REAL-TIME MULTIPLEX RT-PCR ON CIRCULATING TUMOR CELLS

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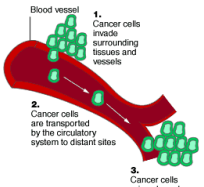
INTRODUCTION



Circulating tumor cells (CTCs) are cancer cells that have detached from solid tumors and entered the blood stream. This can begin the process of metastasis, the most life-threatening aspect of cancer.

Due to the heterogeneity between the primary tumor and metastatic biopsies, characterization of metastases in stead of the primary tumor are preferred to predict patient outcome. But taking biopsies from metastases, if detectable, are cumbersome.

However, to metastasize, CTCs have to travel through the blood before they can take root in another tissue or organ.

The presence of > 5 CTCs in 7.5 ml whole blood was found to predict clinical outcome in metastatic breast cancer (Cristofanilli et al., NJEM, 2004).

Besides quantification of CTCs, characterization of CTCs by gene expression profiling is likely to improve outcome prediction in breast cancer as this may yield better insight into mechanisms underlying dissemination and drug sensitivity.

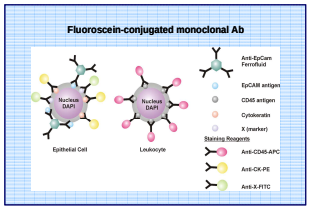
Since one human cell contains 5-10 pg total RNA and traditional real-time RT-PCR requires 5-20 ng RNA for gene analysis, a pre-amplification step is required to analyze expression of multiple genes.

AIM

To establish a method to perform mRNA expression analysis on as little as 5 CTCs isolated from 7.5 ml whole blood.

For this, three linear pre-amplification methods were compared with respect to homogeneous amplification of the starting material, amplification linearity and sensitivity.

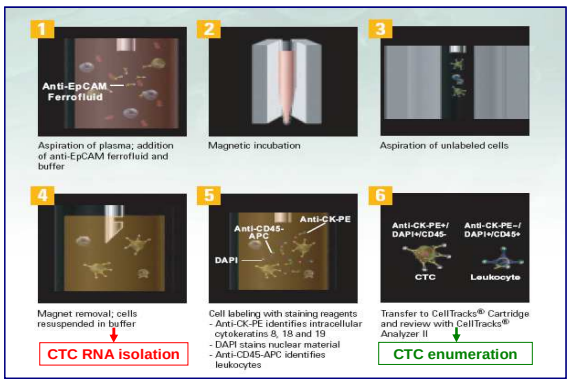
STUDY DESIGN-1: isolation and quantification of CTCs



The CellSearch System (Veridex LLC,Raritan, NJ) was used for the isolation and enumeration of CTC. The system consisted of a semiautomated sample preparation system and the CellSearch Epithelial Cell kit to immunomagnetically enrich cells expressing the epithelial cell adhesion molecule EpCAM (figure 1 to 4).

Isolated cells were then fluorescently labeled with the nucleic acid dye DAPI and labeled monoclonal antibodies specific for leukocytes (CD45) and epithelial cells (cytokeratin 8,18,19-phycoerythrin) (figure 4 and 5).

Identification and enumeration of CTCs was done using the CellSpotter Analyzer (Immunicon), a semiautomated fluorescence microscopy system that permits computer-generated reconstruction of cellular images. CTCs are defined as nucleated cells lacking CD45 and expressing cytokeratin (figure 6).



STUDY DESIGN-2: characterization of CTCs

Two to 20 human breast cancer cells were spiked in 7.5 ml whole blood of healthy donors. Using the CTC kit (CellSearch™), cells that attached to ferrofluids coated with anti-EpCAM Moab were immunomagnetically separated and used for RNA isolation, cDNA synthesis, and subsequent real time mRNA expression analysis before and after pre-amplification.

For this, a selected pilot set of 32 genes was used (see Table 1).

Table 1: Selected pilot gene list

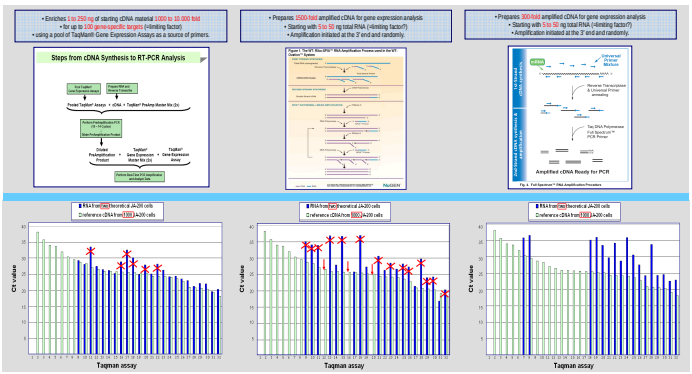
Gene name	Description
control genes for sample loading & RNA quality	
HMBS,	hydroxymethylbilane synthase
GUSB,	glucuronidase, beta
RPL13A,	ribosomal protein L13a
HPRT	hypoxanthine guanine phosphoribosyl transferase
ACTIN	beta-actin
control genes for EpCAM & keratin expression	
TACSTD1	tumor-associated calcium signal transducer 1
TACSTD2	tumor-associated calcium signal transducer 2
KRT19	keratin 19
KRT20	keratin 20
control genes for leucocyte background	
PTPRC (CD45)	protein tyrosine phosphatase, receptor type, C
BST1	bone marrow stromal cell antigen 1
Prognosis related target genes	
MUC1 (EMA)	mucin 1, transmembrane
SCGB2A2 (MGB1)	secretoglobin, family 2A, member 2
ki67	antigen identified by monoclonal antibody Ki-67
EGFR	epidermal growth factor receptor
ERBB2	HER2/NEU
ESR1	estrogen receptor 1
ESR2	estrogen receptor 2
TFF1 (pS2)	trefoil factor 1
TFF3	trefoil factor 3
MET	hepatocyte growth factor receptor
MAGEA3	melanoma antigen family A, 3
CGB	chorionic gonadotropin, beta polypeptide
MDM2	transformed 3T3 cell double minute 2
CXCL1	chemokine (C-X-C motif) ligand 1
SPDEF	SAM pointed domain with ets transcription factor
ALDH1A1	aldehyde dehydrogenase 1 family, member A1
TMSB10	thymosin, beta 10
TWIST	twist homolog 1
TERT	telomerase reverse transcriptase
SERPINB5	serpin peptidase inhibitor, clade B (ovalbumin), 5
GALGT	UDP-N-acetyl-alpha-D-galactosamine: (N-acetylneuraminyl)-galactosylglucosylceramide N-acetylgalactosaminyltransferase (GalNAc-T)

RESULTS-1a: comparing performances of 3 different pre-amplification methods

1: Pre-amp gene-specific amplification method Early Access (Applied BioSystems)

2: Whole Transcriptome Ovation RNA amplification (NuGEN)

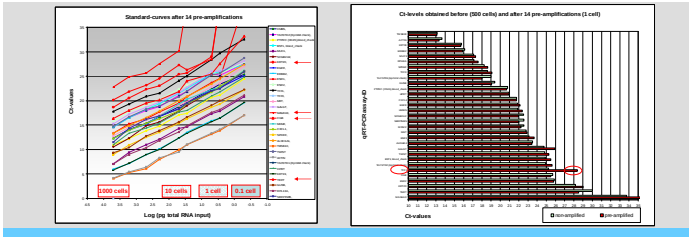
3: Full spectrum RNA amplification (System Bioscience)



RESULTS-1b: performance pre-amp method ABI further evaluated

Pre-amp ABI: Linearity and range

Pre-amp ABI: Homogeneous amplification



Resume performance amplification methods:

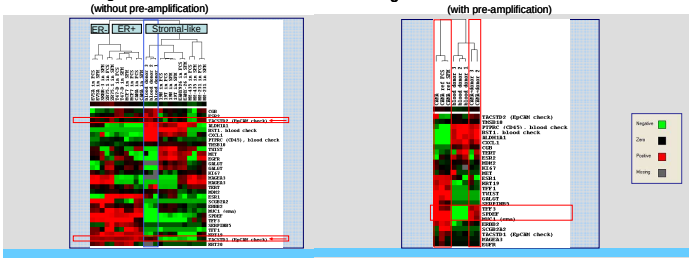
Reliability with respect to conservation of transcript abundance must be checked beforehand for each individual qPCR assay.

The pre-amp method from ABI was superior with respect to homogeneous amplification of small amounts of starting material and ease of protocol. But ABI's pre-amp method appears only reliable for assays that require less then 30 qPCR cycli, thus for the more abundant transcripts.

RESULTS-2: gene expression profiling of cell lines, whole blood, and 5 tumor cells spiked in whole blood

Profiling of cell lines and whole blood (without pre-amplification)

Profiling 5 CAMA-1 cells in 7.5 ml whole blood (with pre-amplification)



Unsupervised two-dimensional hierarchical clustering analysis of breast cells, healthy blood donors and 5 spiked tumor cells.

Each horizontal row represents a gene, and each vertical column corresponds to a sample. Red or green color indicates a transcription level above or below the geometric average expression of the genes across all samples. Green = negative (relatively low expression), red = positive (relatively high expression), black = zero (in-between expression), grey = not done (missing).

Left panel: Gene expression was assessed by qRT-PCR in RNA isolated from 3 different healthy control blood donors and 11 cell lines with different characteristics cultured in complete growth medium (SFM) or in serum-free medium (SFM). 19N = primary fibroblast strain derived from normal breast tissue, 19T = primary fibroblast strain derived from tumor tissue of the same patient, EAHY-926 = immortalized endothelial cell line).

Note that with this pilot set of marker genes the stromal-like cultures (19N, 19T, MM-231, MM-435, EAHy-926) with low or no keratin 19 expression cluster together with the blood donors and are separated from the more epithelial-like TACSTD1 (EpCAM) positive cultures. Also the ER-positive cultures (ZR75.1, T47-D, MCF7 and CAMA) cluster together.

Right panel: Gene expression was assessed by qRT-PCR in 3 different healthy control blood donors before and after spiking with 5 CAMA-1 cells. (refs CAMA-1 in FCS and SFM: n=1000 cells each without pre-amplification, other conditions assessed after pre-amplification).

Note that the pre-amplified CAMA-1 cells cluster together with their non-pre-amplified sister cells. Since in various experiments qRT-PCR data from pre-amplified RNA from 5 tumor cells showed a highly similar expression pattern compared with non-amplified RNA from 1000 tumor cells, pre-amplification did not compromise gene expression profiling.

Although the samples spiked with a few CAMA-1 cells cluster with the blood donors, they are with this pilot set of marker genes already separated without leucocyte specific depletion.

DISCUSSION AND CONCLUSION

This study shows the feasibility of multiple gene expression analysis on RNA isolated from only one tumor cell with a pre-amplification method that does not compromise gene-expression profiling.

With this method, expression analysis of several tumor-specific genes in blood samples containing only 2 tumor cells is already possible.

However, gene expression analysis of EpCAM purified cells isolated from blood of healthy donors showed that some contaminating blood cells in the EpCAM-purified CTC-fraction exhibited mRNA expression of EpCAM and other genes thought to be tumor-specific. The presence of EpCAM or non-specific EpCAM binding to Fc receptors may account for this contamination.

Nevertheless, already several tumor-specific genes (e.g., MUC-1, TFF1, and SPDEF) could be detected in samples containing only 5 tumor cells spiked in 7.5 ml whole blood.

To further optimize CTC characterization, additional purification steps are required to reduce the leukocyte contamination in EpCAM purified CTC fractions.