Endometrial Adenocarcinoma: Analysis of Circulating Tumour Cells by RT-qPCR

ALEXANDRA C. KÖLBL, REBEKKA WELLENS, JULIAN KOCH, BRIGITTE RACK, STEFAN HUTTER, KLAUS FRIESE, UDO JESCHKE and ULRICH ANDERGASSEN

Department of Obstetrics and Gynecology, Ludwig-Maximilians University of Munich, Munich, Germany

Abstract. *Background: Endometrial adenocarcinoma is a frequently occurring cancer in women, accounting for 42,000 deaths every year. Despite treatment with standard therapy, occurrence of remote metastases and local recurrences is high. Through help of RT-qPCR minimal residual disease could be detected and characterized, facilitating therapeutic decision making. Materials and Methods: A number of marker genes were first tested in model systems and genes that performed best, were consequently used for the examination of 13 blood samples from endometrial carcinoma patients. Results: Cytokeratin 19 and MIG7 were chosen for the analysis in patient samples. Both genes were found up-regulated in small tumours and in one large tumour, but no statistical correlations could be revealed between expression levels of these two genes and tumour characteristics. Conclusion: There seems to be a coherence between gene expression and the stage of tumorigenesis, but the number of samples is still too small, to be able to obtain statistical significant differences.*

Endometrial adenocarcinoma is the fourth most frequent cancer entity occurring in women (1). Every year about 11,300 newly diagnosed endometrium carcinomas are registered (2). The risk of sickening from this malignant disease rises by increasing age, most patients are between 75 and 80 years old at primary diagnosis. Although 5-year survival rates are 72% (Europe) and 84% (USA), about 42,000 women die per year from the consequences of this disease. Thereby, the most prominent risk factors are hormone therapies based on estrogen (3), the metabolic syndrome, diabetes mellitus, nulliparity and different former carcinomas (4). Most frequently the first recognizable symptom is the incidence of postmenopausal bleeding (5), as screening and early diagnosis are largely ineffective. Diagnosis can usually only be made histopathologically by analysis of tissue samples from fractionated uterine abrasion (6). Therapy is mostly limited to surgery, followed by radiation in form of a brachy- or teletherapy (7), seldomly a chemotherapeutical concept using platin derivates is applied. After therapy about 25% of patients show up with recurrence or metastasis (8), so that a consequent follow-up care is indispensable (9). Up until now, there is no multiple-line treatment for progression or recurrence of tumour mass (7).

Therefore, the involvement of circulating and disseminated tumour cells as prognostic and predictive factors for disease and therapy outcome would be of great benefit for therapeutic interventions. Circulating tumour cells (CTCs) are single tumour cells, that detach from the primary tumour, invade lymphatic and blood vessels and circulate with liquor stream (10) and are considered as origin of remote metastasis (11). By entering bone marrow, where these tumour cells are able to form tumour reservoirs (12-17), CTCs turn into disseminated tumour cells (DTCs). CTCs have been already discovered as early as 1869 by Thomas Asworth, who found tumour cells in the blood of a person, who died from metastatic cancer (18). Nowadays the occurrence of CTCs and DTCs is already included in international tumour staging systems (19, 20), as their role in epithelial cancer progression and their linkage to worse prognosis had been clarified (10, 21). But still the detection of CTCs is a technical challenge due to the small number of tumour cells in comparison to the surrounding blood cells ($1/10^6$-10^7 blood cells; (22)). The highly sensitive real-time PCR (RT-PCR) could constitute a solution to this problem. The method is based on the fact, that tumour cells, which are of epithelial origin, express a different panel of genes than mesenchymal blood cells. In the on-hand study the even more sensitive method of TaqMan® RT-PCR (23) was used for the analysis of a set of five genes for their benefit in CTC-detection from blood

Correspondence to: Prof. Dr. Udo Jeschke, Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Ludwig-Maximilians-Universität München, Maistrasse 11, 80337 München, Germany. Tel: +89 440054240, Fax: +89 440054916, e-mail: Udo.Jeschke@med.uni-muenchen.de

Key Words: Endometrial adenocarcinoma, circulating tumour cells, real-time PCR, marker genes.

samples of endometrial carcinoma patients. Cytokeratin 20 (CK20) was already described to be expressed in endometrial carcinoma, but not in healthy tissues, and also not in blood cells (24). Cytokeratin 19 (CK19) is generally considered a marker gene for epithelial cells, used also in the APAAP-staining (25, 26). It had already shown relevance for CTC-detection from blood samples of breast cancer patients (27, 28), and its expression is also described in endometrial carcinoma tissue (29). Cyclin E (*CCNE*) is also known to be overexpressed in endometrial adenocarcinomas (30) and had been used already in RT-qPCR detection of CTCs from blood samples of different malignant entities and has been shown to be a useful marker in endometrial carcinoma, as was *MAL2* (31). Furthermore *Mig-7* was described as marker gene for poor prognosis in endometrial carcinoma and had also already been used in RT-qPCR-based detection of CTCs from blood samples of endometrial carcinoma patients (32).

The present study consists of two experimental parts: first, an artificial model system was created by adding cells from endometrial adenocarcinoma cell lines to blood samples of healthy donors. Possible marker genes were tested for their performance in RT-qPCR in this artificial system. In the second part, the genes that seemed to be most suitable for tumour cell detection in the artificial system were subsequently used in RT-qPCR with samples of endometrial adenocarcinoma patients. Their expression was correlated statistically to some tumour characteristics. For this study, five genes were tested as genes that are specific for endometrial cancer. We used different cells from endometrial carcinoma cell lines and added them in different amounts to blood samples. A tumour-specific gene expression is characterized by an increase of gene expression in parallel to an increase of added tumour cells. The most specific genes were used in the analysis of patient samples and marker gene expression was correlated to different tumour characteristics.

Materials and Methods

Cells. Epithelial adenocarcinoma cell lines HEC-1A and RL95-1 (ATCC: HTB-112 and CRL-1671 respectively; ATCC, Wesel, Germany) were cultured according to the distributor´s recommendations. Cells were grown to sub-confluency and fed every third day. For sub-culturing and counting cells had to be detached from the bottom of the cell culture flask by trypsin/EDTA (Biochrom, Berlin, Germany) and ingested in their normal culture media. For the spiking experiments required cell numbers were assembled by diluting the cell solution with PBS (Biochrom, Berlin, Germany). Cells were added to the healthy blood samples after the Trizol-step during RNA-isolation to prevent immunological reactions of the blood cells with the tumour cells. 0, 10, 100 and 1000 cells/ml blood were added to different blood samples.

Blood samples. For the collection of blood sample from endometrial carcinoma patients an ethical vote (LMU 148-12) was on hand, according to the Declaration of Helsinki. Blood was only collected after explaining matters to the patient and obtaining a written consent. The fraction of mononuclear cells, which also contains CTCs, is enriched *via* density gradient centrifugation of the blood sample (33). About 20 ml blood was withdrawn from healthy donors and 13 endometrial carcinoma patients, diluted to about 30 ml with PBS (Biochrom, Berlin, Germany). The diluted blood was carefully layered onto 20 ml Histopaque 1077 (Invitrogen, Darmstadt, Germany) in a Falcon tube and a density gradient centrifugation was carried out at 400 × *g* for 30 min. Then the buffy coat containing lymphocytes, and in case of patient samples also circulating tumour cells, was aspired, transferred to a fresh tube and filled with PBS. Thereby cells were washed during a following centrifugation step at 250 × *g* and 4˚C for 10 min. Supernatant was removed and the harvested cell pellet was air-dried and then kept on –80˚C until further use. For RNA isolation cell pellets were thawn and resuspended in 1 ml Trizol LS (Invitrogen, Darmstadt, Germany). Then 0.2 ml chloroform (Merck, Darmstadt, Germany) were added and suspension was agitated vigorously and centrifugated at 12,000 × *g* and 4˚C for 15 min. After centrifugation three phases could be discerned, of which the clear upper phase containing RNA is carefully aspired and transferred in a fresh tube, 1 ml isopropanol (Merck) was added and the solution was stored at -20˚C overnight. After another centrifugation step at 12,000 × *g*, 4˚C for 10 min supernatant can be carefully removed from the RNA pellet, and pellet is washed in 75% ethanol (Merck) by centrifugation at 12,000 × *g*, 4˚C for 10 min. Then RNA pellet was air dried and resolved in 20 µl DEPC-water. The RNA amount and quality were determined by photometry (Nanodrop, Implen, München, Germany) and denaturing agarose gel electrophoresis, respectively.

For experiments in the artificial system, blood samples from healthy donors were treated as described, only after the Trizol-step, the designated amount of tumour cells (HEC 1A, RL95-2) was added. It is crucial to add the tumour cells at this step of the experimental procedure, to prevent immunologic agglutination of tumour cells with normal blood cells, influencing results of Real-Time qPCR.

Reverse Transcription and RT-PCR. For reverse transcription 4 µg of RNA were interspersed with the reagents from the SuperScript III First Strand Synthesis Super Mix Kit (Invitrogen, Darmstadt, Germany) in the order described in the manual and reaction steps were carried out as stated in the product manual. CDNA samples were then kept on -20˚C until further use.

Two µl of these cDNA are then mixed within the wells of a PCR Plate (MicroAmp® Fast Optical 96-well plate with barcode, ABI, Foster City, CA, USA) with 18 µl of the target gene specific reaction master mix, consisting of 1 µl of gene specific TaqMan-Primer (Hs99999905_m1 for GAPDH, Hs_03003631_g1 for 18S, Hs00761767_m1 for CK19, Hs00300643_m1 for CK20, Hs00180319_m1 for CCNE, Hs00294541_m1 for MAL2, Hs00706258_m1 for Mig7; ABI, Foster City, CA, USA) 10µl TaqMan® Fast Universal PCR Mastermix (ABI, Foster City, CA, USA) and 7 µl distilled water. The plate was sealed with an optical adhesive cover (ABI) and placed into the 7500 Fast Real Time PCR system (ABI). Each gene was analyzed in quadruplicates at RT and water controls were included. 18S and GAPDH were used as internal controls for the reaction. PCR was run in the following cycles: initial denaturation (95˚C for 20 sec), 40 amplification cycles (95˚C, 3 sec; 60˚C, 30 sec). Fluorescence was displayed in the SDS 1.3.1 software.

Table I. *Relative gene expression values of CK19 and Mig7 in comparison to tumour characteristics of 13 endometrial adenocarcinoma patients.*

N	Age (years)	T	N	M	Grading	FIGO	Histology	Menopausal state	*CK19*	*MIG7*
1	57	pT1a	Nx	M0	G1	IA	End. Adenocarc.	Post	0.666	1.238
2	76	pT2	N0	M1	G2-3	IIb	Metast. Adenocarc.	Post	3.041	1.297
3	61	pT1a	N0	M0	G1	IA	End. Adenocarc.	Climacteric	44.902	73.72
4	75	pT2	N0	M1	G2-3	IIb	Metast. Adenocarc.	Post	0.769	0.452
5	54	pT1a	N0	Mx	G1	IA	End. Adenocarc. Recurrence	Post	0.206	0.335
6	32	pT1a	N0	Mx	G2	IA	End. Adenocarc.	Pre	0.003	0.82
7	58	pT2	N2(7/18)	M0	G3	IIIC2	Clear Cell Adenocarc.	Climacteric	1.925	6.134
8	58	pT1a	N0	M0	G1	IA	End. Adenocarc.	Post	168.1	404.10
9	69	pT3a	N1(1/18)	Mx	G3	IIIC	Serous Adenocarc.	Post	0.04	0.026
10	69	pT1a	N0	Mx	G1	IA	End. Adenocarc.	Post	0.666	0.82
11	55	pT4b	pN2(6/8)	M1	G2	IVB	End. Adenocarc.	Climacteric	7969	165.221
12	53	pT1a	pN0	Mx	G1	IA	End. Adenocarc.	Post	16798	215.731
13	65	pT1a	pNx	Mx	G2	IA	End. Adenocarc.	Post	15.692	1.192

Evaluation. Fluorescence intensities were converted to CT, ΔCT, ΔΔCT and RQ-values (RQ-values were generated as described in Livak *et al*. (34)) by the SDS software and could be transferred to Microsoft® Excel™, which was then used to draw respective graphs. Statistical comparison of tumour features and gene expression values was done *via* SPSS V.22.

Results

To save precious patient material, considered marker genes were first analyzed in an artificial system, consisting of blood samples from healthy donors to which a certain amount of HEC 1A and RL95-2 tumour cells are added. For these spiking experiments, both cell lines were used in equal amounts, as a mixture of cell lines has already been shown useful for such experiments in the breast cancer background (28, 35). Unfortunately CK 20 expression could not be detected in these experiments. The other four genes show a more or less strong increase in gene expression by increasing number of tumour cells added to the blood sample (Figure 1). The greatest increase was seen for *Mig7*. Already in the sample of 10 tumour cells added per ml blood an increase in gene expression could be noted, and the expression rises up to 160 in RQ-value in the 100-cells-sample. *CK19* also showed a great boost in the 10 and 100 cells/ml sample, but almost no further increase in gene expression was seen in the 1,000-cells sample. An increase in gene expression for the *CCNE2* gene could be noted in the sample, in which 100 cells were added per ml blood, and the increase for *MAL2* was hardly notable. Therefore, *CK19* and *Mig7* were selected for analysis of patient samples, as increase in gene expression is much lower for the other two genes analyzed.

By comparing gene expression with tumour characteristics (Table I), an interesting fact was found: the highest gene expression values for *CK19* and *Mig7* were found in three pT1a, N0, M0, G1, FIGO IA patients (Patient 3, 8 and 12) and in one patient with advanced disease (patient 11; pT4b, pN2, pM1, G2, FIGO IVB). Besides that finding, no coherence could be found between gene expression and tumour characteristics.

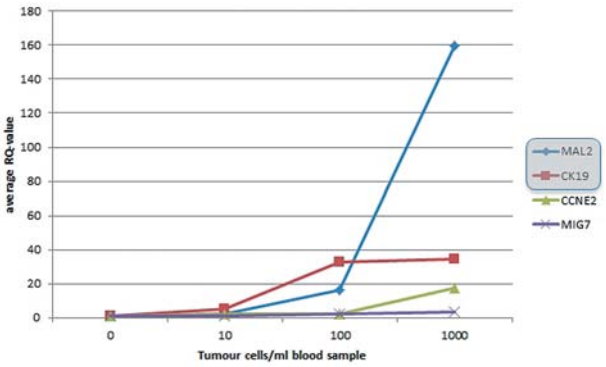

Figure 1. *Increase of relative quantification values as a function of cell number added to blood samples of healthy donors.*

An analysis of gene expression in different patient subgroups concerning patient age at primary diagnosis (*p*-values 0.7206 and 0.1741 for *CK19* and *Mig7* respectively), tumour size (p=0.98922 for *CK19* and 0.6601 for *Mig7*), lymph node affection (p=0.9913 for CK19 and 0.9384 for Mig7), metastatic status (*p*-values: 0.3974, 0.8656 for *CK19* and *Mig7* respectively), grading (p=0.5672 and 0.3524 for *CK19* and *Mig7*) and FIGO stage (*p*-values: 0.9581 for *CK19*, 0.6732 for *Mig7*) did not reveal any statistical significant coherences (Table II).

Table II. *Statistical comparison of gene expression in different patient subgroups. No significant differences were found.*

Feature compared	*CK19* (*p*-value)	*Mig7* (*p*-value)
Patient age (<60 years *vs.* >60 years)	0.7206	0.1741
Tumour size (pT1 *vs.* pT2-4)	0.9892	0.6601
Lymph node affection (N0 *vs.* N1/2)	0.9913	0.9384
Metastatic status (M0 *vs.* M1)	0.3974	0.8656
Grading (G1 *vs.* G2/3)	0.5672	0.3524
FIGO stage (I *vs.* II-IV)	0.9581	0.6732

Discussion

As it has already been described in the literature, 20% of endometrial adenocarcinoma patients have an occurrence of circulating tumour cells in the blood (36), and it has been shown by immunomagnetic/immunofluorescence assays, that they were associated with myometrial invasion and lymph node positivity (37). Therefore, CTCs could be rather useful blood biomarkers for a non-invasive diagnosis of endometrial adenocarcinoma (38) and for the prediction of distant metastasis in patients with endometrial adenocarcinoma, what until now did not succeed with other methods (39-41).

The preliminary experiments of the on-hand study, using blood samples from healthy donors spiked with tumour cell line cells represent a good model system to examine marker genes and their respective RT-PCR primers. *CK20*, which has been described to be a good marker gene (24), did not work in our experiments, probably due to a non-optimal performing primer. Another primer for CK20 should be tested in further experiments. The advantage of this artificial system is that no precious patient material is wasted. For a closer relation of gene expression values and tumour characteristics and to receive statistically more meaningful results, a greater number of patient samples would have to be analyzed. Additionally it would make sense, to have one of the gold-standard methods in CTC-detection, like the CellSearch® system (42, 43) running simultaneously, to be able to state more precise conclusions. Furthermore, more marker genes, like *NUCKS*, which has been shown to be a risk factor for poor prognosis and recurrence in endometrial cancer (44), or estrogen receptor α (*ERα*), which has been identified as a predictive biomarker for endometrial adenocarcinoma by tissue microarrays (45), would help refine analysis, in order to not only detect, but also characterize tumour cells from blood samples, what could then in turn have a prognostic value and influence treatment strategies (46).

Acknowledgements

The Authors thank T. Thormeyer for helping with statistical analysis.

References

1 AGO: Empfehlungen für Diagnostik und Therapie des Endometriumkarzinoms - Aktualisierte Empfehlungen der Kommission Uterus auf Grundlage der S2k Leitlinie (V 1.0, 1.6.2008). AWMF Leitlinien Register Nr 0320/342013.
2 GEKID GdeKiDeV: Cancer in Germany 2005/2006. Incidence and Trends. 2006.
3 Emons G and Heyl W: Hormonal treatment of endometrial cancer. J Cancer Res Clin Oncol *126*: 619-623, 2000.
4 Hecht JL and Mutter GL: Molecular and pathologic aspects of endometrial carcinogenesis. J Clin Oncol *24*: 4783-4791, 2006.
5 Moodley M and Roberts C: Clinical pathway for the evaluation of postmenopausal bleeding with an emphasis on endometrial cancer detection. J Obstet Gynaecol *24*: 736-741, 2004.
6 Marsden DE and Hacker NF: Optimal management of endometrial hyperplasia. Best Pract Res Clin Obstet Gynaecol *15*: 393-405, 2001.
7 Tumorzentrum M: Malignome des Corpus uteri. (PD Dr. Christian Dannecker, Kolben PDM, Kürzl PDR eds.). München Wien New York: W. Zuckerschwerdt Verlag, 2007.
8 Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E and Vergote I: Treatment modalities in endometrial cancer. Curr Opin Oncol *19*: 479-485, 2007.
9 Beckmann K, Iosifidis P, Shorne L, Gilchrist S and Roder D: Effects of variations in hysterectomy status on population coverage by cervical screening. Aust NZ J Public Health *27*: 507-512, 2003.
10 Franken B, de Groot MR, Mastboom WJ, Vermes I, van der Palen J, Tibbe AG and Terstappen LW: Circulating tumor cells, disease recurrence and survival in newly diagnosed breast cancer. Breast Cancer Res *14*: R133, 2012.
11 Pantel K and Brakenhoff RH: Dissecting the metastatic cascade. Nat Rev Cancer *4*: 448-456, 2004.
12 Bragado P, Sosa MS, Keely P, Condeelis J and Aguirre-Ghiso JA: Microenvironments dictating tumor cell dormancy. Recent Results Cancer Res *195*: 25-39, 2012.
13 Diel IJ, Solomayer EF, Costa SD, Gollan C, Goerner R, Wallwiener D, Kaufmann M and Bastert G: Reduction in new metastases in breast cancer with adjuvant clodronate treatment. N Engl J Med *339*: 357-363, 1998.
14 Pantel K and Woelfle U: Micrometastasis in breast cancer and other solid tumors. J Biol Regul Homeost Agents *18*: 120-125, 2004.
15 Riethdorf S and Pantel K: Disseminated tumor cells in bone marrow and circulating tumor cells in blood of breast cancer patients: current state of detection and characterization. Pathobiology *75*: 140-148, 2008.
16 Ring A, Smith IE and Dowsett M: Circulating tumour cells in breast cancer. Lancet Oncol *5*: 79-88, 2004.
17 Smerage JB and Hayes DF: The measurement and therapeutic implications of circulating tumour cells in breast cancer. Br J Cancer *94*: 8-12, 2006.
18 Asworth T: A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Med J Aust *14*: 146-147, 1869.
19 Hermanek P, Sobin LH and Wittekind C: How to improve the present TNM staging system. Cancer *86*: 2189-2191, 1999.
20 Singletary SE, Patel-Parekh L and Bland KI: Treatment trends in early-stage invasive lobular carcinoma: a report from the National Cancer Data Base. Ann Surg *242*: 281-289, 2005.

21 Graves H and Czerniecki BJ: Circulating tumor cells in breast cancer patients: an evolving role in patient prognosis and disease progression. Patholog Res Int *2011*: 621090, 2011.
22 Ghossein RA, Bhattacharya S and Rosai J: Molecular detection of micrometastases and circulating tumor cells in solid tumors. Clin Cancer Res 5: 1950-1960, 1999.
23 Holland PM, Abramson RD, Watson R and Gelfand DH: Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci USA *88*: 7276-7280, 1991.
24 Klein A, Fishman A, Zemer R, Zimlichman S and Altaras MM: Detection of tumor circulating cells by cytokeratin 20 in the blood of patients with endometrial carcinoma. Gynecol Oncol *78*: 352-355, 2000.
25 Kurec AS, Baltrucki L, Mason DY and Davey FR: Use of the APAAP method in the classification and diagnosis of hematologic disorders. Clin Lab Med *8*: 223-236, 1988.
26 Noack F, Schmitt M, Bauer J, Helmecke D, Kruger W, Thorban S, Sandherr M, Kuhn W, Graeff H and Harbeck N: A new approach to phenotyping disseminated tumor cells: methodological advances and clinical implications. Int J Biol Markers *15*: 100-104, 2000.
27 Andergassen U, Hofmann S, Kolbl AC, Schindlbeck C, Neugebauer J, Hutter S, Engelstadter V, Ilmer M, Friese K and Jeschke U: Detection of Tumor Cell-Specific mRNA in the Peripheral Blood of Patients with Breast Cancer— Evaluation of Several Markers with Real-Time Reverse Transcription-PCR. Int J Mol Sci *14*: 1093-1104, 2013.
28 Zebisch M, Kolbl AC, Schindlbeck C, Neugebauer J, Heublein S, Ilmer M, Rack B, Friese K, Jeschke U and Andergassen U: Quantification of breast cancer cells in peripheral blood samples by real-time rt-PCR. Anticancer Res *32*: 5387-5391, 2012.
29 Stewart CJ, Crook ML, Lacey J and Louwen K: Cytokeratin 19 expression in normal endometrium and in low-grade endometrioid adenocarcinoma of the endometrium. Int J Gynecol Pathol *30*: 484-491, 2011.
30 Cassia R, Moreno-Bueno G, Rodriguez-Perales S, Hardisson D, Cigudosa JC and Palacios J: Cyclin E gene (CCNE) amplification and hCDC4 mutations in endometrial carcinoma. J Pathol *201*: 589-595, 2003.
31 Obermayr E, Sanchez-Cabo F, Tea MK, Singer CF, Krainer M, Fischer MB, Sehouli J, Reinthaller A, Horvat R, Heinze G, Tong D and Zeillinger R: Assessment of a six gene panel for the molecular detection of circulating tumor cells in the blood of female cancer patients. BMC Cancer 10: 666, 2010.
32 Phillips TM and Lindsey JS: Carcinoma cell-specific Mig-7: a new potential marker for circulating and migrating cancer cells. Oncol Rep *13*: 37-44, 2005.
33 Andergassen U, Kolbl AC, Hutter S, Friese K and Jeschke U: Detection of Circulating Tumour Cells from Blood of Breast Cancer Patients *via* RT-qPCR. Cancers *5*: 1212-1220, 2013.
34 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. Methods *25*: 402-408, 2001.
35 Zebisch M, Kölbl A, Andergassen U, Hutter S, Neugebauer J, Engelstädter V, Günthner-Biller M, Jeschke U and Friese K: Detection of circulating tumour cells on mRNA levels with established breast cancer cell lines. Biomed Rep *1(2)*: 231-234, 2013.
36 Alonso-Alconada L, Muinelo-Romay L, Madissoo K, Diaz-Lopez A, Krakstad C, Trovik J, Wik E, Hapangama D, Coenegrachts L, Cano A, Gil-Moreno A, Chiva L, Cueva J, Vieito M, Ortega E, Mariscal J, Colas E, Castellvi J, Cusido M, Dolcet X, Nijman HW, Bosse T, Green JA, Romano A, Reventos J, Lopez-Lopez R, Salvesen HB, Amant F, Matias-Guiu X, Moreno-Bueno G, Abal M and Consortium E: Molecular profiling of circulating tumor cells links plasticity to the metastatic process in endometrial cancer. Mol Cancer *13*: 223, 2014.
37 Bogani G, Liu MC, Dowdy SC, Cliby WA, Kerr SE, Kalli KR, Kipp BR, Halling KC, Campion MB and Mariani A: Detection of circulating tumor cells in high-risk endometrial cancer. Anticancer Res *35*: 683-687, 2015.
38 Nisenblat V, Bossuyt PM, Shaikh R, Farquhar C, Jordan V, Scheffers CS, Mol BW, Johnson N and Hull ML: Blood biomarkers for the non-invasive diagnosis of endometriosis. Cochrane Database Syst Rev *5*: CD012179, 2016.
39 Koskas M, Fournier M, Vanderstraeten A, Walker F, Timmerman D, Vergote I and Amant F: Evaluation of models to predict lymph node metastasis in endometrial cancer: A multicentre study. Eur J Cancer *61*: 52-60, 2016.
40 Koskas M, Huchon C and Amant F: Characteristics and prognosis of patients with early-stage endometrial cancer who refuse adjuvant radiotherapy. Gynecol Oncol, 2016. doi: 10.1016/j.ygyno.2016.03.016. [Epub ahead of print].
41 Koskas M, Uzan J, Vanderstraeten A, Vergote I and Amant F: External validation of non-imaging models for predicting distant metastasis in patients with endometrial cancer. Gynecol Oncol, 2016. doi: 10.1016/j.ygyno.2016.05.008. [Epub ahead of print]
42 LLC: Veridex Cell Search Web site, 2010.
43 Muller V, Riethdorf S, Rack B, Janni W, Fasching PA, Solomayer E, Aktas B, Kasimir-Bauer S, Pantel K and Fehm T: Prognostic impact of circulating tumor cells assessed with the CellSearch System and AdnaTest Breast in metastatic breast cancer patients: the DETECT study. Breast Cancer Res *14*: R118, 2012.
44 Liu T, Tan S, Xu Y, Meng F, Yang C and Lou G: Increased NUCKS expression is a risk factor for poor prognosis and recurrence in endometrial cancer. Am J Cancer Res *5*: 3659-3667, 2015.
45 Backes FJ, Walker CJ, Goodfellow PJ, Hade EM, Agarwal G, Mutch D, Cohn DE and Suarez AA: Estrogen receptor-alpha as a predictive biomarker in endometrioid endometrial cancer. Gynecol Oncol *141*: 312-317, 2016.
46 Lester-Coll NH, Park HS, Rutter CE, Corso CD, Young MR, Ratner ES, Litkouhi B, Decker RH, Yu JB and Damast S: Who benefits from chemoradiation in stage III-IVA endometrial cancer? An analysis of the National Cancer Data Base. Gynecol Oncol, 2016. doi: 10.1016/j.ygyno.2016.04.544. [Epub ahead of print].

Received April 18, 2016
Revised May 20, 2016
Accepted May 23, 2016