

Quantitative PCR marker genes for endometrial adenocarcinoma

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Abstract. Endometrial adenocarcinoma is a common malignancy in women worldwide, with formation of remote metastasis occurring following oncological treatment. Circulating tumor cells (CTCs) are regarded to be the origin of haematogenous metastasis formation. The present study aimed to identify suitable marker genes using a quantitative polymerase chain reaction (qPCR) approach to detect CTCs from blood samples of patients with endometrial carcinoma. Therefore, RNA was isolated from endometrial adenocarcinoma cell lines and from healthy endometrial tissue and reverse transcribed to cDNA, which was then used in qPCR on a number of marker genes. Cytokeratin 19 and claudin 4 were identified as suitable marker genes for CTCs in endometrial adenocarcinoma, due to their high expression in the majority of the cell lines investigated. The expression values of the genes examined varied widely between the different cell lines, which is similar to the variation in the patient samples. Therefore, the necessity for a set of genes for CTC detection and not one single marker gene is demonstrated. qPCR is a fast, cost-efficient and easy to perform technique, which may be used in the detection of CTCs. Investigation of the occurrence of CTCs in cancer patients would aid in the prevention of metastasis and thereby refine treatment.

Introduction

Endometrial adenocarcinoma is the fourth most frequent gynecological malignancy in Germany (1), ~11,300 women are newly diagnosed with this cancer annually (2). The risk of developing endometrial carcinoma increases with age, and may be augmented by estrogen-based hormonal therapies (3), diabetes mellitus, nulliparity and former carcinomas (4). The most common symptom is sudden bleeding, in peri- and postmenopausal women (5). In contrast to other tumors, preventive screening is often ineffective. A specific diagnosis may only be made by histological tissue examination subsequent to hysteroscopy (6). Therapeutic interventions primarily consist of surgery, followed by radiation therapy (7). Chemotherapy is rarely administered (8). Regardless of medical treatment, in ~25% of all cases, patients develop remote metastasis (8), resulting in necessary follow-up care (9).

The aim of the present study is to predict the formation of metastasis, in order to prevent the processes associated with metastatic outgrowth. Metastasis develop from single cells that have dissociated from the primary solid tumor, circulate through lymph vessels and the blood stream before settling in different sites of the body, thus allowing for metastatic formation. These single tumor cells are termed circulating tumor cells (CTCs) (10). In cases where CTCs infiltrate the bone marrow, they are able to persist for years without doing any harm and become disseminated tumor cells (DTCs) (11-16). The occurrence of CTCs and DTCs is often an indicator of an unfavorable prognosis for patients (10,17), and therefore are included in international tumor staging systems (18,19).

The detection of DTCs is exhausting and a time-consuming procedure for patients, whereas the detection of CTCs from blood samples is advantageous, as this biomaterial is more easily accessible. One disadvantage in the detection of CTCs from blood samples is that the number of CTCs obtained is small in comparison to the surrounding white blood cells (20); therefore, highly sensitive methods for detection are required. The primary detection system currently available is Cell Search® System, distributed by Veridex LLC (Raritan, NJ, USA), which is approved by the US Food and Drug Administration for metastatic breast cancer. It is based on an immunomagnetic enrichment of tumor cells with fluorescent staining of tumor-specific cell surface epitopes. However, this method is expensive and laborious.

The current study aimed to identify a method for the detection of CTCs using the quantitative polymerase chain reaction (qPCR) method. This is based on the fact that as the

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Abbreviations: CCNE, cyclin E1; CK19, cytokeratin 19; CLDN-4, claudin-4; CTCs, circulating tumour cells; DEPC, diethylpyrocarbonat; DTCs, disseminated tumour cells; GPER, g-protein coupled estrogen receptor; Her-2, human epithelial growth factor receptor; LHCGR, luteinizing hormone/choriogonadotropin receptor; MAL2, T-cell differentiation protein 2; MGL, mamoglobin; MIG7, migration inducing gene 7; VEGFR, vascular endothelial growth factor receptor

Key words: endometrial adenocarcinoma, quantitative PCR, marker genes, CTC, metastasis, cell lines

primary tumor is of epithelial origin and expresses an epithelial gene panel. CTCs may also express these epithelial genes. Therefore, they may be distinguished from blood cells, which are mesenchymal cells, by gene expression. The advantage of this method is that it is more sensitive, less expensive than other methods in the field and may be performed quickly in nearly every laboratory. However, it is challenging to find suitable marker genes for the detection of CTCs, which are able to distinguish between tumor and blood cells (21-23). Therefore, the expression of a set of genes was compared between cells isolated from healthy endometrium and endometrial adenocarcinoma cell lines. To the best of our knowledge, there are only a few studies describing the presence of CTCs in patients with endometrial adenocarcinoma. In particular, a high-risk group of patients with high-grade endometrial adenocarcinomas (24), demonstrated a correlation of the occurrence of CTCs with stemness was demonstrated in a previous study and CTCs were recognized as possible therapeutic targets (25).

The marker genes used in the present study were cyclin E1 (CCNE), cytokeratin 19 (CK19), claudin 4 (CLDN-4), G-protein coupled estrogen receptor (GPER), human epidermal growth factor receptor 2 (Her-2), luteinizing hormone/choriogonadotropin receptor (LHCGR), T-cell differentiation protein 2 (MAL2), mammaglobin (MGL), migration inducing protein 7 (MIG7) and vascular endothelial growth factor receptor 2 (VEGFR2) (Table I). CCNE is often overexpressed in endometrial adenocarcinomas (26) and has been used in qPCR detection of CTCs from blood samples of different malignant entities. CCNE and MAL2 have been identified as useful markers in endometrial carcinoma tissue (27). MIG7 was described as a marker gene, which often predicts poor prognosis in patients with endometrial adenocarcinoma (28). CK19 is a marker of epithelial cells and is also used in the alkaline phosphatase-anti-alkaline phosphatase test, which is routinely used in cancer diagnosis (29,30). CLDN-4 has been recognized as a biomarker in the treatment of patients with endometrial adenocarcinoma (31). MGL is frequently used as a marker gene for breast malignancies; however, it may have an important function in malignant endometrial tissues (32), and was therefore also selected for the set of marker genes to be tested by the present study. The expression of GPER is dysregulated in malignant endometrium and is involved in steroid hormone signaling (33). Her-2 is particularly prevalent in early endometrial tumorigenesis, in coherence with Cox-1 and -2 (34). VEGFR2 is an important factor in metastasis formation and neoangiogenesis, ensuring blood supply in the newly growing tumor mass (35). LHCGR is also associated with tumor staging (36). It is also important for cell proliferation (37,38) and may be correlated with grading of endometrial carcinomas (39).

Materials and methods

Cell lines and subcultivation. The endometrial adenocarcinoma cell lines AN3CA (HTB-111), HEC-1-A (HTB-112), HEC-1-B (HTB-113), KLE (CRL-1622) and RL-95-2 (CRL-1671) were purchased from American Type Culture Collection (Manassas, VA, USA), Ishikawa (cat. no. 99040201) from European Collection of Authenticated Cell Cultures (Salisbury, UK). All cell lines were established from endometrial carcinoma

patients (see Table II) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom, Ltd., Berlin, Germany), supplemented with 10% fetal calf serum (FCS; Biochrom, Ltd.) and 1% penicillin/streptomycin (P/S) (Biochrom, Ltd.), except KLE, as they required DMEM-F12 (Biochrom, Ltd.), 10% FCS and 1% P/S as culture medium. Cells were subcultured as indicated by the supplier's protocol.

For RNA isolation, cells were washed with phosphate-buffered saline (Biochrom, Ltd.), and TRIzol LS (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added. Cells disrupted by the addition of TRIzol were wiped off the cell culture bottle by a cell scraper (Corning Inc., Corning, NY, USA).

Isolation of endometrial stromal cells from tissue samples. Tissue samples of healthy endometrium were obtained from 10 patients undergoing endometrial examination in scope of fertility and are included in this examination following an unsuspicious pathological result as a negative control group. Patients were informed and had signed consent, following the Declaration of Helsinki (ethical vote LMU 148-12). Samples were maintained in DMEM-F12, 10% FCS and 1% P/S at 4°C overnight or processed immediately. Extraction of stromal cells was performed as described in Fernandez-Shaw *et al* (40) and Zhang *et al* (41). Briefly, tissue was cut in 2-3 mm pieces with a scalpel and incubated with 1 mg/ml collagenase (Invitrogen; Thermo Fisher Scientific, Inc.) in complete DMEM medium for 2 h at 37°C. The suspension was then filtered through 250 µm tissue strainers (Thermo Fisher Scientific, Inc.). The liquid phase was placed onto 40 µm cell strainers (Falcon; Thermo Fisher Scientific, Inc.). Stromal cells were maintained in the liquid phase and spun down at 300 x g for 10 min at 4°C. The supernatant was discarded, and RNA was isolated from the cell pellet by addition of 1 ml TRIzol LS.

RNA isolation. Cell suspensions were already in TRIzol LS, and 0.2X volume of chloroform (Merck Millipore, Darmstadt, Germany) was added for RNA isolation. The cell suspension was then vigorously vortexed and centrifuged at 12,000 x g and 4°C for 15 min. The clear liquid phase was carefully aspirated and transferred into a fresh reaction tube. Isopropanol (0.5 ml; Merck Millipore) was added to each sample, vortexed again and incubated overnight at -20°C.

The next day the suspension was centrifuged at 12,000 x g and 4°C for 10 min, the supernatant discarded and the RNA pellet washed by the addition of 1 ml 75% ethanol (Merck Millipore) and centrifuged at 12,000 x g and 4°C for 10 min. The ethanol was subsequently removed, the pellet air-dried for 15 min and dissolved in diethylpyrocarbonate-treated water. The concentration and ratio of the isolated RNA was determined photometrically at wavelengths 260 and 280 nm. Only RNA with a ratio of 1.7-1.9 is used for further experiments.

Reverse transcription. A total of 4 µg of the isolated RNA in a maximum volume of 6 µl were used for reverse transcription. Reverse transcription was performed using SuperScript III First Strand Synthesis Super Mix kit by Invitrogen (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, 1 µl Oligo-dTs and 1 µl First Strand buffer were added to the RNA and incubated at 65°C for 5 min. Next,

Table I. Characterization of used marker genes and their respective Taq-Man qPCR primers (Thermo Fisher Scientific, Inc.).

Gene	Chromosomal location	Primer cat. no.	Characteriaztion	Function
CCNE	19q12	Hs_00180319_m1	Regulator of CDK2	Overexpression results in chromosome instability
CK19	17q21.2	Hs_00761767_m1	Intermediate filament protein	Structural integrity of epithelial cells
CLDN-4	7q11.23	Hs_00976831_s1	Integral membrane protein	Component of tight junctions
GPER	7p22.3	Hs_00173506_m1	Binds estrogen, important for cellular signaling	Some splice variants are known
Her-2	17q12	Hs_00170433_m1	Proto-oncogene	Overexpression results in development and progression of aggressive cancer types
LHCGR	2q16.3	Hs_00174885_m1	G-protein coupled receptor	Male secondary sexual character development
MAL2	8q24	Hs_00294541_m1	Multispan transmembrane protein	Involved in polarized transport
MGL	11q12.3	Hs_00419570_m1	Belongs to family of secretoglobulins	Involved in cell signalling, immune response and chemotaxis
MIG7	1p22.1	Hs_00706258_m1	Involved in cell signalling	Limited to embryonic/fetal cells and epithelial cancer cells
VEGFR2	4q12	Hs_00911700_m1	Mediator of VEGF-induced endothelial proliferation, survival, migration and morphogenesis	Angiogenesis and vascular development

CCNE, cyclin E1; CK19, cytokeratin 19; CLDN-4, claudin 4; GPER, G-protein coupled estrogen receptor; Her-2, human epidermal growth factor receptor 2; LHCGR, luteinizing hormone/choriogonadotropin receptor; MAL2, T-cell differentiation protein 2; MGL, mammary-globin; MIG7, migration inducing protein 7; VEGFR2, vascular endothelial growth factor receptor 2; CDK2, cyclin-dependent kinase 2; VEGF, vascular endothelial growth factor.

10 μ l 2X Reaction Mix and 2 μ l reverse transcriptase were added and the solution was incubated at 42°C for 50 min. Reverse transcriptase was subsequently heat-inactivated by an incubation at 85°C for 5 min. The cDNA produced was stored at -20°C until it was used in qPCR.

qPCR. For qPCR, 2 μ l of the respective cDNA sample was pipetted into each well of a 96-well plate (Thermo Fisher Scientific, Inc.). A mastermix for each gene was prepared, for the number of samples, which were analyzed. Therefore, for each reaction, 10 μ l reaction mix (Thermo Fisher Scientific, Inc.), 7 μ l water and 1 μ l of the respective gene-specific probe (Table I) were mixed and 18 μ l of this mixture was added to the cDNA, giving a total reaction volume of 20 μ l. The plate was sealed and centrifuged at 315 x g for 1 min. and placed in a qPCR machine. The cycles were run in the following scheme: 20 sec at 95°C as an initial denaturation, followed by 40 cycles consisting of 3 sec at 95°C and 30 sec at 60°C. Fluorescence was determined at the end of each amplification cycle and the relative quantification values (RQ values) were calculated using SDS-software (version 1.3.1) by the $2^{-\Delta\Delta C_q}$ method (42). 18S was used as an internal reference, and gene expression values of endometrial adenocarcinoma cell lines were set in

reference to healthy stromal cells isolated from tissue samples as aforementioned. The reaction assays for each gene and cell line were performed as quadruplicates.

Results

In order to identify a suitable set of marker genes for CTC-detection from blood samples of patients with endometrial adenocarcinoma, qPCR was performed on mRNA/cDNA obtained from 6 human endometrial adenocarcinoma cell lines (AN3 CA, HEC-1-A, HEC-1-B, Ishikawa, KLE, RL95-2) and from healthy endometrial tissue, with 10 different genes, which were previously described in the literature as qPCR marker genes or were associated with endometrial carcinoma and metastasis formation.

RQ values of >1 were deemed to be a indicator of upregulation of gene expression in comparison to the expression levels of the same gene in the reference tissue (healthy endometrium). RQ values <1 indicated that the gene was expressed at a lower level compared with the control sample.

The present study found low RQ values for LHCGR, VEGFR2 and MGL, which in all cell lines have RQ values <1, with the exception of VEGFR2 in KLE-cells, where the RQ

Table II. Endometrial adenocarcinoma cell lines.

First author, year	Cell line	Tumour type	Donor age (years)	Donor ethnicity	Depositor	Cell line characteristics	Refs
Dawe, 1964	AN3CA	Adenocarcinoma	55	Caucasian	CJ Dawe	Isolated from metastatic lesion in lymph nodes	(43)
Kuramoto, 1972	HEC-1-A	Adenocarcinoma	71	Caucasian	H Kuramoto	Cells from well differentiated adenocarcinoma (grade II), expresses platelet activated growth factor and c-fos, tumourigenic in nude mice	(44)
Kuramoto, 1972	HEC-1-B	Adenocarcinoma	71	Caucasian	H Kuramoto	Substrain of HEC-1-A, more flattened growth, tumourigenic in nude mice, diploid to tetraploid	(44)
Lessey, 1996	Ishikawa	Adenocarcinoma	39	Asian	A Taylor	Induces cancer in nude mice, expresses ER and PR	(45)
Hendricks, 1997	KLE	Adenocarcinoma	64	Caucasian	GR Richardson	Tumourigenic in nude mice, cells have microvilli and junctional complexes, no formation of glands observed	(46)
Way, 1983	RL-95-2	Carcinoma	65	Caucasian	DL Way	Expresses ER, possesses α -keratin, cells have junctional complexes and surface microvilli	(47)

value is 4.742. In RL95-2 cells, LHCGR had a very low expression level, and even after 40 cycles of PCR no PCR-product was fluorescently detected, resulting in a not detected (nd) RQ value. The remaining seven genes tested via qPCR had different expression levels in the various cell lines tested. High expression levels were observed for CLDN-4 and CK19, especially in RL95-2 cells (317.51 and 501.911, respectively). This was also evident in the remaining five cell lines; however, not to the same extent. For Her-2, CCNE and MAL2 intermediate expression levels were found. GPER and MIG7 were upregulated in comparison with healthy tissues in the majority of the cell lines investigated, with exception of MIG7 in RL95-2 cells (0.897) and GPER in Ishikawa (0.211) and KLE (nd) (Fig. 1; Table III).

Discussion

In the present study, markedly different gene expression values were observed between the different genes, and also between the cell lines. The differences observed for one gene among the different cell lines to a certain degree reflects the situation, that would occur when using patient samples. For example, the gene expression levels in the HEC-1-A and HEC-1-B cell lines are similar, possibly due to their common origin. By contrast, the Ishikawa cell line has a different gene expression pattern, which is rather different compared with the remaining cell lines. This could be due to it being the only cell line that was obtained from an Asian patient, whereas the rest were from Caucasian patients; therefore, it is possible that the carcinomas developed in different genetic backgrounds. Furthermore, the donor of the Ishikawa cell line was younger, possibly premenopausal, whereas the donors of the other cell lines were older and presumably

postmenopausal, and this may additionally have contributed to the variations in genetic background for tumorigenesis. Therefore, in order to successfully detect CTCs from blood samples of patients with endometrial adenocarcinoma, the primary challenge is to establish a suitable set of marker genes, which would enable the detection of CTCs with a high sensitivity. These results are in accordance with previous findings using breast cancer cell line cells and blood samples from patients with breast cancer for CTC detection (21-23).

CLDN-4 and CK19 were identified to be highly suitable, confirming the recent results of Pan *et al* (31), which used CLDN-4 as a biomarker for endometrial adenocarcinoma. By contrast, CK19 is an established marker gene and has been used in tumor cell diagnostics routinely (29,30). CK19 is a typical epithelial marker, therefore it is not unusual that it is expressed in CTCs. Furthermore, Her-2 and MAL2 may also be suitable marker genes, with high expression levels in the majority of cell lines investigated in the present study. MAL2 was also previously identified as a marker gene in endometrial adenocarcinoma (27). A previous study has determined that the expression levels of CCNE are upregulated in endometrial adenocarcinoma (26). Additionally, as Her2 also exhibits high expression levels in the majority of the cell lines, and may be easily detected by qPCR, it represent a potential marker gene in patient samples, as it has been previously identified as a marker of early tumorigenesis (34).

LHCGR, VEGFR2 and MGL exhibited a consistently lower expression across the tumor cell lines investigated. However, this does not mean that these genes are not important for tumorigenesis and the formation of remote metastasis. It is possible that the mRNAs of those genes are degraded quickly following protein translation and therefore that their expression may not be determined using qPCR. In order to

Table III. Relative quantification values for all genes and cell lines examined.

	AN3 CA	HEC-1-A	HEC-1-B	Ishikawa	KLE	RL95-2
CCNE	0.045	8.938	40.558	15.215	9.807	2.582
CK19	2.716	3.069	40.581	3.305	45.305	501.911
CLDN-4	7.063	0.345	86.115	42.771	11.693	317.510
GPER	10.682	1.002	2.345	0.211	nd	9.547
Her-2	10.095	68.220	59.618	4.491	78.536	5.297
LHCGR	0.005	0.016	0.201	0.018	0.328	nd
MAL2	0.003	58.343	55.510	15.292	30.297	12.726
MGL	0.015	0.010	0.003	0.008	0.005	0.011
MIG7	4.513	4.974	8.131	1.781	7.839	0.897
VEGFR2	0.002	0.003	0.003	0.010	4.742	<0.001

CCNE, cyclin E1; CK19, cytokeratin 19; CLDN-4, Claudin 4; GPER, G-protein coupled estrogen receptor; Her-2, human epidermal growth factor receptor 2; LHCGR, luteinizing hormone/choriogonadotropin receptor; MAL2, T-cell differentiation protein 2; MGL, mammaglobin; MIG7, migration inducing protein 7; VEGFR2, vascular endothelial growth factor receptor 2; nd, not detected.

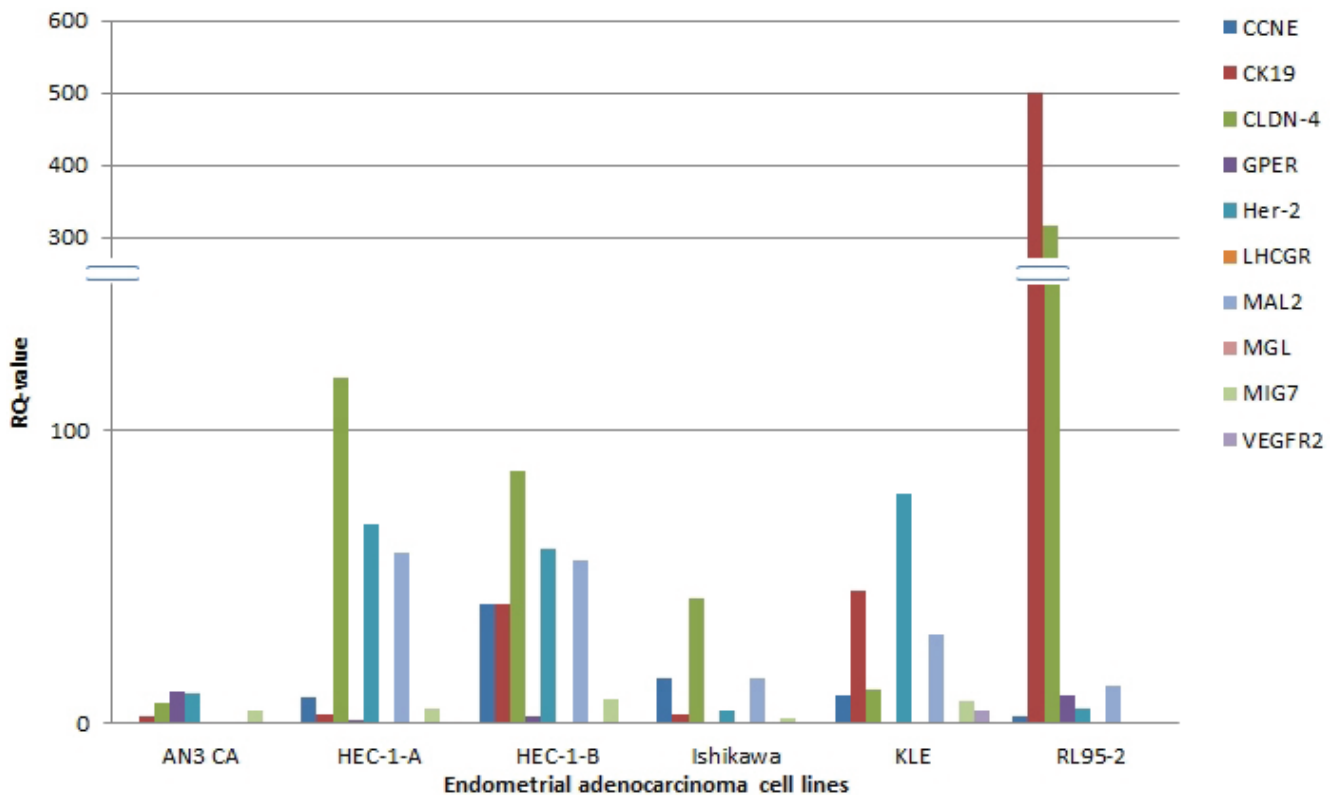


Figure 1. Comparison of the expression of the different genes in various endometrial adenocarcinoma cell lines. CCNE, cyclin E1; CK19, cytokeratin 19; CLDN-4, Claudin 4; GPER, G-protein coupled estrogen receptor; Her-2, human epidermal growth factor receptor 2; LHCGR, luteinizing hormone/choriogonadotropin receptor; MAL2, T-cell differentiation protein 2; MGL, mammaglobin; MIG7, migration inducing protein 7; VEGFR2, vascular endothelial growth factor receptor 2.

overcome this obstacle, a potential approach may be to investigate marker genes with long RNA half-lives, thus increasing the time available for detection.

In conclusion, qPCR is a potential method for the diagnosis of CTCs in endometrial adenocarcinoma. Therefore, it may aid in the refinement of treatment options, and indicate whether a patient has a particular potential for metastasis, as CTCs are present in the blood stream. The present study indicates

a potential set of reliable marker genes which may be used with this methodology, however, further studies are required to confirm and expand upon this. It will be important to clarify whether particular levels of gene expression can be correlated to specific numbers of tumor cells in a blood sample. Therefore standard curves would be required to be generated, using varying quantities of tumor cells diluted in blood samples from healthy individuals, an approach already in use for breast

cancer (23). A potential limitation of the method is that for particular genes additional methods may be required, in order to clarify their expression, as identified by the present study for LHCGR, MGL and VEGFR2. qPCR is a fast, cost-efficient and easy to perform method, which may be conducted in the majority of laboratories, and may be a useful tool for CTC detection in various types of cancer.

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