Circulating rare cells enable highly efficient cancer detection

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Abstract

We intended to develop a protocol combining the novel micro-fluidic Parsortix technology (ANGEL 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) patients2 and in 24% of ovarian cancer (OC) patients with no malignancy diagnosis1. 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 studies1,2. Likewise, we observed 71% mRNA-positive cases in metastatic BC patients, which is more than twice as high as in earlier studies using molecular1 or immuno-magnetic CTC detection2,3. 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 ANGEL 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 immunostaining, but has also been developed to allow a release (harvest) of the captured cells.

The system is based on a unique design that combines the efficiency of enrichment and enrichment with a release system. The cells are separated from the sample by centrifugation. The captured cells are then released using a release buffer. The released cells can then be analyzed immediately or stored for further analysis.

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

- The efficiency of the Parsortix technology to capture tumor cells has been evaluated using breast cancer cell lines with a mean diameter of 10μm. As captured cancer cells are much smaller in size (11-15μm), we performed side-by-side experiments to assess the ability of the Parsortix device to capture small tumor cells as well.

Leukocytes are sufficiently depleted for downstream analysis of CTCs

- We investigated whether the Parsortix device and qPCR analysis of the enriched sample. Blood samples from healthy donors were obtained using the Parsortix device. To evaluate whether the amount of residual leukocytes was dependent on the volume of blood processed, we started from 0.5, 1, 2, and 4 ml of blood, and from a cell fraction, which had been pre-enriched from 10ml blood with density gradient centrifugation. The gene expression levels of BEAM, leukocyte-specific CD45, and the total number of leukocytes spiked into the qPCR-positive CTC markers were assessed using qPCR.

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 screening. 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 study4. The value of CTCs detected by Parsortix/qPCR to monitor responsiveness to 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.

Parsortix enabled an accurate estimate of CTC presence in cancer patients

The analysis of an initial 7-gene set (EpCAM, PPI, TUSC3, EMP2, LAMB1, FN1, and MAL2) showed negligible variability in healthy donor blood. The analysis of this background level of the CTC markers investigated was just 0/1 (0%) for rrBSO. When comparing these results with clinical analysis, the specificity of recurrent disease was defined to provide 100% specificity. There were no false positives for rrBSO 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. 20% and 35% 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 patients1. By combining the extremely sensitive qPCR with the label-free Parsortix enrichment, we found as much as 71% mRNA-positive BC patients.

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 Cq value in healthy donor samples as the cut-off value. Utilising the 30 gene markers, we observed that the threshold sets 100% of 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.

4-20ml blood samples from BC patients were analyzed following enrichment using the Parsortix technology. The enriched fractions of CTC were then analyzed for CTC gene expression using qPCR. The results demonstrated that the presence of CTCs can be detected in all study patients. The detection of CTCs was confirmed by multi-marker analysis using antibodies to EpCAM and CD45. Counting was performed using anti-CD45 and nuclei staining with DAPI.

References

4. www.gynonc.eu