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The detection of circulating tumor cells expressing E6/E7 HR-HPV oncogenes in peripheral blood in cervical cancer patients after radical hysterectomy

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The aim of this study was to establish the sensitive, specific and clinically acceptable method for detection of tumor cells (TCs) circulating in peripheral blood (PB) of cervical cancer patients without the clinically detectable risk of disease progression.

The 7.5 ml of PB of healthy donor was spiked with 5 to 100 cells from SiHa or HeLa cell lines. The spiked tumor cells were collected without gradient centrifugation, by standard gradient centrifugation or by modified gradient centrifugation combined with immunomagnetic separation using EpCAM antibody with affinity for epithelial cell adhesion molecule. The number of collected TCs was determined by EpCAM-FITC-staining and their viability was detected by nested RT-PCR amplifying E6/E7 HR-HPV 16 or HR-HPV 18 oncogenes. For the technical validation of this approach the TCs separation and RT-PCRs were repeated several times.

The recovery of viable TCs was reproducibly higher using modified gradient centrifugation combined with immunomagnetic separation in comparison with standard approach. The recovery of TCs in low number of spiked TCs (range from 5 – 20 TCs in 7.5 ml of PB) using modified gradient centrifugation was not reproducible. The recovery of TCs in higher number of spiked TCs (25 TCs and more in 7.5 ml of PB) was reproducible with average recovery about 50 %.

The sensitivity of nested RT-PCR amplifying E6/E7 oncogenes was decisively influenced by the number of recovered TCs and the amount of cDNA introduced to RT-PCR, as well.

Using this approach we were allowed to detect circulating TCs (CTCs) in cervical cancer patients without metastases, thus this procedure might become a tool to early estimation of disease progression. According to our knowledge, this is the first report describing the use of EpCAM antibody for CTCs detection in cervical cancer patients.

Key words: HPV, tumor circulating cells, immunomagnetic separation

One of the most frequent causes of death in cancer patients is the metastases formation in distant places. Even at the time of primary diagnosis and surgery the occult metastases frequently occure. The clinically detectable metastases formation during disease progression is preceded by circulation of tumor cells (CTCs) in peripheral blood (PB). The detection of CTCs is under the sensitivity limit of actually used standard laboratory methods. The introduction of more sensitive and specific molecular approaches could reveal tumor cells (TCs) dissemination at early stage of disease progression, so the CTCs examination may have therapeutic and prognostic implications.

One of the first information about TCs detection in PB of cancer patient after death was published in 1869 [1]. The CTCs detection gained greater attention between 1955 and 1965, when several thousand patients with different types of solid tumors were tested for CTCs by cytological methods. The cytological methods were shown to be false-positive, because of confusion between megakaryocytes and tumor cells. Improved cytological methods allowed cytology to detect true CTCs by light microscopy in 1965 [2]. However, the sensitivity of light microscopy was very low, so routine CTCs examination was in 1965 abandoned.

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About 20 years later, the more sensitive immunohistochemical methods were developed to detect TCs in bone marrow and PB in patients with different types of cancer [3, 4]. The immunohistochemistry declared the detection sensitivity of 1 TC among 10,000-100,000 mononuclear cells, but inspite of this, it could not contribute to routine cancer staging.

In late 1980s and mainly in 1987, when the new highly sensitive and specific methods of DNA analysis and the polymerase-chain reaction (PCR) technique were developed, the identification of CTCs and metastases widespread in leukemias, lymphomas and later also in solid tumors [5].

The process of metastases formation consists of different steps – metastatic cascade - and the metastases are developing after each step in metastatic cascade is succesfully completed.

The metastatic cascade begins with stimulation/inhibition of different gene casettes in tumor cells and in different tissues, which allow the tumor cells: 1. leaving the primary tumor, 2. entering the lympho-vascular circulation (intravasation) and survival in circulation, 3. fixing in a small capillaries, 4. passage through vessels into tissue in a new places (extravasation) and, in the end, 5. dormancy folloved by proliferation/ apoptosis equilibrium of the tumor cells in a new organ. When proliferation dominates apoptosis the metastasis formation becomes clinically detectable [6].

The circulating tumor cells (CTCs) proceeding metastatic cascade are clinically undetectable, however, they are decisive in later phase of disease progression.

Human papillomaviruses with high oncogenic risk (HR-HPV) were detected nearly in all cervical cancer cases (99,7 %) [7]. In a meeting of the International Agency for Research on Cancer (Lyon, France in 2005) the working group concluded that there is sufficient evidence about causative role of 13 genotypes of HPVs in cervical cancer initiation [8]. The genomes of 13 HR-HPV genotypes can integrate into the cellular genome, while the cellular expression machinery contributes to the expression of viral E6 and E7 oncogenes [9]. Kessis and others found out, that HR-HPV viral oncogene E6 can bind to cellular tumor suppressor protein p53 causing its ubiquitination. By this way, viral E6 protein can participate in either cell cycle deregulation or transcription activation of different genes regulated by p53, as well [10–12]. Moreover, E6 contains a cystein dublet (C-x-x-C) suggesting, that protein contains zinc finger and is able to bind to DNA and/ or to interact with another proteins [13]. The role of E6 oncoprotein in cell immortalization and transformation seems to be complex.

The HR-HPV E7 protein contains within its N-terminal region conserved motif essential for binding to retinoblastoma tumor suppressor protein pRB and pRB-related proteins [14]. Phosphorylated pRB was detected during cell moving towards S phase, on the other hand, hypophosphorylated pRB was associated with cell cycle arrest. In HR-HPV transformed epithelial cells the binding of E7 to pRB avoid dephosphorylation of pRB and cell continue the cycle. Moreover, the E7 oncoprotein N-terminal region contains two serins, which are substrate for phosphorylation. Mutations in this region reduce the transforming ability of E7 [15]. It seems, that binding of E7 to pRB is not the only event sufficient for cell transformation and that the E7 function is similarly to E6 oncoprotein, more complex.

Up till now, there is a number of articles dealing with the detection of epithelial TCs circulating in PB in patients with breast, ovarian, prostate, lung or colorectal cancer and melanoma [16–21]. However, only a small number of reports is focused on detection of TCs circulating in cervical cancer patients although 10–15 % of patients with early stage of cervical cancer with histologically negative lymph nodes and clear resection margins after ablative approach will develop recurrence and are at high risk of cancer death [22–24]. Due to the most frequent detection of HR-HPV 16 and HR-HPV 18 infection in cervical cancer and precancerous lesions (together more than 70% of cases) the E6/E7 oncogene expression was chosen as unique biological marker for detection of viabile TCs circulating in PB in cervical cancer patients.

Patients and methods

At first, we compared the sensitivity and specificity of different approaches of recovering CTCs in PB in experimental conditions. The 7.5 ml samples of PB of healthy volunteer were collected to EDTA containing tubes. The PB samples were diluted 1:1 with PBS and spiked with different number of cells from SiHa or HeLa cell lines.

The 7.5 ml samples of PB of 10 cervical cancer patients after Wertheim-Meigs hysterectomy were collected to EDTA tubes and the number of CTCs was determined. The E6/E7 HR-HPV oncogenes expression detection was preceded by positive HR-HPV DNA detection in PB for at least 1 year.

Spiking of PB samples. Briefly, the SiHa or HeLa cells were cultured in Petri dishes containing RPMI 1640 supplemented with 10% fetal calf serum at 5% CO_2 environment and were harvested using trypsin. The viability of SiHa and HeLa cells was determined by trypan blue. After harvesting the cell suspension was diluted by RPMI 1640 to approximately 5 viabile cells in 20 µl volume. The number of cells in 20 µl volume of cell suspension was determined by microscopic quantification with cell chamber. The cell quantification in 20 µl aliquots was repeated several times by at least two workers. After repeated counting the average cell number in 20 µl aliquot of cell suspension was determined. Different volumes of cell suspension containing different cell number were spiked to PB samples. Samples of PB analysed in one day (one procedure), were spiked with 5, 10, 20, 25, 40 or 100 SiHa or HeLa cells respectively. Each spiked PB sample was analysed in parallel. To each procedure one PB sample without spiked TCs was added as negative controle. Totally, 17 repeats of procedure were performed during 12 months. The procedures were performed by two different operators.

Enucleated cell depletion. The enucleated red elements were depleted from PB samples spiked with CTs using three approaches: 1. centrifugation without gradient, 2. centrifugation through standard Ficoll gradient (Ficoll-Paque Plus, AmershamBiosciences) and 3. centrifugation through Ficoll gradient modiefied in our laboratory.

The first approach to nucleated cells separation was based on incubation of whole PB samples for 10 min. in RT in lysis buffer (145 mmol/l NH_4Cl and 100 mmol/l $NaHCO_3$) leading to depletion of the red elements prior to centrifugation in 3000 g without Ficoll gradient. The pellet of nucleated cells was resuspended in 200 µl of cell lysis buffer (RNAqueous, Ambion).

The second approach was based on enucleated cells depletion by gradient centrifugation through Ficoll. Besides standard gradient centrifugation we used a modified Ficoll gradient centrifugation, as well to increase the number of recovered TCs. The modified Ficoll gradient centrifugation was based on different centrifugation conditions (time and g). The centrifugation at 500 g for 15 min. at RT we found as the best effective modification of gradient centrifugation. Briefly, after modified gradient centrifugation the entire liquid volume without red element pellet (opalescent Ficoll, diffuse cell ring and serum) was harvested, several times washed with PBS according the manufacturer´s instruction, centrifuged at 1 800 g for 10 min. at RT and final pellet of nucleated cells was resuspended into 200 µl volume of Hank´s salt solution (Hank's BSS, Cambrex Research Products). The most of white blood cells and contaminating enucleated red elements were then depleted by following immunomagnetic separation of spiked TCs (SiHa or HeLa). To enriche the cell suspension with the TCs the EpCAM antibody with affinity for epithelial cell adhesion molecule was used according the manufacturer´s instruction (Human EpCAM Positive Selection Kit, StemCell Technologies). After immunomagnetic TCs enrichment the recovered cells were resuspended in 200 µl of cell lysis buffer and stored at -24°C untill total RNA extraction (RNAqueous, Ambion).

The effectivity of three different approaches was determined by comparison of a number of recovered TCs and E6/E7 HR-HPV 16 or HR-HPV 18 oncogene expression.

The total RNA extraction, cDNA synthesis and nested RT-PCR conditions. The 200 µl lysates from cell suspensions after centrifugation without gradient and through Ficoll gradient (standard and modified) followed by immunomagnetic enrichement were used for total RNA extraction. To avoid possible DNA contamination we used the RNA separation approach combined with DNaseI incubation according manufacturer´s instruction (TURBO DNA-free, Ambion).

The first strand of cDNA was reversely transcribed from total volume of extracted RNA (from 7.5 ml of spiked PB) by using the Ready-To-Go You-Prime First-strand kit according manufacturer´s instruction (GE Healthcare). The total volume of cDNA was 30 µl.

The E6/E7 HR-HPV 16 and 18 oncogene expression was determined by nested RT-PCR using 4 or 10 µl of cDNA in total 25 µl volume of PCR.

The cDNA integrity and biological activity was determined by 18S rRNA RT-PCR using 2µl of cDNA in total 25 µl volume of PCR. The primer sequences for 18S rRNA RT-PCR were published earlier [25].

RT-PCR reaction mixtures for E6/E7 HR-HPV 16 and HR-HPV 18 using 4µl of cDNA contained: 1x reaction buffer recommended for DyNAzyme EXT, 200 µmol/l $MgCl_2$, 25 µmol/l each dNTP (Finnzymes) and 20 pmol of sense and antisense primers for target gene respectively and 1U DyNAzyme EXT (Finnzymes).

RT-PCR reaction mixtures for E6/E7 HR-HPV 16 and HR-HPV 18 using 10 µl of cDNA contained only 1x reaction buffer recommended for DyNAzyme EXT without $MgCl_2$, 20 pmol of sense and antisense primers for target gene respectively and 1U DyNAzyme EXT in total 25 µl volume.

The primer sequences for E6/E7 HR-HPV 16 and 18 and for another HR-HPV genotypes detected in patient PB samples, profile of cycles for the first and second step of nested RT-PCRs and size of PCR products including E6/E7 HPV splice variants are summarized in Table 1. The set of primers detecting E6/E7 HR-HPV 39, 51 and 66 are ommited, because up till now we have not detected CTCs infected with these strains in PB of any of examined patients.

The uniqueness of E6/E7 HR-HPV primer sequences was designed according HR-HPV complete genome maps (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The primer selection programme (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used to avoid hairpin structure and self-annealing events. The PCR products were analysed in 2% agarose gel with ethidium-bromide staining with molecular weight standard using photo-documentation system ULTRA–LUM with analytical softwares SCION and Gel-Pro Analyzer, version 3.0 (Media Cybernetics). The specificity of E6/E7 PCR products were confirmed by sequence analysis (AbiPrism 3130, Applied Biosystems – data not shown).

Microscopic detection of TCs . The TCs in totally ten PB samples spiked with 50 SiHa or HeLa cells with modified gradient centrifugation were conjugated with anti-EpCAM FITC antibody (StemCell Technologies) according the manufacturer´s instruction. The rates of recovered FITC-conjugated TCs were microscopically determined by differential interference contrast (DIC) and immunofluorescence detection using Olympus IMT-4 microscope (Olympus, Hamburg, Germany). Images were taken with colour camera ColorView III (Soft Imaging System, Műnster, Germany) using image analysis software AnalySIS^D (Olympus Biosystems, Hamburg, Germany). Briefly, the immunomagnetically enriched cell suspensions were incubated for 15 min. at RT with anti-EpCAM FITC antibody and the entire volume of cell suspensions was transferred into the cell culture chambers Flexiperm (Heraeus Biotechnologie, Hanau, Germany). Two PB samples without spiked TCs were used as a negative control.

Table 1. List of primers and PCR conditions

Names of primer paires	Sequence 5' > 3'	PCR profiles (°C / sec)	Size of PCR products (bp)
18S rRNA – house-keeper		94 / 20 58 / 45	141
18S-F	GTA ACC CGT TGA ACC CCA TT	72 / 45	
18S-R	CCA TCC AAT TCG GTA GTA GCG	45 cycles	
1. step		94/20	
HR-HPV 16		62/45	*566 / 384 / 267
16E6-A1	ATG TTT CAG GAC CCA CAG GAG C	72/45	
1635E7-A1	C**W**T CCT CCT CCT CTG AGC TG	45 cycles	
2. step		94 / 20	
HR-HPV 16		58 / 45	*508 / 326 / 209
16E6-A2	AGT TAC CAC AGT TAT GCA CAG AG	72 / 45	
1635E7-A2	AAT TGC TCA TAA CAG TA**K** AG**R** TCA G	45 cycles	
1. step		94 / 20	
HR-HPV 18		60 / 45	*448 / 266
18E6-C1	CAC TTC ACT GCA AGA CAT AGA AAT AAC	72 / 45	
18E7-C1	TTG TGT TTC TCT GCG TCG TTG G	45 cycles	
2. step		94 / 20	
HR-HPV 18		60 / 45	*341 / 159
18E6-C2	TAT TGC AAG ACA GTA TTG GAA CTT AC	72 / 45	
18E7-C2	CTG TCG TGC TCG GTT GCA G	45 cycles	
1.step		94/20	
HR-HPV 33+58		62/45	575
3358E6-A1	AAA CCA CG**R** ACA TTG CAT GAT TTG TG	72/45	
3358E7-A1	CTT GTC CAT CTG GC**S** GGT C	45 cycles	
2.step		94/20	
HR-HPV 33+58		62/45	410
3358E6-A2	CTT TGC A**R**C GAT CTG AGG TAT ATG	72/45	
3358E7-A2	GGT CAG TTG GTT CAG GAT **R**TA AAT C	45 cycles	
1.step		94 / 20	
HR-HPV 31		60 / 45	606
31E6-A1	ATG TTC AAA AAT CCT GCA GAA AGA C	72 / 45	
31E7-A1	TAA TTG GAT GTG TCC GGT TCT G	45 cycles	
2.step		94/20	
HR-HPV 31		62/45	*475 / 273
31E6-A2	TCG GCA TTG GAA ATA CCC TAC G	72/45	
31E7-A2	ACA GTG GAG GTC AGT TGC CTC	45 cycles	
1.step		94 / 20	
HR-HPV 52		60 / 45	718
52E6-A1	GTT TGA GGA TCC AGC AAC ACG	72 / 45	
52E7-A1	TTG TAA TGT GCC CAA CAG CAG	45 cycles	
2.step		94/20	
HR-HPV 52		61/45	*574 / 297
52E6-A2	ACC CTG CAC GAA TTG TGT GAG	72/45	
52E7-A2	TTG TGG CTT GTT CTG CTT GTC C	45 cycles	
1.step		94/20	
HR-HPV 56		61/45	580
56E6-D1	AGC CAC AAT TCA ACA ATC CAC AG	72/45	
56E7-D1	CAT CCT CAT CCT CAT CCT CTG	45 cycles	
2.step		94/20	
HR-HPV 56		63/45	518
56E6-D2	AAC GTC CAC GAA GCC TGC AC	72/45	
56E7-D2	TTG CAC TGT AGG TCA ATT TCT GTT TG	45 cycles	
1.step		94/20	
HR-HPV 59		63/45	636
59E6-C1	CTT TGA GGA TCC TAC ACA ACG AC	72/45	
59E7-C1	GCT AGT AGC AAA GGA TGA TTA ACT C	45 cycles	
2.step		94/20	
HR-HPV 59		61/45	562
59E6-C2	TGC CTG ATT TGA GCA CAA CAT TG	72/45	
59E7-C2	TTC ATT CTC GGA GTC GGA GTC	45 cycles	

* Splice variants, **W,K,R,S** – degenerated primers

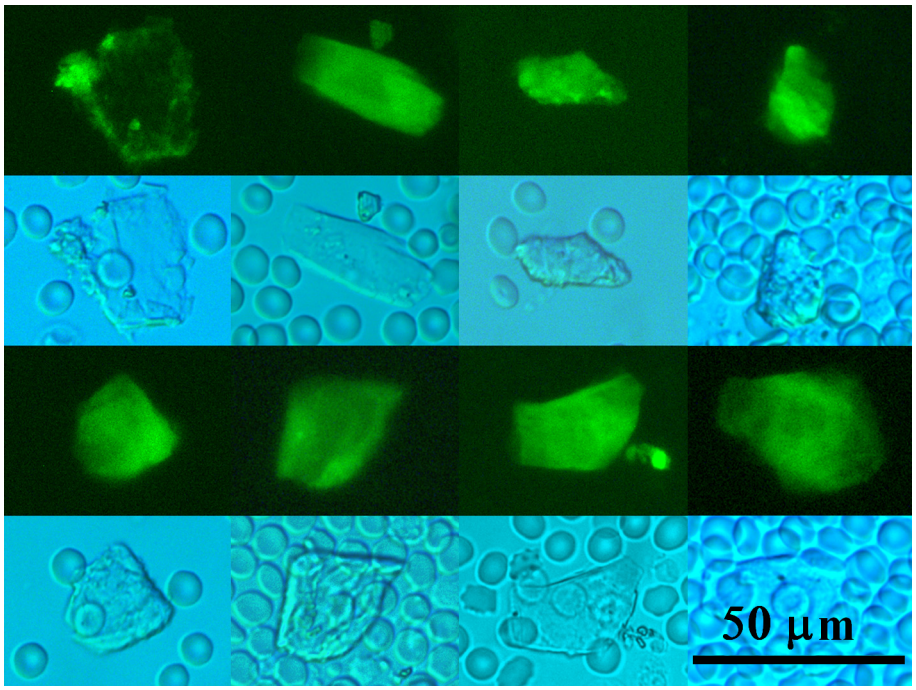

Fig. 1. The gallery of recovered TCs shapes. The TCs were microscopically detected by two methods. First and third rows – immunofluorescence detection with anti-EpCAM FITC antibody, second and fourth rows – differential interference contrast (DIC) visualization using Olympus IMT-4 microscope (Olympus, Hamburg, Germany).

Results

The 7.5 ml PB samples were spiked with varying number of SiHa or HeLa cells from the cell lines derived from cervical carcinomas (ATCC). The TCs were separated without gradient, by standard or modified Ficoll gradient combined with immunomagnetic separation. The presence of TCs was confirmed by the E6/E7 HR-HPV 16 or HR-HPV 18 expression detection using nested RT-PCR with 4 or 10µl of cDNA.

The E6/E7 expression detection in spiked PB samples. In the case of PB samples spiked with less than 100 TCs separated without gradient centrifugation we did not detect any E6/E7 expression if 4µl of cDNA was used in the first step of nested RT-PCR. The use of 10µl of cDNA in the first step of nested RT-PCR led to improved sensitivity of PCR, however, the positive E6/E7 expression detection was not reproducible and varied from procedure to procedure.

In the case of PB samples spiked on the average with 40 TCs and separated through standard Ficoll gradient followed by immunomagnetic enrichment we detected the E6/E7 expression if 10 µl volume of cDNA was used in the first step of RT-PCR. In spite of increased sensitivity of TCs separation compared with procedure described abow, the positive E6/E7 expression detection was not reproducible if 4 µl of cDNA was used in the first step of nested RT-PCR. If 10 µl volume of cDNA was used in the first step of RT-PCR, the E6/E7 expression detection was reproducible.

In the case of PB samples spiked with 5 to 20 TCs separated with modified Ficoll gradient followed with immunomagnetic enrichment we detected E6/E7 expression if 10 µl of cDNA was used in the first step of nested RT-PCR. However, reproducibility was achieved if on the average 25 TCs were spiked to 7.5 PB samples and 10 µl of cDNA was used in the first step of nested RT-PCR.

Microscopic detection of recovered TCs. The rates of recovered TCs after modified Ficoll gradient separation and immunomagnetic enrichment were determined and the different shapes of detected TCs images were summarized in Fig. 1. In two PB samples without spiked TCs (negative control) we did not detect any FITC-stained cells.

Sensitivity, specificity and reproducibility. According to our results, the combination of modified Ficoll gradient separation followed by immunomagnetic enrichement of TCs using anti-EpCAM antibody and nested RT-PCR using at least 1/3 of total cDNA volume reversely transcribed from total RNA volume separated from 7.5 ml of spiked PB is the most sensitive, specific and reproducible „home-made“ approach.

The inter-assay variability using negative controls (two operators and several assays in paralelle during 1 year) led to the recovery rate of TCs spiked to 7.5 ml of PB on the average 50%. The sensitivity of compared approaches of CTs separation is summarized in Table 2.

The specificity of primers for E6/E7 HR-HPV 16 or HR-HPV 18 were tested in the nested RT-PCR amplifications

Table 2. The sensitivity of compared approaches

	Number of spiked SiHa or HeLa cells					
	5	10	20	25	40	100
cell separation without gradient	-	-	-	-	-	posit
cell separation with standard gradient	-	-	-	-	posit	posit
cell separation with modified gradient	±	±	±	posit	posit	posit

± not reproducible results

Table 3. Patient characteristics

Patient	Age at therapy	FIGO stage	Lymph node status hystologically	Lymph node status DNA analysis	SCCA[1)]	CA 125[1)]	Genotype in cervical cell suspension	Genotype in CTCs from peripheral blood	E6/E7 expression[1)]
A	50	pT1b1 pN0 pM0	negat	negat	1.2	10	HR-HPV 58 + 33	HR-HPV 58 + 33	no
B	47	pT1a2 pN0 pM0	negat	negat	0.3	21.1	HR-HPV 58	HR-HPV 58	no
C	52	pT1b1 pN1 pM0	MTS in 2 LN	negat	0.7	4.6	HR-HPV 58	HR-HPV 58	no
D	50	pT1b1 pN0 pM0	negat	negat	2.3	8.8	HR-HPV 16	HR-HPV 16	no
E	41	pT1b1 pN0 pM0	negat	negat	0.1	16	HR-HPV 16 + 33	HR-HPV 16 + 33	no
F	68	pT2 pN1 pM0	MTS in 1 LN	HR-HPV 58 in 1 LN	0.8	52	HR-HPV 58	HR-HPV 58	no
G	37	pT1b pN0 pM0	negat	nt	0.8	10.2	HR-HPV 16 + 58	HR-HPV 16 + 31	HR-HPV 16
H	42	pT1 N0 Mx	negat	nt	3.9	105.8	nt	HR-HPV 16	HR-HPV 16
I	39	T1b1 N0 M0	negat	HR-HPV 16 + 18	1.1	18.2	nt	HR-HPV 16 + 58	no
J	48	pT1b1 pN1 pM0	MTS in 1 LN	HR-HPV 18	0.2	11.1	nt	HR-HPV 18	no
K	64	pT1b pN0 pM0	negat	HR-HPV 58 in 1 LN	0.5	17.7	nt	HR-HPV 58	no
L	43	pT1b pN0 pM0	negat	nt	2.4	11.4	HR-HPV 16	HR-HPV 35	no
M[2)]	61	IIIA	-	-	1.2	10.9	nt	HR-HPV 35	HR-HPV 35

[1)] at the time of CTCs detection

[2)] inoperable cervical carcinoma (CTC detected 3 years after RAT and hysterectomy)

nt not tested

using 10 µl volume of cDNA transcribed from RNA extracted from uninfected cells or cells infected with another types of HR-HPV (data not shown). In no case we detected positive E6/E7 HR-HPV 16 or HR-HPV 18 expression, respectively.

The modified Ficoll gradient approach was found as the most sensitive among three tested separation techniques and was used for detection of CTCs in 7.5 ml PB samples from 13 patients with cervical cancer after radical hysterectomy (HYS) or radiotherapy/hysterectomy (RAT/HYS). The data on patient characteristics (age, FIGO staging, histological lymph node status, lymph node status after DNA analysis, serum markers SCCA and CA 125 level, HR-HPV type presence and E6/E7 HR-HPV oncogene expression) are listed in Table 3. The CTCs containing HR-HPV DNA was detected in all 13 cervical cancer patients 3 to 5 years after HYS or RAT/HYS. The E6/E7 oncogene HR-HPV 16 positive expression was detected in 2 patients and E6/E7 oncogene HR-HPV 35 positive expression was detected in 1 patient.

Discussion

The metastases formation is the most critical event that influences the prognosis of the disease and the survival of cancer patients. Cancer cells can spread from the primary tumor through peritoneum and lympho-vascular circulation, so the detection of disseminated CTCs in PB can represent the higher risk of cancer disease progression [6].

The large number of articles is dealing with detection, characterization and clinical significance of CTCs in PB of cancer patients [16-24]. The efficiency of the CTCs recovery varies from one approache to another and taking together, the sensitivity and specificity of different "home-made" and semiautomated approaches in CTCs detection is influenced by: 1. the determination of number of exactly viable TCs spiked to PB during validation in experimental conditions (the source of possible false-negativity during biological characterization of recovered TCs), 2. the different methods of CTCs separation preserving the cell viability (the conditions of gradient cell separation directly influence the number of recovered CTCs), 3. poor differentiation between nucleated non-malignant and malignant cells, and the most important is 4. the confirmation of viability of recovered TCs by their further genetic characterization (distinguishing between viable and died TCs using non-hematological cell specific genetic markers or tumor cell specific genetic markers and poor inter-laboratory comparison because of the use of different PCR primer sets).

The American Society of Clinical Oncology published the update of recommendations for the use of tumor markers in breast cancer, however, the CTCs detection found insufficient to support routine use in clinical practice [26].

This decision could be acceptable in the case of tumors characteristic by high clonal capacity like breast cancer is. The genetic analysis of recovered CTCs from PB of breast cancer patients could not reflect the biological activity of primary tumor, so interpretation of CTCs analysis results could be at least controversial. For example, Wülfing P et al, 2006 [27] detected HER2-positive CTCs in PB of 12 patients with breast cancer while the primary tumor showed a neagtive score for HER2 (≤ 10% HER2-positive cells). They explained this discrepancy as a result of at least two factors: 1. the selection of tumors with favourable prognosis (predominantly small tumors well or moderately differentiated with nodal negativity) and 2. the different sensitivity of methods (HER2 overexpression in CTCs versus immunohistochemical detection of HER2 level in breast cancer tissue). On the other hand, the probable explanation of this discrepancy could be selection of a small number of HER2-positive cells during tumor progression and cell dissemination. Moreover, it has been reported, that significant portion of HER2-negative tumor cells during disease progression develops high concentration of serum HER2, so the HER2 amplification could be considered as the result of cancer progression [28].

The Breast Cancer Intergroup of North America currently plans to study the role of CTCs in a prospective randomized trial (S0500). The primary end point of this trial will be evaluation of overall survival [29].

Although the modern methods of molecular genetics are able to introduce more data from small number of cancer cells and can influence the progress in CTCs era, the genetic characterization of CTCs in PB remains serious problem due to difficult determination of abnormal expression of cancer associated genes in small number of TCs in excess of non-tumor cells [5]. The most powerfull technique is RT-PCR amplification using reversely transcribed cDNA from RNA/mRNA and its modifications. However, inspite of the number of PCR modifications, the PCR technique has some limitations. The first is the false-positive results due to illegitimate gene expression (expression of any gene in any cell type). Although, this inappropriate gene expression is in non-malignant cells low, it could lead to false-positivity because of high sensitivity of RT-PCR [30]. The second, PCR amplification can lead to false-negative results due to a very small number of real viable CTCs in PB sample and, in the end, the third limitation is the presence of RT-PCR inhibitors in some patient tissues. The two last limitations could be resolved by immunomagnetic enrichment techniques during CTCs separation [16–21].

Up till now, with the exception of several "home-made" separation techniques the semi-automated systems (OnkoQuick and CellPrep system) have been described [31–33]. The CellPrep system has been found as more sensitive than the OnkoQuick [34]. According to our results, the sensitivity of described "home-made" TCs separation approach based on modified Ficoll gradient centrifugation combined with immunomagnetic enrichment and HR-HPV E6/E7 oncogene expression detection could be sensitive enough to detect disease progression several months before its clinical symptoms. Although the declared average recovery of TCs using the CellPrep system is ≥85%, and the recovery of TCs of the approach described in this article is ≥50%, the uniqueness of chosen genetic marker (the HR-HPV E6/E7 oncogene expression) and high sensitivity of modified RT-PCR amplification allows us to detect about 25 recovered viabile TCs. According to some authors the CTCs number in metastatic carcinoma patients ranged from 0 to 23,618 CTCs per 7.5 ml of PB (mean, 60 – 693 CTCs per 7.5 ml) [17]. Moreover, the healthy or non-malignant disease subjects could have about 2 circulating epithelial cells in 7.5 ml of PB, so in the light of these results, the enumeration of recovered TCs with low detection limit (about 2-5 CTCs in 7.5 ml of PB) looks less important than the further genetic characterization of CTCs. Encouraging are the attempts to establish global gene expression analysis of CTCs [16].

The E6/E7 HR-HPV oncogene expression of 13 accepted HR-HPV genotypes provides the most specific genetic marker distinguishing the non-tumor epithelial cells from cervical cancer tumor cells with integrated HR-HPV DNA circulating in PB [8]. Tseng et al. (1999) [22] detected the presence of HR-HPV 16 and HR-HPV 18 E6 oncogene expression in PB in patients with bulky tumor volume (≥4 cm) and pelvic lymph node metastasis. Patients with positive E6 oncogene expression were after a median follow-up of 22 months at a significantly higher risk of recurrence than those who were E6 oncogene expression negative. In our study we have found the E6/E7 oncogene HR-HPV 16 in 2 patients and E6/E7 oncogene HR-HPV 35 in 1 patient of 13 cervical cancer patients after radical hysterectomy (HYS) or radiotherapy/hysterectomy (RAT/HYS) without clinically determined progression at the time of PB sampling. About 2 years after HYS or RAT/HYS all three patients became HR-HPV DNA positive in PB, although the level of currently examined tumor markers SCCA and CA125 were in physiological limit. From three to four years after HYS or RAT/HYS all three patients with continuous HR-HPV DNA positivity became also HR-HPV E6/E7 oncogene expression positive. The level of SCCA and CA125 started to progressively elevate about 6 months later only in 1 case E6/E7 HR-HPV expression positivity. The progressive elevation of tumor markers level can indicate the higher risk of clinically determined cervical cancer progression. Those patients, whose serum marker level is higher than physiological limit and E6/E7 HR-HPV expression is negative, will be examined in standard clinically determined interval.

Using the described approach to the CTCs detection in PB we could determine the HR-HPV E6/E7 oncogene expression, which preceded the progressive elevation of currently examined tumor markers and the clinical determination of cancer progression, as well.

According to our results we can conclude, that the HR-HPV E6/E7 oncogene expression in CTCs in PB can offer unique genetic marker useful for early detection of the occult

phase of metastatic cascade and could allow the consideration of higher risk of disease progression and possible individual therapy of cervical cancer patients.

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