gene detection (numbers indicating the patients are consistent with Table III)

prevents the activated lymphocytes or any other blood cells showing illegitimate expression of detected marker from affecting the results.

### Isolation of RNAs and reverse transcription

RNAs were extracted using Trizol reagent (Invitrogen) according to manufacturer's recommendations. cDNA synthesis was performed from total RNA at a total volume of 60 μl using ImProm-II™ reverse transcriptase (Promega, Madison, WI).

### Real-time PCR

Real-time RT-PCR was performed using Corbett Research RG-3000 machines. Sequences of primers used, annealing and detection temperatures are presented in Table I. All reactions were performed in duplicate. Detection of PCR products was performed with SYBR® Green I using qPCR Core kit for SYBR® Green I (Eurogentec, Belgium).

### Quantification of telomerase activity

The assays were performed using TeloTAGGG Telomerase PCR ELISA$^{PLUS®}$ kit (Roche, Germany) according to manufacturer instructions. TA was measured twice in independent experiments on 1 μg of proteins or on 20 μl of cell extracts when insufficient amounts of proteins were available. Furthermore, because telomerase has essential RNA component, aliquots of samples were treated with RNase to assess the specificity of reaction.

## Preliminary results

### Quantification of TA by using TeloTAGGG Telomerase PCR ELISA$^{PLUS®}$ kit

Results obtained from TA PCR ELISA assay are gathered in Table II.

### Real-time RT-PCR & electrophoresis

End results of real-time RT-PCR experiments (shown in Table III) were also confirmed by electrophoresis of these reactions' products. An example of such an analysis of detection of CK-19 is shown in Figure 1.

## Discussion

Neither revolutionary discoveries in human genomics and proteomics, nor great advancement in techniques applied in laboratories worldwide (like introducing of microarray technology or use of real-time PCR) fulfilled the hopes for understanding and overcoming cancer diseases. Although we know more and more each year, no significant changes towards treatment and diagnosis of malignancies have been made so far. To surmount that state, researchers survey for new molecular markers appropriate for easy and credible detection of living cancer cells in the blood. In view of the fact that OC appears to be genetically heterogeneous, scientists lean to the opinion of necessity to use more than one marker in order to elevate the specificity and sensitivity of their assays [43]. That is why we decided on the four genes: cytokeratin 19 (*CK19*), catalytic subunit of telomerase (*hTERT*), steroidogenic acute regulatory protein (*StAR*) and creatine kinase B (*CKB*). In our opinion, real-time PCR-based detection of these genes amongst PBMC-fraction of blood collected from patients with epithelial OC would prove to be reliable and sufficient to prove the presence of CTCs in patients. Apart from that, we also wanted to evaluate usefulness of one particular commercial kit (TeloTAGGG Telomerase PCR ELISA$^{PLUS®}$) designed for assessment of TA. Most researchers assessed TA in tissue samples or peritoneal washings collected intraoperatively. There were only a few similar surveys to ours, in which CTCs of OC were isolated by using immunomagnetic enrichment (for example [44] and [45]). Stimpfl et al. [44], compared detection of CTCs in PB by using flow cytometry with RT-PCR of immunomagnetic-enriched fraction of blood cells. The authors assumed that detection of MUC-1 mRNA in cells isolated from PB is sufficient to show that ovarian carcinoma cells are present in the PB of examined OC patients. The selected marker did not prove to be specific, because of a weak expression of MUC-1 found in samples collected from healthy persons [44] (see also [5] and *Introduction*). The survey described in [45] was very similar to our PCR-ELISA assay. Detection of TA in PB of OC patients (stages III and IV) was preceded by immunomagnetic isolation of epithelial cells (both positive and negative methods were applied). TA was then evaluated with TRAPeze ELISA Telomerase detection kit (Intergen, Purchase, NY), which working principle is identical with that of the kit used in our study. TA was detectable in cells from all stage IV patients (8/8 patients; 100%) and 7 of the 20 stage III patients (35%) [45]. No significant differences between the results obtained from either positive or negative isolation methods were observed, which meant that there was no loss of

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CTCs due to the lack of expression of surface antigens used as targets for mAbs [45]. Low sensitivity of assay in stage III patients was explained with the reasoning that at this stage the disease spreads mainly through lymphatic routes. These results and conclusions are in disagreement with ours, because in our study 5 patients were at stage III and TA assessed was significantly higher than in negative controls. Moreover, in one patient TA in PB was higher than in the peritoneal fluid. In [46], Saygan-Karamursel et al. wanted to estimate TA in malignant, benign and normal gynaecological tissue samples by using the Telomerase-PCR ELISA kit. The mean level of TA of the malignant tumour samples was significantly higher than the benign pathologies and normal tissues. However, TA measured in benign diseases was few-fold higher than in normal tissues [46]. These results are in accordance with our results from two patients with cysts (TA in these samples was comparable with that in cancer patients), but together with previously cited obtained in [45], prove that this technique cannot be used in diagnosis of gynaecological diseases. Moreover, Roche® has recently withdrawn TeloTAGGG Telomerase PCR ELISA$^{PLUS®}$ kit from sale without publicising any reasons for that. Results obtained in real-time RT-PCR assays are more promising. Although expression of CK-19 was reaffirmed in all samples (even from patients afflicted with cysts), these were the positive results we expected after scrutinizing the literature (CK-19 was found to be inaccurate RT-PCR marker in PB in [47]). In addition, our hTERT real-time RT-PCR assay proved to be more reliable than PCR ELISA kit. However, two genes selected to show tissue specificity (CKB and StAR) did not turn out to be as selective and sensitive among the samples from patients with gynaecological diseases as we thought, at least in this preliminary report.

## Conclusions

Both results of our study and the small number of articles on molecular markers suitable for detecting CTCs in PB of patients with OC show that this aim will be hard to achieve by using standard molecular biology tools, like PCR or even real-time PCR. However, these attempts should not be terminated, because if any of them succeeds, the diagnostic value of this technique will be great.

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