Circulating Rare Cells Enable Highly Efficient Cancer Detection

Eva Obermayr[1,2], Elisabeth Maritschnegg[1], Paul Speiser[1], Christian Singer[1], Eva Schuster[1], Barbara Holzer[1], Sabine Danzinger[1], Nina Pecha[1,2], Andrew Newland[3], Michael O'Brien[3], Robert Zeillinger[1,2]

[1] Department of Obstetrics and Gynecology, Medical University of Vienna, Vienna (AT)
[2] Ludwig Boltzmann Gesellschaft - Cluster Translational Oncology, Vienna (AT)
[3] ANGLE plc, Guildford, Surrey GU2 7YD (UK)

Abstract # LB-197
Poster presented at the American Association for Cancer Research Annual Meeting | Philadelphia, PA 2015

Abstract

We intended to develop a protocol combining the novel micro-fluidic Parsortix technology (ANGLE plc.) and qPCR for the molecular analysis of CTCs.

Recently we identified CTC-specific mRNA markers allowing CTC detection in 29% of breast cancer (BC) patients[1] and in 24.5% of ovarian cancer (OC) patients at diagnosis[2].

However, the detection of cancer cells was hampered by the large number of contaminating leukocytes from existing CTC enrichment technologies, which provide only low purity. By improving the purity of cancer cells and PCR analysis we sought to increase both sensitivity and specificity of the diagnostic procedure.

Following a technical validation phase, we chose 7 out of 30 pre-selected RNA markers as candidates for the detection of CTCs in cancer patients (OC: n=24, cervical cancer CC: n=6, endometrial cancer EC: n=5, BC: n=7). A total of n=42 cancer patients and n=23 controls (healthy normal volunteers HNV) were studied. In OC patients, the resulting detection rates of CTC-related transcripts were significantly higher (at diagnosis: 80%, at relapse: 78%) than in our previous studies[1,2]. Likewise, we observed 71% mRNA-positive cases in metastatic BC patients, which is more than twice as high as in earlier studies using molecular[1] or immuno-magnetic CTC detection[3]. We identified mRNA-positive cases in other cancer types of the female reproductive tract as well (EC: 75%, CC: 33%); however, the sample size was small.

The addition of the remaining 23 RNA markers identified an increased level of 92% of the cancer patients (n=13) and 100% of the OC patients (n=7) correctly, utilising a cut-off threshold value to retain 100% specificity even though there were detectable gene expression levels of some of these markers (e.g. vimentin) in leukocytes.

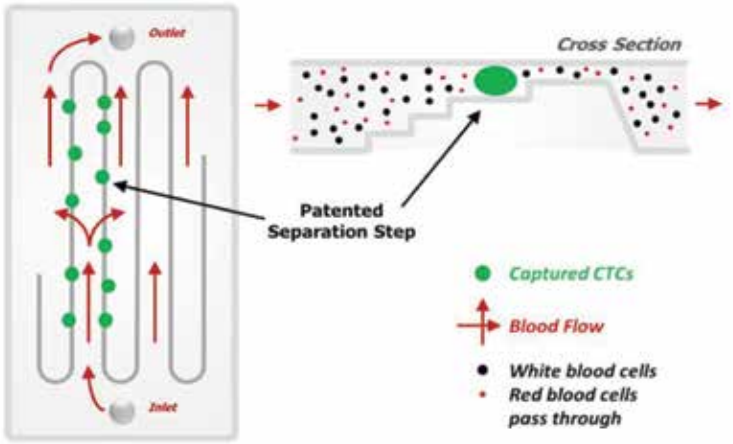
Finally, our sensitive approach correctly identified that an asymptomatic woman at high risk of developing OC/BC was already affected by the disease, when conventional diagnosis (ultrasound, serum tumor marker) failed to detect the disease.

Thus, we have taken a major step forward by combining a novel micro-fluidic cell enrichment and molecular analysis, which will allow the implementation of 'liquid biopsies' in cancer detection studies and as a companion diagnostic in clinical trials.

The Parsortix Technology

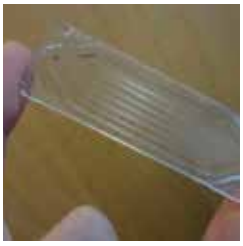
The Parsortix system from ANGLE uses a patented micro-fluidic technology to isolate rare cells (e.g. CTCs) based on their less deformable nature and larger size compared to other blood components.

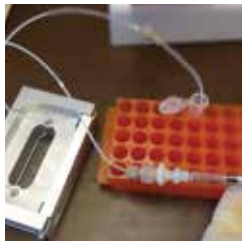
The system allows the identification of the captured cells within the device by in-cassette immuno- staining, but has also been developed to allow a release (harvest) of the captured cells.



CTCs are caught on a 10µm-step that criss-crosses the microscope slide sized disposable cassette. Blood cells pass through and are washed away by rinsing the cassette with buffer.

The cassette is inserted into a clamp which is connected to the device. Whole blood or pre-enriched blood cell fractions are pumped through the disposable cassette. After the separation procedure, staining reagents can be pumped through the cassette in a semi-automated way. Alternatively, lysis buffer can be injected into the cassette to retrieve the enriched sample with high yield for subsequent gene expression analysis.





Technical Validation

The Parsortix device captures small tumor cells from up to 20ml blood

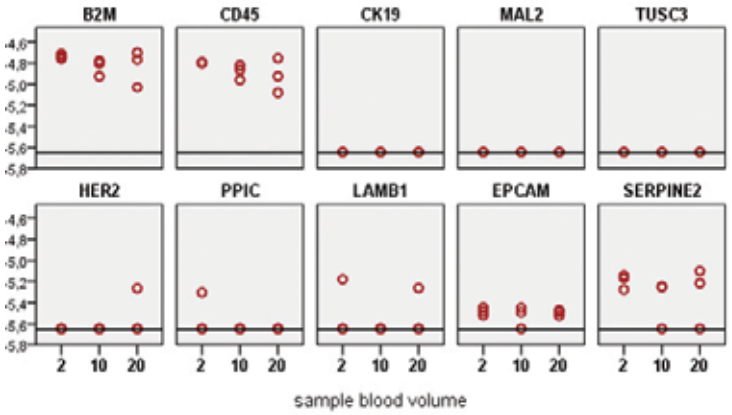
cell line	ml blood	# cells spiked	# cells captured	capture rate
TOV21G	2	10	2	20 %
	2	20	12	60 %
	2	~200	47	24 %
	2	466	40	9 %
CaOV3	2	10	8	80 %
	2	20	14	70 %
	2	188	123	65 %

The efficiency of the Parsortix technology to capture tumor cells had been evaluated using breast cancer cell lines with a mean diameter of >15µm. As cultivated ovarian cancer cells are much smaller in size (~11.5µm), we performed spike-in experiments to assess the ability of the Parsortix device to capture small tumor cells as well.

cell line	ml blood	# cells spiked	# cells captured	capture rate
TOV21G	2	10	-	2
	10	50	-	7/8
	20	100	yes	6/23/33
CaOV3	2	10	-	8
	10	50	-	15
	20	100	yes	5/9/11/13/7

As the number of CTCs in ovarian cancer can be extremely low, we tested the feasibility of the Parsortix technology to process up to 20ml blood. In order to speed up the processing time, 10ml blood samples were run at increased flow rates, and 20ml blood samples were pre-enriched using density gradient centrifugation. Although these adaptions of the separation procedure led to cell loss, the absolute number of captured cells can be increased by starting from a larger sample volume.

Leukocytes are sufficiently depleted for down-stream analysis of CTCs



Pre-enrichment of 20ml blood using density gradient centrifugation

We investigated whether the Parsortix technology was appropriate for subsequent qPCR analysis of the enriched sample. Blood samples from healthy donors were processed using the Parsortix device. To evaluate whether the amount of residual leukocytes was dependent on the volume of blood processed, we started from 2ml, 10ml, and from a cell fraction, which had been pre-enriched from 20ml blood with density gradient centrifugation. The gene expression levels of B2M, leukocyte-specific CD45, and of published CTC markers was analysed using qPCR.

The number of residual leukocytes was independent from the volume of blood processed, and the gene expression background level of the CTC markers investigated was substantially reduced.

Pilot Study

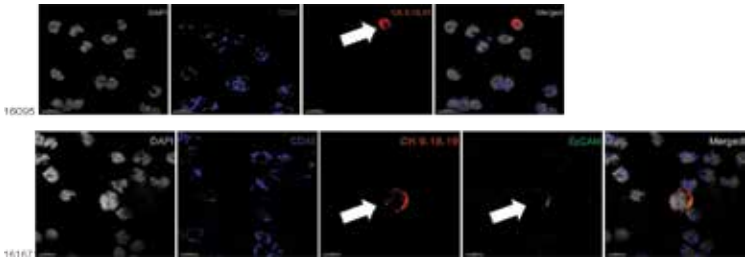
Parsortix enabled an accurate estimate of CTC presence in cancer patients

The analysis of an initial 7-gene set (EpCAM, PPIC, TUSC3, EMP2, LAMB1, FN1, and MAL2) showed negligible background in healthy donor blood. The cut-off threshold values were defined to provide 100% specificity. There were no false positives for n=23 HNV controls. This is a dramatic improvement over all existing diagnostic approaches.

By combining qPCR analysis with the 7-gene set and Parsortix enrichment we achieved substantially higher detection rates of CTC-related transcripts in OC as compared to our previous studies (80% and 78% vs. 24%)[2]. Even in other cancer types, like endometrial and cervical cancer, which are diagnosed at less advanced stages in general, we observed a large number of positive cases, although the small sample size may impair the significance of this finding. In contrast to OC, BC primarily spreads via the hematogenous route and thus is one of the most studied cancer types in the CTC field. Nevertheless, standard CTC diagnostic which relies on the expression of epithelial markers (e.g. EpCAM) detected CTCs in just about 40% metastatic BC patients[3]. By combining the extremely sensitive qPCR with the label-free Parsortix enrichment, we found as much as 71% mRNA-positive BC patients.

qPCR-positive blood samples from cancer patients

Type of disease	Newly diagnosed	Relapse/progression
Ovarian cancer	12/15 (80%)	7/9 (78%)
Endometrial cancer	3/4 (75%)	0/1 (0%)
Cervical cancer	2/6 (33%)	-
Breast cancer	-	5/7 (71%)



20ml blood samples were pre-enriched using density gradient centrifugation (Oncoquick d=1,065 g/ml, Greiner Bio-One). The pre-enriched cell fractions were passed through the Parsortix separation cassettes. The captured cells were lysed within the cassette. Total RNA was extracted from the lysates (Rneasy Micro Kit, Quiagen) and reverse transcribed into cDNA (M-MLV RT, Promega). We performed gene specific pre-amplification (TaqMan pre-Amp Master Mix, Applied Biosystems) and qPCR on up to 30 putative CTC markers (VIAA-7, Applied Biosystems). The presence of CTCs was confirmed by multi-marker staining using antibodies to CKs and EpCAM. Counterstaining was performed using anti-CD45 and nuclear staining with DAPI.

A multi-marker approach to further increase sensitivity

The gene-specific pre-amplification allows the analysis of up to 100 gene markers. In a small set of Parsortix-enriched blood samples taken from cancer patients (n=13) and healthy donors (n=9), we measured the gene expression levels of a total of 30 gene markers. As some of these additional markers (e.g. vimentin) are expressed in leukocytes as well, we normalized the resulting Cq values to CD45 mRNA levels as a measure of leukocyte contamination, and chose the maximum ΔCq in healthy donor samples as the cut-off value. Utilising the 30 gene markers, with the threshold set for 100% specificity, we achieved exceptionally high sensitivity with 92% of the cancer patients classified correctly by this multi-marker analysis. It was notable that for ovarian cancer, both primary and relapse, (n=7) 100% sensitivity was achieved and all cancer patients were correctly classified.

Parsortix enrichment allows for the sensitive detection of rare cells

Utilising the exceptional sensitivity of the approach, we were able to measure increased gene expression levels in the blood of an asymptomatic woman at high risk for ovarian cancer.

A 20ml blood sample taken from a BRCA1/2 mutation carrier before undergoing risk-reducing salpingooophorectomy (rrBSO)

was analyzed using Parsortix/qPCR system for the presence of CTC-related markers. Neither elevated levels of the tumor marker CA-125 nor abnormal ultrasound indicated the presence of a suspicious tumor mass. Nevertheless, the patient tested positive with the Parsortix/qPCR system and was subsequently diagnosed with OC during rrBSO. In addition, the presence of cancer cells was further confirmed in the patient's uterine lavage sample by mutation-specific digital PCR. The Parsortix/qPCR system was successful in detecting ovarian cancer where all existing diagnostic procedures failed.

Conclusions and Outlook

- Molecular methods provide not only an extremely sensitive technology for detecting rare events, but also allow high-throughput analysis, quantitative results, and user- independency.
- The Parsortix technology contributes to the unprecedented specificity of the overall approach by providing a high purity CTC sample. Parsortix is a label-free technology, and as such may become the gold standard for the unbiased enrichment of CTCs independent from their epithelial phenotype.
- By combining the Parsortix technology with qPCR analysis, we achieved an unprecedented high detection rate of CTC-related transcripts, even in early stage patients, when conventional diagnostic methods failed.
- Further technical improvements will be made in order to establish a robust methodology for rare cell detection.
- We plan to validate our results in a large cohort of ovarian cancer patients provided by a multi-center study[4]. The value of CTCs detected by Parsortix/qPCR to monitor responsiveness to a new drug will be evaluated.
- We will investigate the role of CTCs for the differential diagnosis of ovarian cancer versus benign ovarian diseases. CTC diagnosis may help to decide which patients will benefit from treatment in specialized cancer centers.

The retrieval of a high purity CTC population from a large blood volume may advance the implementation of CTCs as liquid biopsies and as a companion diagnostic tool in clinical trials.

References

1. Obermayr E, et al. (2010). 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.
2. Obermayr E, et al. (2013). Molecular characterization of circulating tumor cells in patients with ovarian cancer improves their prognostic significance - A study of the OVCAD consortium. Gynecologic Oncology 128 (1): 15-21.
3. Miller MC, et al. (2010). Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. Journal of Oncology 2010: 617421.
4. www.gannet53.eu

ANGLE

ANGLE NORTH AMERICA INC.
3711 Market Street
University City Science Center, 8th Floor
Philadelphia, PA 19104 USA

T: 215-966-6240
E: enquiries@angleplc.com

ANGLE EUROPE LTD.
3 Fredrick Sanger Road
Surrey Research Park
Guildford, Surrey GU2 7YD United Kingdom

T: +44 (0) 1483 685830
E: enquiries@angleplc.com

www.angleplc.com

PR1-SD-B 2016-005