7

Circulating Tumour Cells: Implications and Methods of Detection

Nisha Kanwar and Susan Done
Department of Laboratory Medicine and Pathobiology, University of Toronto
Campbell Family Institute for Breast Cancer Research,
University Health Network, Toronto
Canada

1. Introduction

1.1 Breast cancer and metastasis

Despite progressive advances in the fields of screening, radiation and chemotherapy, metastasis remains the leading cause of more than 90% of breast cancer related deaths. In metastasis, a small, select group of cells develops the capacity to disseminate from the primary tumour, and circulate via the blood or lymphatic system to distant organs, developing tumours at these new sites. Metastasis is a highly inefficient process, where less than 0.01% of tumour cells are able to successfully seed at secondary organs (Chiang & Massagué, 2008). In order to emerge as metastatic, tumour cells must progress through all of the steps of metastasis: invasion of tissues surrounding the primary tumour, intravasation into the circulatory system, survival in the circulation by evasion of the immune system or apoptosis, arrest in a distant capillary, extravasation, and finally proliferation in a distant organ (Fidler, 2003). These cells have often been referred to as decathlon athletes because of their aggressive biology. Once metastasis has occurred, conventional therapies are rendered less effective due to the nature of these cells. They are heterogeneous in their metastatic potentials, growing at different rates, with different invasive abilities and varying responses to drugs and treatment. It is also particularly difficult to detect the early stages of metastasis and assess the presence of minimal residual disease after treatment, owing to the microscopic routes of progression. Current methods used to predict the risk of metastasis and determine suitable treatment regimens are based on evaluation of tumour characteristics such as size, histological grade, lymph node involvement, and expression of treatment response markers like ER, PR and Her2 receptors (Weigelt et al., 2005). These practices however, are invasive and limited in their prognostic value. They are able to identify only 30% of breast cancer patients with a high risk for metastasis (Weigelt et al., 2005). There is a significant need for the development of new early prognostic markers for metastasis. Ultimately, if the spread of cells from the primary tumour could be stopped, then deaths from metastasis could be prevented. Researchers continue to try and shed more light on the molecular alterations in tumour cells which lead to metastasis.

1.2 Circulating tumour cells

The first observation of circulating tumour cells (CTCs) was made in 1867 by an Australian physician, Thomas R. Ashworth. He showed that cells found in the circulation were identical to those found in the original cancer; and that they may be able to explain the origin of multiple metastatic tumours (Ashworth, 1867). Current research has established that primary tumours themselves have a gene expression signature that is predictive of metastasis, and that the shedding of neoplastic cells into the circulation begins early on (van de Vijver et al., 2002). Furthermore, these tumour cells are able to develop the capacity to metastasize independently from the primary tumour, with a unique set of genetic aberrations (Schardt et al., 2005; Schmidt-Kittler et al., 2003). Targeting only the primary and metastatic tumours is insufficient to tackle breast cancer in a systemic manner. It appears that the 'vehicles' that are responsible for cancer spread may provide the supplementary molecular targets urgently needed. Metastasis has been known to develop in patients with small primary tumours, and even in 2-4% of rare cases of undetectable primary tumours (Weigelt et al., 2005; Hüsemann et al., 2008). Early breast cancer mouse models, as well as human samples show evidence of the spread of circulating tumour cells at early stages, completely independent of tumour size (Hüsemann et al., 2008). The number of tumour cells that enter circulation in mouse models is highest immediately after initial transformation, and these cells will accumulate additional mutations over time to eventually be selected for malignant growth in new organs (Hüsemann et al., 2008). However, not all patients with detectable spread of tumour cells will go on to develop metastasis (36-50% of patients show no detectable metastatic disease up to 22 years later) (Graves & Czerniecki, 2011). Many confounding factors in the secondary organ microenvironment will affect the transformation of these cells from dormant to metastatic. None the less, these findings are indicative of metastasis being an early event. Circulating tumour cells (CTCs) might be the earliest detectable cells with metastatic abilities and are emerging as a promising biomarker for breast cancer progression. CTCs may affect cancer prognosis years before the onset of overt metastasis, and therefore improve risk assessment and help identify patients in need of additional treatment. The cells themselves may provide new targets for therapy to prevent their spread to distant sites.

2. Clinical relevance of CTCs

CTCs are found in the peripheral blood and are separate from disseminated tumour cells or DTCs, which are found in the bone marrow. DTCs are an independent prognostic marker for poor prognosis in breast cancer. In a meta-analysis of prospective studies with at least 10 years of follow up data on 4703 metastatic breast cancer patients, 30.6% of patients had DTCs which were associated with tumour stage, lymph node involvement, tumour grade, and hormone receptor negative status (Braun et al., 2005). The acquisition of DTCs from bone marrow, however, is an invasive procedure, requiring a high standard of quality control for repeated draws from the iliac crest of patients (Pantel et al., 2008). Several studies have shown an association between the occurrence of DTCs and CTCs in early and metastatic breast cancer (Graves & Czerniecki, 2011). Therefore, the collection of blood samples from patients for analysis of CTCs without surgical intervention, is a more attractive alternative for clinical purposes.

CTCs have been extensively characterized as epithelial tumour cells, large in size, with a high nuclear to cytoplasmic ratio, irregular nuclear shape, non-proliferative and in some

cases, apoptotic (Pantel et al., 2008; Aktas et al., 2009; Fehm et al., 2010; Ignatiadis et al., 2011; Wikman et al., 2008). These are not exclusive characteristics however, and a great degree of phenotypic heterogeneity exists within this population of cells.

CTCs have been reported in 70-100% of patients with distant metastatic spread and 46-71% of patients with local nodal involvement (Cristofanilli et al., 2004). A multicenter prospective study of 177 patients compared quantities of CTCs in relation to outcome and showed that metastatic breast cancer patients with more than 5 CTCs per 7.5mL of blood had a shorter progression-free and overall survival (2.7 months vs. 7.0 months and 10.1 months vs. >18 months) (Cristofanilli et al., 2005). In a follow up study with the same group of patients, it was reported that the number of CTCs was a better indicator of disease progression than traditional techniques such as imaging with PET, CT or MRI scans (Cristofanilli et al., 2007; Bidard et al., 2008; Nelson, 2010; Liu et al., 2009). Similar associations have been found in patients with colon and prostate cancer. Notably, decreased levels of CTCs are seen in response to chemotherapy suggesting a role for the measurement of CTCs in treatment monitoring. Chemo-resistant CTCs have been shown to be Her2 positive, although they originated from Her2 negative primary tumours (Flores et al., 2010; Fehm et al., 2010). A proportion of these patients had metastatic tumours which were also Her2 positive. Such discrepancies were also found with ER, PR and EGFR status suggesting a critical role for CTCs in stratifying patients for treatment (Fehm et al., 2009). Furthermore, epithelial-mesenchymal transition (EMT) and stem cell characteristics, such as expression of Twist, and ALDH1 are detectable in CTCs of metastatic breast cancer samples (Aktas et al., 2009). Such markers might be associated with a stem-cell like subpopulation persisting after treatment, capable of survival, self-renewal and aggressive propagation of new tumours. Further exemplifying this hypothesis, it has been shown that some CTCs have a multidrug resistance protein (MRP) expression profile which correlates with ALDH1 expression. CTCs from patients who expressed more than 2 MRPs, had shorter progression-free survival, and resistance to chemotherapy (Gradilone et al., 2011). Both DTCs and CTCs were included in tumour marker assessment for breast cancer by the American Society of Clinical Oncology in 2007 (Harris et al., 2007). The report was based on studies rated at Level of evidence I and II (prospective randomized controlled trials, prospective therapeutic trials, or meta-analyses testing the utility of a marker). While acknowledging the immense research interest and publications in the field, the clinical utility of CTCs and DTCs is yet to be established and requires further large scale studies, especially in early breast cancer (Harris et al., 2007). Clinically, the mere presence of CTCs is indicative of a negative prognostic impact and highlights a role for these cells as biomarkers of disease progression and drug response.

3. Methods for enrichment of CTCs from blood

CTCs are a rare cell population (approximately 1 in 100 million nucleated blood cells of breast cancer patients). Metastatic breast cancer patients have been reported to have a range of 0 to a few thousand CTCs per 1-10 mL of blood (Flores et al., 2010; Talasaz et al., 2009) , compared to early stage breast cancers which from the limited publications to date as well as results from our own laboratory, report a range of 0 to less than 10 CTCs per 7-20 mL of blood (Ignatiadis et al., 2011; Lang et al., 2009; Shaw et al., 2011). A major obstacle is obtaining a sufficient number of CTCs, void of contaminating white blood cells, for downstream purposes such as genome, transcriptome or proteome analysis. Enrichment is usually a pre-requisite to any detection or isolation protocol described. After enrichment,

detection of cells is improved at least 10,000-fold, depending on the technique. Several methods for enrichment of CTCs have been described – immunomagnetic bead separation; density centrifugation separation, size based exclusion, and flow cytometric separation. Each of these methods allows for either positive selection by targeting CTCs with epithelial markers (cytokeratin, EpCAM), or negative selection by targeting white blood cells with leukocyte markers (CD45). There is however, a common and significant caveat in these procedures; CTCs are enriched from blood using normal epithelial cell markers due to lack of CTC specific malignant cell markers. Also, there is the possibility that in the process of tumourigenic progression, some CTCs may in fact lose their epithelial markers during epithelial-mesenchymal transition, and thus would not be selected for. To address these shortcomings, different methods of enrichment are combined (double selection) in order to compliment each other, and a panel of markers is used to best ascertain the identity of cells being enriched for.

3.1 Immunomagnetic bead enrichment

In February 2008, the FDA approved the CellSearch® System (developed by Veridex, LLC - Johnson & Johnson, NJ) as the first, validated method to accurately detect and enumerate CTCs in order to monitor metastatic disease in cancer patients (Riethdorf et al., 2007). This system incorporates the interaction of target cells with antibodies conjugated to magnetic nanoparticles or 'immunomagnetic beads'. Once cells are bound to the magnetic beads, they are passed through a magnetic core, where they are selectively retained, while unbound cells flow through. They are then fixed and labeled with fluorescent tags for subsequent analysis and enumeration. The advantage of this system is that it allows the use of multiple markers for CTCs as well as non-CTCs to enable more reliable identification. The standard panel includes CTC labeling antibodies such as EpCAM, and cytokeratins, as well as leukocyte labeling antibodies such as CD45. DAPI stains the nuclei to enable characterization of the multi-lobed nuclei, or low nuclear to cytoplasmic ratio seen in white blood cells compared to mostly high nuclear to cytoplasmic ratio seen in CTCs (Figure 1).

Recently, the Her2 antibody was added to the panel of test markers (Ignatiadis et al., 2011). The entire system is automated, including computerized image analysis and acquisition (Figure 2). It appears to be the gold standard of CTC detection to date, with more studies being published, each validating its sensitivity which has been established as a median of 5 CTC per 7.5mL of blood (Kraan et al., 2011; Ignatiadis et al., 2011; Savitri Krishnamurthy et al., 2010; Sandri et al., 2010; Van der Auwera et al., 2010; Bidard et al., 2010).

Recently, a modified version of this system was released, known as the CellSearch® Profile kit. This system incorporates a fewer number of steps, providing only enrichment of cells with the immunomagnetic beads, followed by elution. This allows for isolation and further processing of viable cells for other purposes such as cytometric or molecular analyses such as IHC, TUNEL assays, FISH, RT-PCR or DNA assays (Flores et al., 2010). Fewer processing steps has also resulted in a greater than 20-fold increase in the yield of CTCs with a range of 4 - 2432 CTCs per 7.5mL of blood (Flores et al., 2010). In addition, a group of researchers at Stanford University, CA have developed their own system based on the immunomagnetic principle, called the Magsweeper (Talasaz et al., 2009). This device incorporates magnetic rods with plastic sheets, which undergo repeated capture-wash-release cycles to enhance specific binding of target cells while non-specifically bound cells are gently washed off.

Another innovative device that has branched out from this technology is the CTC-chip, recently developed by a group of researchers at Harvard University, MA(Figure 3). The CTC-chip uses micro-fluidic principles for the capture of viable CTCs from small volumes of blood. Immunomagnetic nanoparticles are replaced by 78,000 microposts over a surface of 970 mm^2, each coated with antibodies for EpCAM (Nagrath et al., 2007). Capture of CTCs from blood by the microposts is dependent on strict laminar flow and volume conditions, with optimal capture occurring at a flow rate of less than 2.5ml per hour (Nagrath et al., 2007). Once bound to the posts, CTCs may be processed in a similar fashion as the CellSearch® system by fixation and fluorescent labeling for analysis. It appears to be an efficient system, enriching CTCs in the range of 5 - 1281 cells per 1mL of blood (Nagrath et al., 2007).

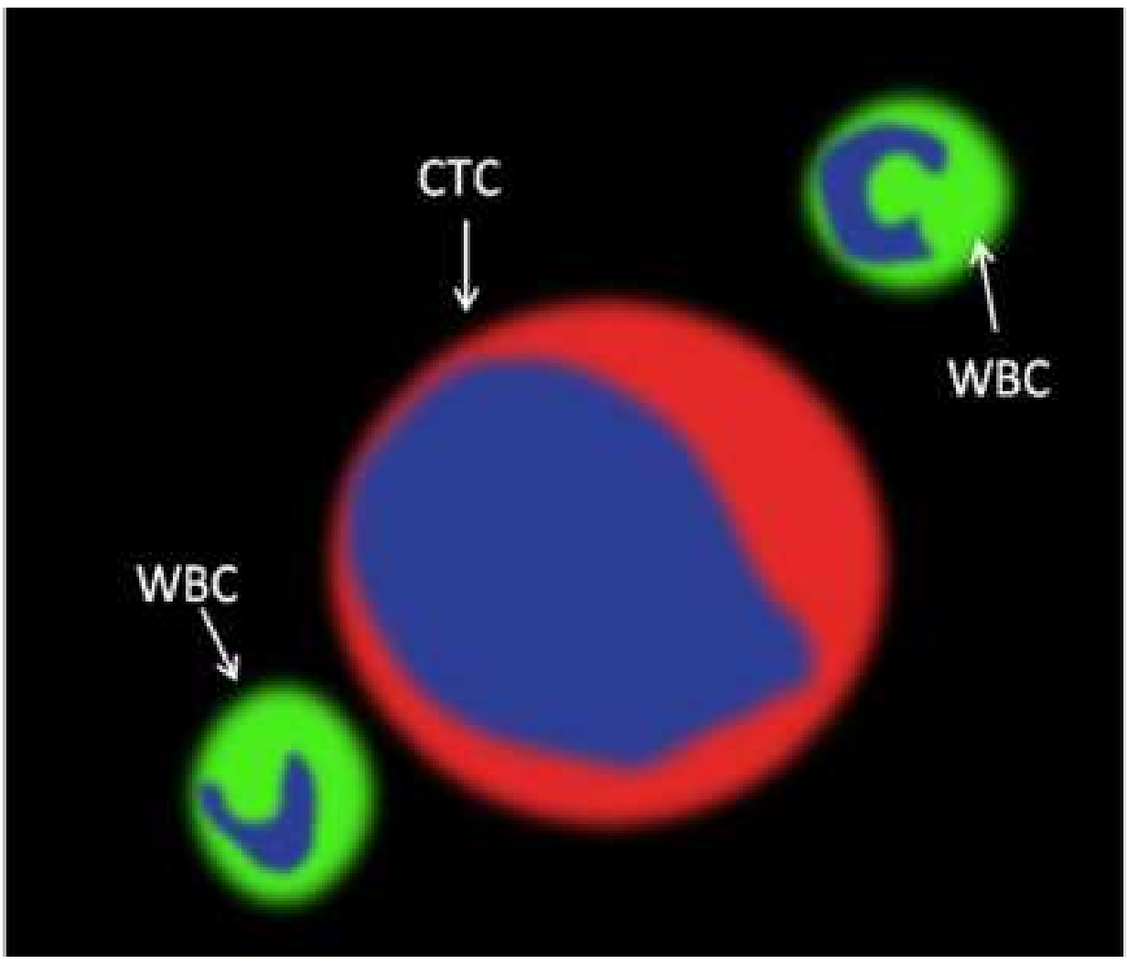


Fig. 1. Schematic of immunofluorescent staining of a CTC (cytokeratin: red, CD45: green, DAPI: blue). Tumour cells (CTC) are identified by high nuclear to cytoplasmic ratio and cytokeratin expression in the cytoplasm. White blood cells (WBC) are identified by smaller cellular size and CD45 expression.

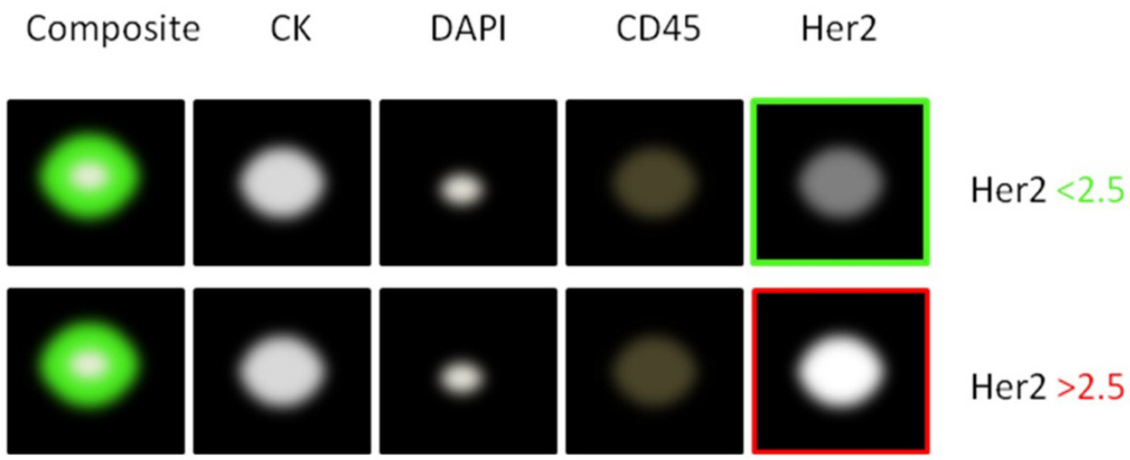


Fig. 2. Schematic of typical results obtained from the CellSearch® analysis of CTCs isolated from metastatic breast cancer patients. Composite analysis of 2 CTCs is displayed; with positive labeling for cytokeratin (CK), DAPI and Her2 (two intensities shown, >2.5 is diagnosed as Her2 positive) and negative labeling for CD45 (leukocyte marker).

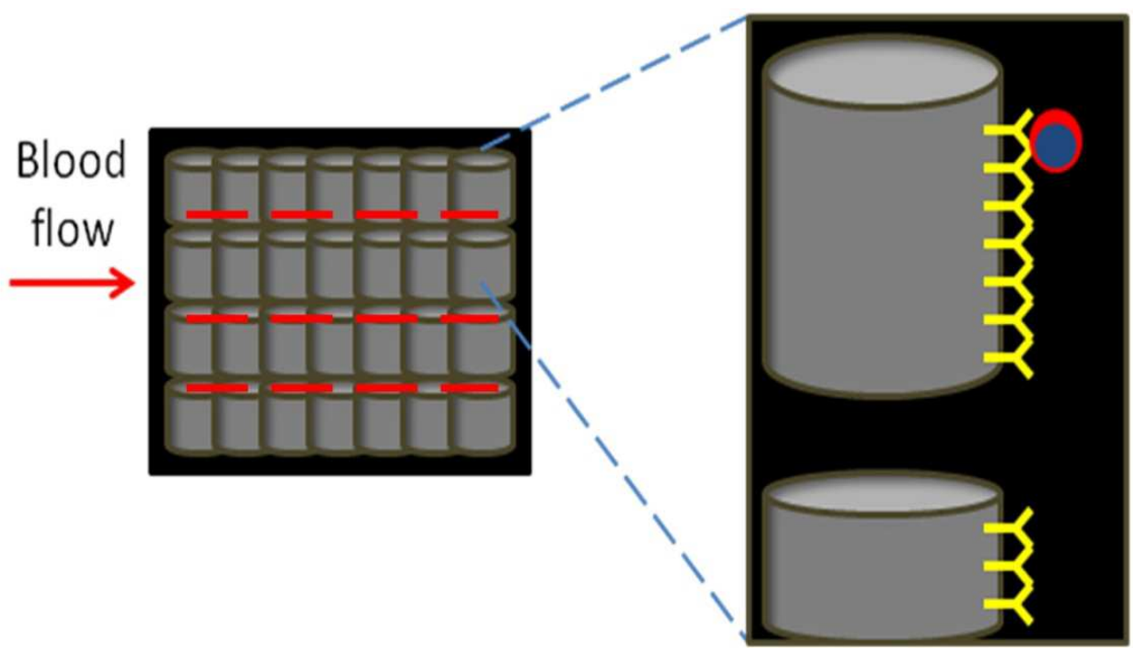


Fig. 3. CTC-chip with microposts; blood flows over and through microposts at designated flow rate and volume for capture of CTCs. Enlarged schematic shows micropost coated with EpCAM antibodies (yellow) interacting with a complementary epitope on the CTC.

The next generation of the CTC-chip is the Herringbone chip, with enhanced capture (26% improvement from the CTC-chip) and higher flow rates (4.8mL of blood per hour) (Figure 4). The improved performance is the result of a platform consisting of angular flow paths or microvortices which allow for passive mixing of blood as it flows through the chip, significantly increasing the number of interactions between CTCs and the antibody coated

chip surface (Stott et al., 2010). In addition, the platform of the chip is made up of a transparent material, allowing for improved imaging options, both for transmitted light IHC staining as well as fluorescent staining (Stott et al., 2010).

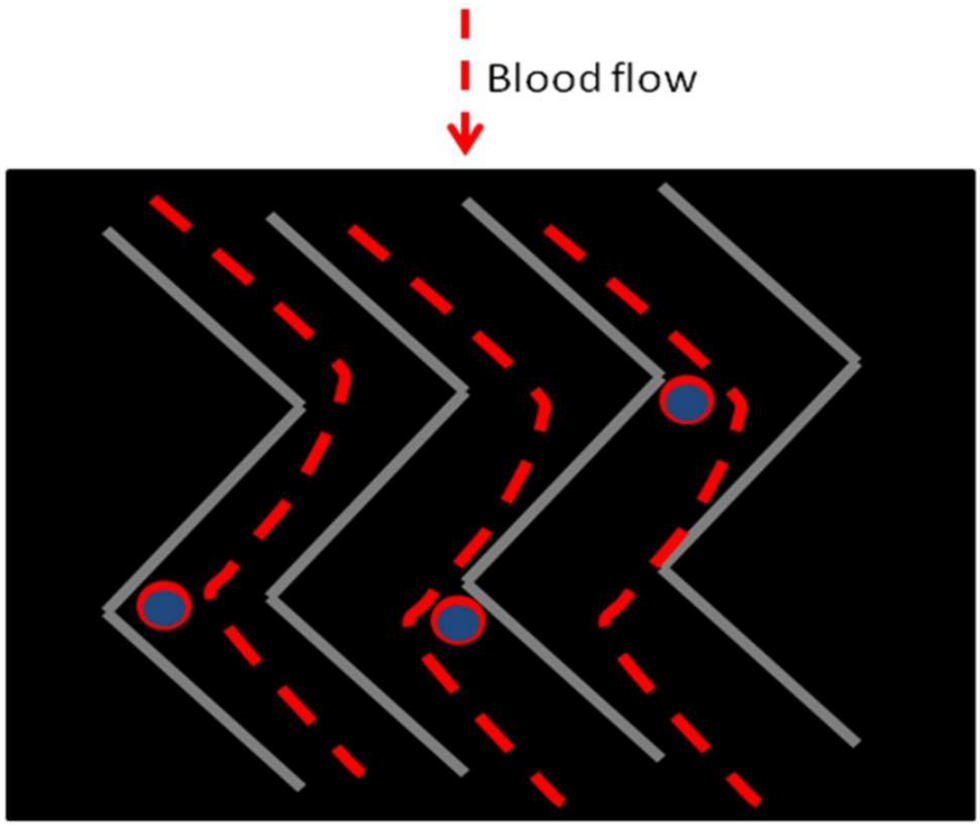


Fig. 4. Magnified representation of the asymmetric, grooved surface of the Herringbone CTC chip. Microvortices allow for enhanced number of interactions between CTCs and the EpCAM antibody coated surface of the chip.

Despite the promise of performance of microfluidic devices, there remain two significant disadvantages to these systems. Firstly, since cells are not fixed, samples must be processed within 2 hours of collection to maintain cell viability and capture by the chip. This complicates multicenter studies or the clinical utility of this system worldwide until this device is made commercially available. Secondly, once captured, CTCs are bound to the chip, and any subsequent analysis must be performed on the chip itself. Although this is beneficial for visualization and enumeration type assays, it is not transferrable to nucleic acid based assays such as RT-PCR or DNA analysis.

3.2 Physical properties based enrichment

CTCs are also enriched based on their physical properties such as density and size. Protocols have been developed for density based separation where tetrameric antibodies aid in the formation of aggregates of white blood cells and red blood cells, called 'immunorosettes' (Gertler et al. 2003). These aggregates are complex structures with a higher density than single CTCs. Centrifugation allows for a fast and convenient pelleting of

the blood cells, enriching CTCs in the interphase of blood serum and a separating medium such as Percoll™ or Ficoll™ (Gertler et al., 2003). Size based separation protocols utilize the property of tumour cells being at least twice the size of the majority of leukocytes (>20μm versus 8 - 10μm). Blood is passed through filters which, with the help of a gentle vacuum pressure, exclude CTCs and a small number of larger white blood cells such as macrophages and monocytes (Pinzani et al., 2006).

One obvious advantage of this type of separation is that CTCs are not being selected for by epithelial cell surface markers which might be lost during the aforementioned EMT processes. While both of these methods of separation provide the advantage of enriching for viable CTCs, which may be collected and stained for cytopathological analysis or subjected to RNA or DNA analyses, the technology is quite elementary and imprecise, and fails to give reproducible data. Also, not all CTCs are greater than 8μm, and there will remain a proportion that will be missed by this technique as well.

3.3 Evaluation of two prevalent methods of CTC enrichment

In our laboratory, we have evaluated immunomagnetic and density centrifugation based methods of CTC enrichment using spiking experiments. Quantities of 100, 1000, 10^4 and 10^5 cells from the MCF7 breast cancer cell line were spiked into 10 mL of peripheral blood from healthy volunteers. RossetteSep™ (density centrifugation separation) and EasySep™ (immunomagnetic bead separation) kits from StemCell Technologies Inc. were used for negative selection of spiked MCF7 cells from blood. The RossetteSep™ kit resulted in a purer cell eluate compared with the EasySep™ kit which contained interfering residual magnetic beads, and white blood cell clumps. The disadvantage however, was the lack of sensitivity and automation of both methods. The level for retrieving spiked cells was determined to be less than 60% of 10^4 and 10^5 spiked cells. Cells spiked below this threshold were not retrieved by either method.

We have since validated a method of CTC enrichment from blood using the autoMACS™ cell sorter (Miltenyi Biotec). This machine incorporates negative selection of CTCs and immunomagnetic, automated separation. Immunolabeled cells pass through a magnet containing a column made up of a porous matrix dense enough to allow single cell flow through (Gijs, 2004). Each single cell is analyzed and sorted as labeled (CD45 positive leukocyte) or unlabelled (CD45 negative CTC), and eluted through its respective port. This method parallels the principle of the FDA approved CellSearch® System. Spiking experiments showed a 1000-fold increase in sensitivity of cells recovered compared to previously tested techniques in our laboratory. Sensitivity of the assay was dependent on the volume of blood processed, which corresponded to the number of white blood cells present in the sample. Addition of a red blood cell lysis step served two purposes; (1) to reduce background of interfering red blood cells and enhance immunomagnetic bead-white blood cell interactions; and (2) to pellet the remaining cellular fraction and allow exclusion of non-specific free RNA/DNA. MCF7 breast cancer cells spiked into 3 ml of blood from healthy volunteers showed 70-90% recovery of 10-100 spiked cells, respectively (R=0.8959).

4. Genetic profiling of CTCs

Enriched CTCs are subjected to two types of analyses – immunocytometric or RT-PCR based assays. Both methods allow for multiplexing of markers to increase specificity. Markers include cytokeratins, Her2, MET, MUC1, ESR1, TWIST, ALDH1, EGFR, Ki-67 and many

more. RT-PCR assays are the most frequently reported method of CTC detection, and have the highest sensitivity (1 CTC in over 1-10 million nucleated blood cells) (Ring et al., 2005; Gerges et al., 2010). RT-PCR assays also have the advantage of high-throughput efficiency and allow for the analysis of larger panels of genes ranging from a few up to 16 genes as was recently published (Sieuwerts et al., 2008). This study showed that the proof of principle experiment was successful for analysis of a panel of 96 genes in one single tumour cell. This result however is affected by a high background of white blood cell RNA in patient samples, and thus was limited to an exploratory set of 16 genes capable of differentiating expression profiles of CTC positive breast cancer patients from CTC negative breast cancer patients (Sieuwerts et al., 2008). Another study aimed to determine the global expression pattern of CTCs, using the Affymetrix GeneChip™ platform, and once again concluded that a different panel of 16 genes was expressed specifically in CTCs from metastatic breast cancer patients (Smirnov et al., 2005). Noticeably, this study also looked at CTCs originating from different cancer tissues, and found that CTCs derived from different cancers had unique expression patterns as well (Smirnov et al., 2005).

The disadvantages of these methods of profiling are false expression signatures created from non-specific primers or staining, lack of expression of the marker of interest on CTCs in the sample, pseudogenes, and low expression of the marker of interest on non-malignant cells. Work done in our own laboratory demonstrated the use of RT-PCR techniques to enumerate CTCs in blood by correcting for a percentage of these types of false signatures (Iakovlev et al., 2007). Blood from healthy human volunteers was spiked with 1-10,000 tumour cells from breast cancer cell lines. Also, free RNA from these same cell lines was spiked separately to determine false positive results from free RNA, and genomic DNA released from dying cells in blood. We were able to show that there is a linear relationship between the number of CTCs in blood, and the amount of CK19 RNA, with a reliable detection limit of 5 cells per mL of blood with Ct values <40 to correct for false positives. We also determined that by including a centrifugation step to pellet CTCs and separate them from other fractions of blood, most of the contaminating free RNA and DNA can be excluded. These techniques may prove useful for high throughput comparative quantification or analyses of CTCs in individual patients during treatment and subsequent follow up for clinical management purposes.

RNA is highly unstable and is readily degraded once it is released from the cell. Comparatively, DNA based assays are much more reliable, robust and less technically demanding. Genomic characterization of CTCs has however been unachievable thus far. Even the most sensitive technique of CTC enrichment from blood does not provide a pure sample void of white blood cell contamination. Furthermore, even if isolation of a pure CTC sample was achieved, genomic analysis would be quite challenging owing to the low numbers of cells in circulation. If the whole genome of CTCs can be profiled, we could identify novel genomic alterations specific to CTCs which may be utilized in the development of specific markers for CTCs in blood. Also, regions of altered DNA in CTCs from early breast cancer patients hold valuable prognostic genetic information for the progression of early breast cancer to metastasis.

4.1 Isolation of single CTCs for DNA extraction

Our laboratory has been involved in the testing and validation of various technologies to address the problem of specificity during isolation of CTCs in order to genomically characterize these cells. We have designed a protocol to isolate single CTCs using immunocytochemical staining and single cell laser capture microdissection (LCM), followed

by whole genome amplification (WGA) of DNA for high density copy number analysis (Figure 5).

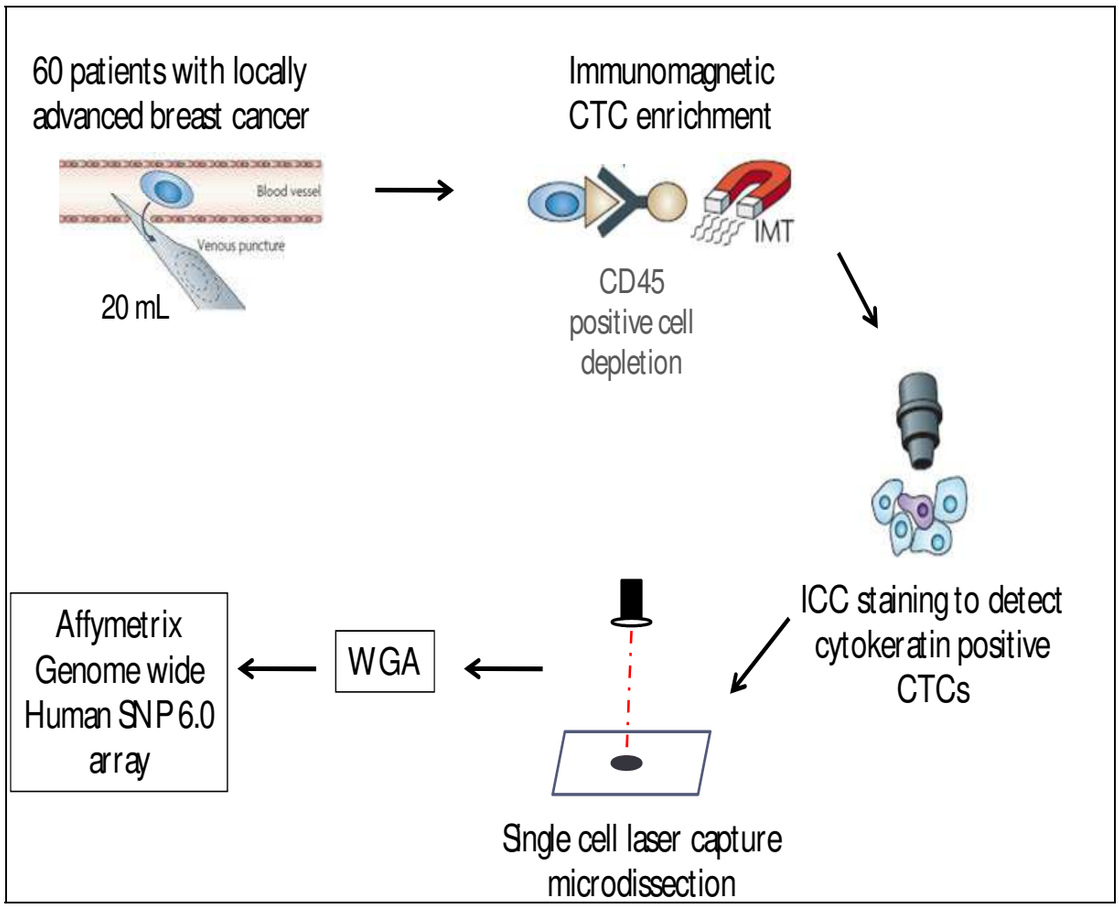


Fig. 5. Flowchart of steps involved in the isolation of single CTCs from the blood of patients with locally advanced breast cancer, for purposes of whole genome amplification (WGA) and high resolution copy number analysis.

We used the autoMACS™ separator for immunomagnetic based enrichment for CTCs from 20mL of blood drawn from patients with locally advanced breast cancer. Each blood sample is processed in 3mL aliquots for optimal enrichment of CTCs from blood, as determined from spiking experiments described earlier. Each aliquot is lysed for red blood cells, pelleted, and then resuspended in separation buffer containing magnetic beads conjugated to CD45 antibodies. The aliquot is then processed, selecting for the CD45 negative fraction which consists of enriched CTCs. The enriched CTC suspension is transferred onto a glass slide using a Cytospin centrifuge. It is then fixed and stained with a pan-cytokeratin AE1/AE3 antibody. This mixture of cytokeratin antibodies recognizes the vast majority of both low and high molecular weight keratins; CK 1-19; except for CK17 and CK18. Once the primary and secondary antibodies are bound, the method of detection that we use is a third antibody conjugated to the enzyme glucose oxidase which is not expressed in mammalian cells (Hard et al., 1989). This property abolishes any non-specific staining that occurs with horseradish peroxidase or alkaline phosphatase methods of detection. These enzymes are expressed

endogenously in white blood cells and thus could react with the detection substrate resulting in false positives (Figure 6). Glucose oxidase, however, is absent from white blood cells, and will react with the detection substrate positively staining only those cells that were bound to the primary and secondary antibody. This crucial modification of the staining protocol allows for the certainty of true positives. Positively stained CTCs are then lifted off the slide using single cell laser capture microdissection (Figure 7).

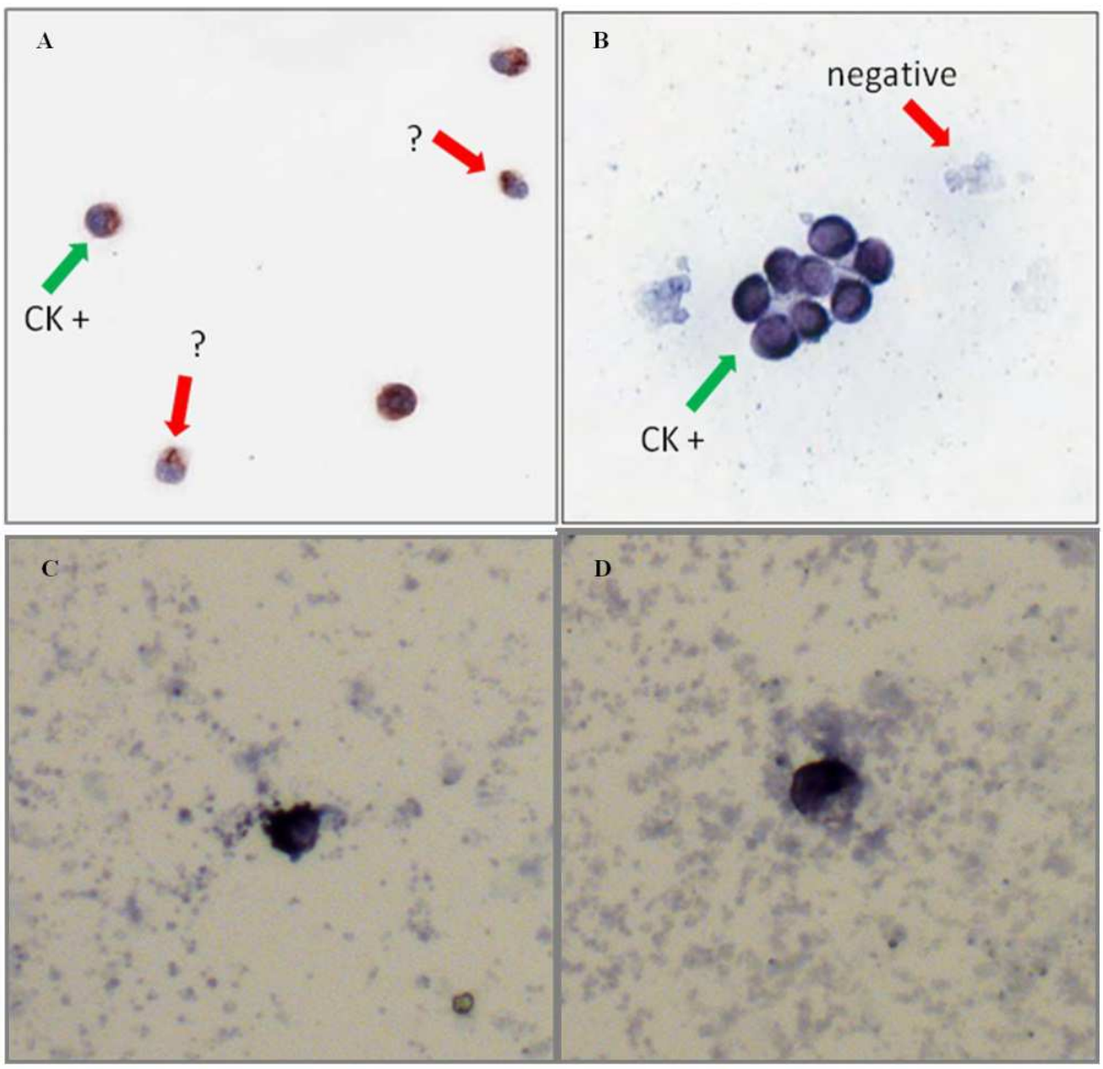


Fig. 6. Enriched cancer cells after spiking of MCF7 cells into blood from healthy human volunteers. (A) Ambiguous staining of CK positive tumour cells and residual white blood cells using horseradish peroxidase detection of antibodies. (B) Specific staining of CK positive tumour cells and negative background of white blood cells using glucose oxidase detection of antibodies. (C) and (D) Enriched single CTCs from locally advanced breast cancer patients using the validated protocol.

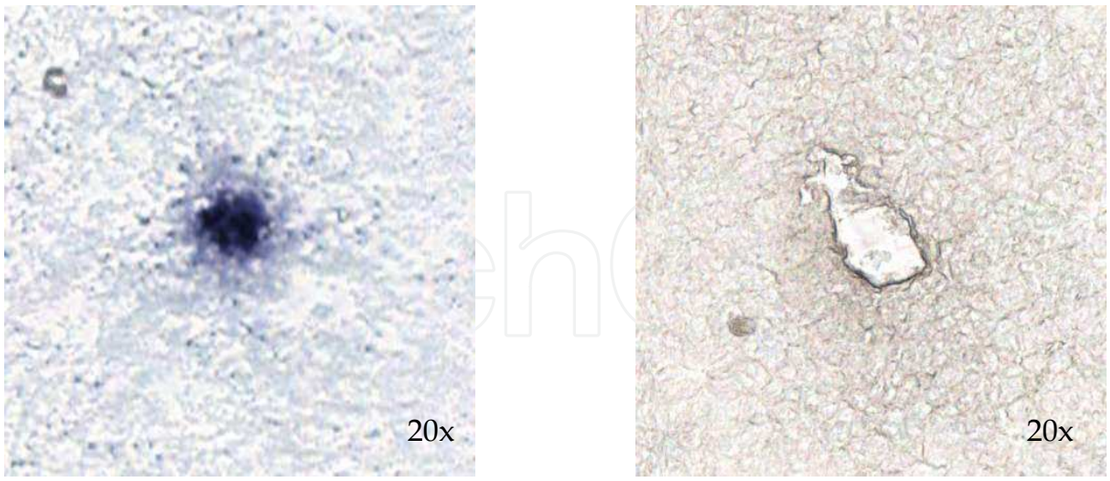


Fig. 7. Cytokeratin positive CTC isolated from the blood of patient with locally advanced breast cancer, before and after lifting off slide using single cell laser capture microdissection.

4.2 Whole genome amplification of single cells

While there are numerous studies in the literature that have successfully isolated and enumerated CTCs; there is no knowledge of the genomic alterations of CTC DNA. Our laboratory has been successful at amplifying the whole genome of single CTCs in order to obtain sufficient amounts of representative DNA for microarray analysis. Most commercial platforms require between 700ng – 1000ng of good quality genomic DNA. We have established a protocol for whole genomic amplification of single cells using a modified protocol of the WGA4 kit (Sigma). We obtained between 1.5 - 3µg of amplified genomic DNA from single cells, which is sufficient for the Affymetrix Genome-Wide Human SNP 6.0 array. This platform currently provides the highest resolution of copy number and loss of heterozygosity data with coverage of greater than 2 million copy number and SNP probes across the entire genome. Our protocol has been validated using MCF7 cell line DNA from as few as 2 single cells. Microarray data was analyzed using Genotyping Console software. Segmented data was analyzed separately for copy number losses and gains (SNP-FASST segmentation algorithm) to identify significant genomic alterations. To begin analysis, we first noted regions of genomic aberrations in the MCF7 cell line that were previously described, and then compared this existing data to regions we obtained with the Affymetrix SNP 6.0 array (Shadeo et al., 2006 & Przybytkowski et al., 2011). Analysis shows expected aberrations in the MCF7 cell line were conserved across samples with amplified single cell starting material when compared to unamplified DNA ($p<0.000001$, 75% concordance). We improved this protocol by introducing a ligation step after genomic amplification. The average size of fragments that probes on the Affymetrix microchip bind to are 700-1000bp long. After WGA however, most DNA fragments are less than 500bp. Ligating these small amplified DNA fragments produces larger fragments of DNA for processing on the microchip, which in turn allows for more SNP and copy number calls to be made. Concordance was improved to greater than 90% after optimization ($p<0.000001$) (Figures 8-10 and Table 1).

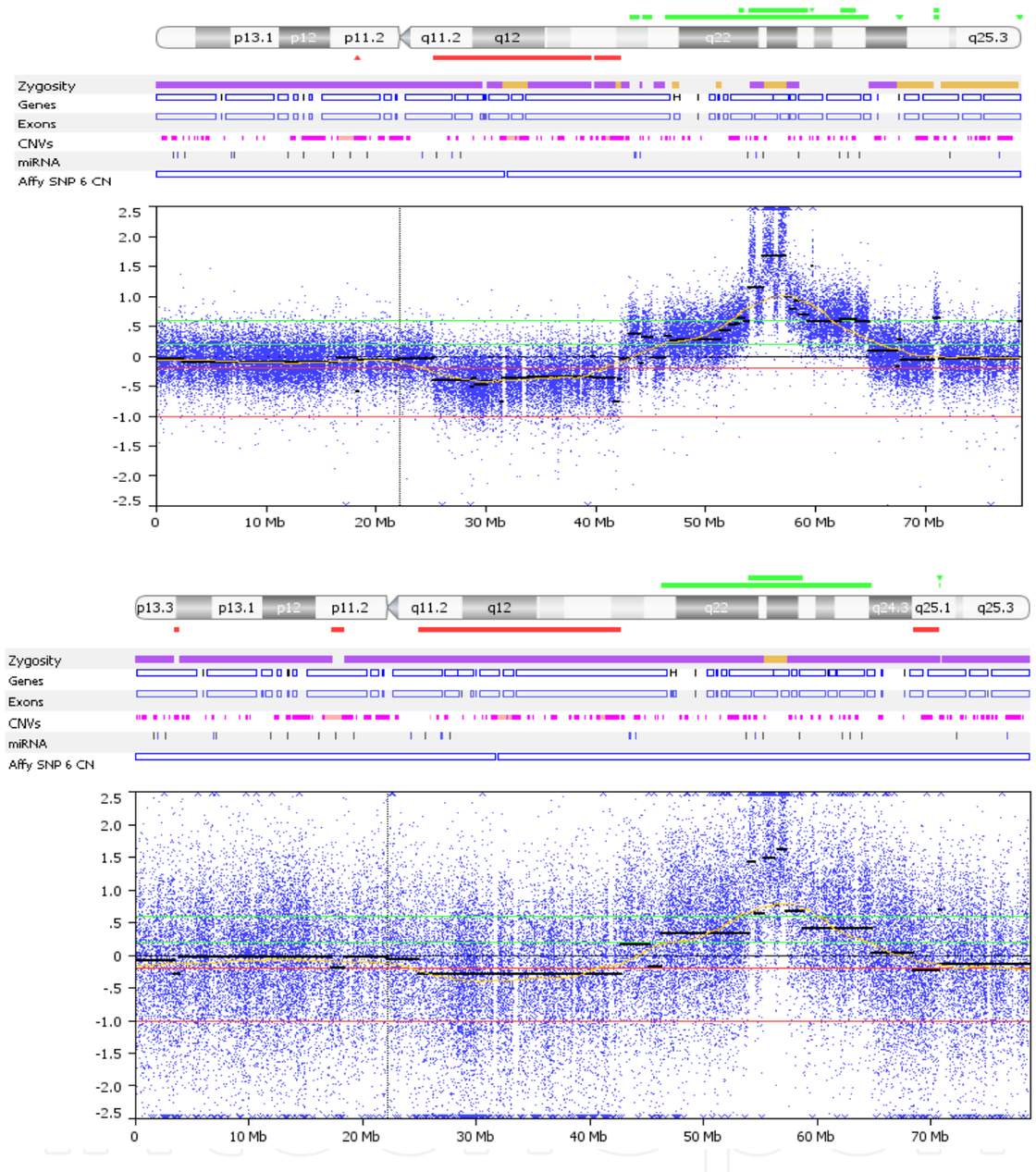
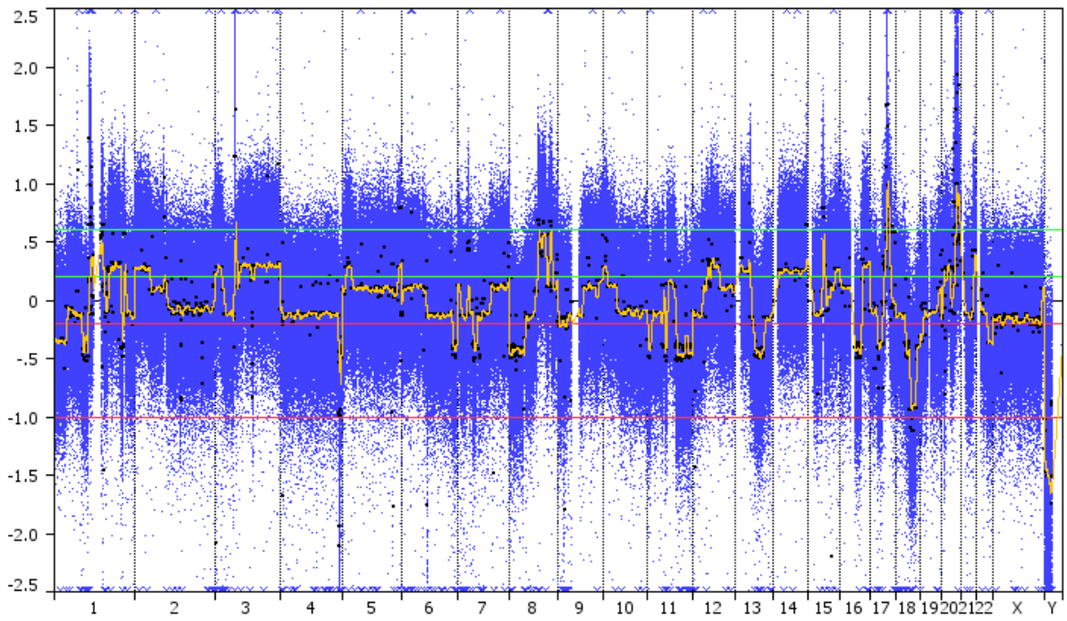


Fig. 8. Comparison of Log2 ratios of copy number between unamplified genomic DNA from MCF7 breast cancer cells (top) and amplified DNA from 2 single MCF7 cells (bottom). Noticeably, although single cells produce more noisy probe calls (blue dots), copy number gains (green) and losses (red), as well as segmentation (black horizontal lines) are significantly conserved across single cell amplification, with 92% concordance. Analysis was performed using Nexus Copy Number software with the following parameters: significance threshold p<1.0E-6; maximum contiguous probe spacing = 1Mbp; minimum probes per segment = 10; Log2 ratios: gain >0.2; high gain > 0.6; loss < -0.2; high loss < -1.0.

Unamplified genomic DNA



Amplified DNA from 2 single cells

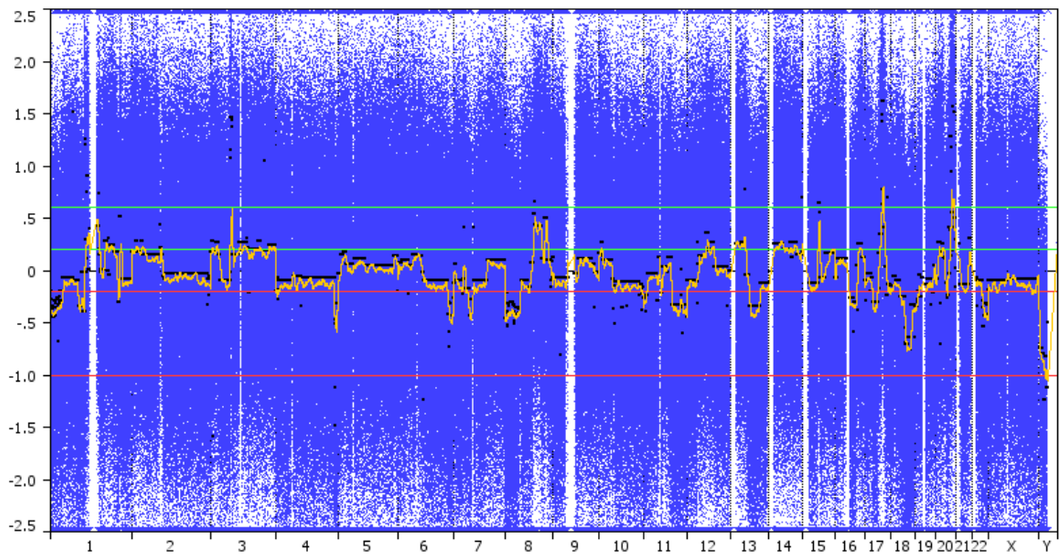


Fig. 9. Moving average of probes (1000 Kb) shows reproducible data between unamplified DNA and amplified single cell DNA from MCF7 breast cancer cells. Analysis was performed using Nexus Copy Number software with the following parameters: significance threshold p<1.0E-6; maximum contiguous probe spacing = 1Mbp; minimum probes per segment = 10; Log2 ratios: gain >0.2; high gain > 0.6; loss < -0.2; high loss < -1.0.

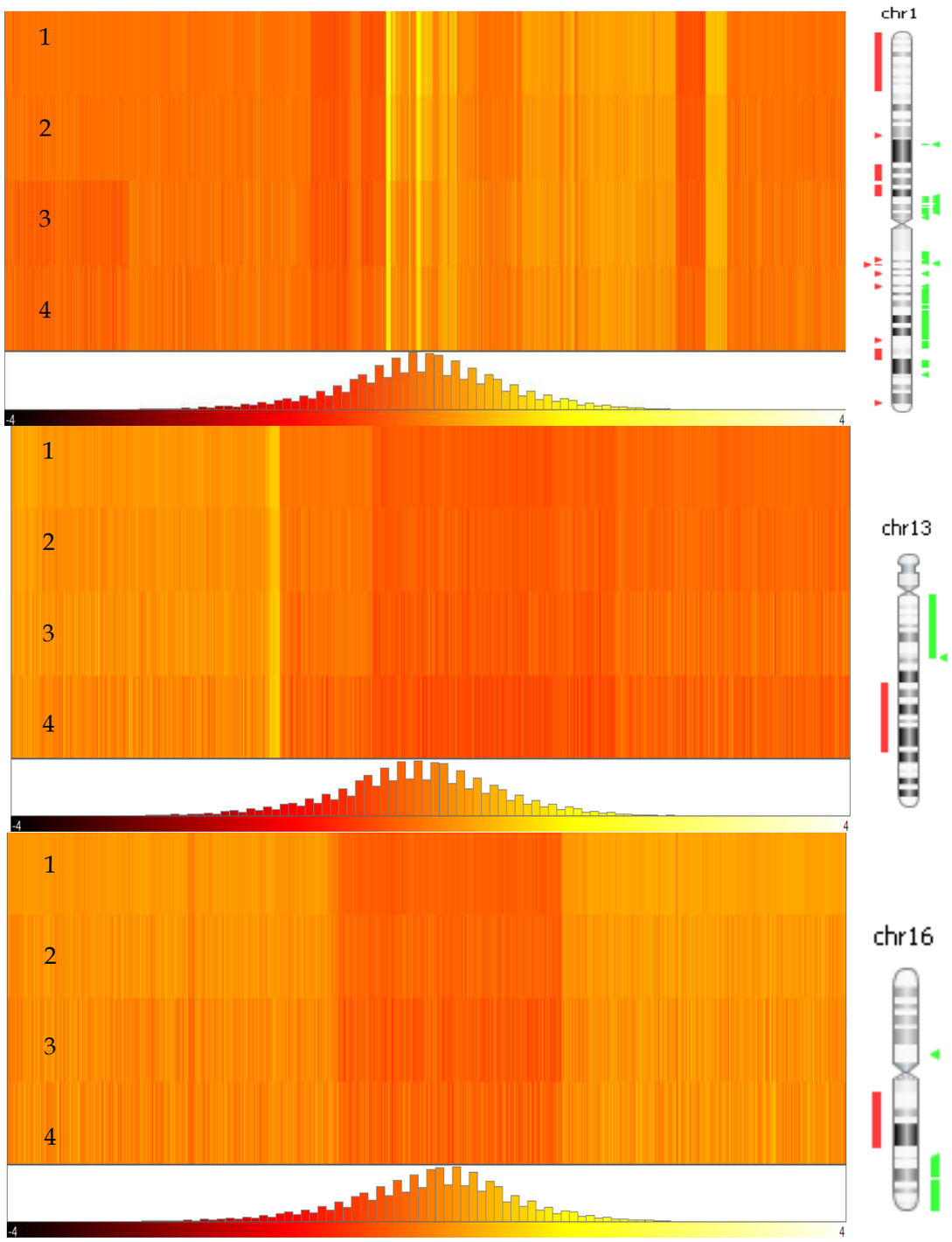


Fig. 10. Copy number heat maps of chromosomes 1, 13 and 16 illustrating genomic gains (yellow) and losses (red) in the MCF7 breast cancer cell line; corresponding chromosomal view showing genomic gains (green) and losses (red), shown to the right. Each row represents one sample (Rows correspond with - 1: unamplified genomic DNA, 2-4: amplified DNA from 2, 3 and 5 single cells, respectively). Analysis was performed with Genotyping Console software.

Subsequently, CTCs isolated from patients with locally advanced breast cancer were processed using this protocol. This is an interesting subgroup of breast cancer patients, as they have locally invasive, larger primary tumours (>5cm) and the cancer has usually metastasized to the lymph nodes but not beyond. Thus they are a subgroup of early metastatic patients, however the cancer is advanced enough for there to be a high likelihood of the presence of detectable numbers of CTCs for genetic analyses. From a total of 31 patients, CTCs were detected in 58% with a range of 1-20 cells per 20 mL of blood. We are currently performing microarray data analyses on these samples, with the goal of addressing some of the research questions discussed below.

Comparison 1	Comparison 2	# SNPs called	# SNPs concordant	SNP concordance (%)
Unamplified genomic	Amplified genomic	733884	700714	96
Amplified genomic	Amplified 2 cells	687896	633305	92
Amplified genomic	Amplified 3 cells	706094	660446	94
Amplified genomic	Amplified 5 cells	663709	567244	86
Amplified 2 cells	Amplified 3 cells	680912	629454	93
Amplified 3 cells	Amplified 5 cells	656417	568087	87
Amplified 2 cells	Amplified 5 cells	644176	558169	87

Table 1. Concordance results of SNP calls made between samples of MCF7 breast cancer cells. SNP call concordance is accurately conserved between the unamplified genomic and amplified genomic samples at 96%. Single cell samples are compared to the amplified genomic sample for concordance so as to control for variability introduced by the WGA protocol. SNP call concordance is accurately conserved in amplified single cell samples (amplified genomic vs 2, 3, and 5 cells). A test for precision and reproducibility of the WGA protocol was also performed by comparing concordance of SNP calls within amplified single cell samples (2vs3, 3vs5, and 2vs5). Analysis performed with Genotyping Console software.

Breast cancer is a heterogeneous disease which can be classified into 5 accepted molecular subtypes. Luminal A tumours tend to be ER+/PR+/Her2-, respond well to hormone therapy and have the most favourable outcome; Luminal B tumours tend to be ER+/PR+/Her+; Her2+ tumours are ER-/PR-; Basal tumours which are ER-/PR-/Her- tend to be the most aggressive, demonstrate the worst prognosis and have limited therapeutic options; and lastly the normal-like tumours are not clearly defined, and display a putative-initiating stem cell phenotype (Perou et al., 1999, 2000; Sørlie et al., 2001, 2006). Using the genomic profiles generated from CTCs, copy number signatures could be configured to determine the predictive power of CTC genomic profiles in the subtyping of breast cancer based on clinical parameters of the primary tumour. Additionally, genomic profiles of matched primary and CTC samples could be analyzed for common and unique regions, to identify alterations involved in disease progression. The utility of CTC genomic profiles could also be extended to the monitoring of patient response to chemotherapy and other treatments. Decrease in CTC numbers is an already established phenomenon in good response and prognosis of patients undergoing treatments. It would be interesting to investigate the idea that CTCs themselves have a genetic signature capable of stratifying patients into responders and non-responders to various treatment regimens. The

characterization of genomic signatures of CTCs could pave the way to the development of biomarkers of disease subtype, progression to metastasis and response to treatment. In the future, a simple blood based assay could detect high risk patients, guide treatment options, identify new drug targets, and thus illuminate the process of metastasis.

5. Future directions: Challenges and applications

Although there is substantial evidence showing the prognostic value that CTCs hold in metastatic breast cancer, their clinical significance, especially in early breast cancer remains unclear. There are no reports or studies on the presence of CTCs in ductal carcinoma in situ (DCIS) (Graves & Czerniecki, 2011). Numerous ongoing trials and studies are attempting to shed more light on this field. The Southwest Oncology group aims to determine the clinical value of CTC analyses for monitoring therapies. They are addressing the question of how beneficial serial CTC counts are for non-responding patients to the first-line of chemotherapy to determine if they should be placed on an alternative treatment regimen. If elevated CTCs are indeed informative, then enumeration assays could potentially replace the existing option of waiting for clinically detectable evidence of progressive disease. The DISMAL project hopes to identify new prognostic or therapeutic targets via the molecular characterization of CTCs and primary tumours. They aim to determine if there is a correlation between the profiles of primary tumours and the occurrence of CTCs, and if so are these profiles informative for processes responsible for early tumour cell dissemination? The SUCCESS trial indicates that CTCs do in fact play a prognostic role in early breast cancer, where the presence of just one single CTC has the ability to predict poor disease-free survival, distant disease-free survival, as well as overall survival. Various other ongoing trials are asking questions such as what similarities CTCs have with matched primary cancers, and whether the Her2 status of CTCs can predict a patient's response to trastuzumab, especially in those cases where the primary tumour has been removed and CTCs persist (Pantel et al., 2008)?

The current state of technologies in this field does not allow for the differentiation between CTCs of prognostic value or metastatic potential from those that are merely in circulation for a few hours destined for apoptosis or destruction by the immune system. Less than 0.01% of CTCs in circulation will lead to overt metastasis. CTCs that escape immune surveillance, or adjuvant treatments are believed to be a more aggressive subpopulation of chemoresistant, stem-cell like (CD44+/CD24-) tumour cells. There have been reports on the low concordance between markers such as Her2, ER/PR and EGFR expression in CTCs compared to primary tumours from the same patient. Furthermore, EMT and stem cell characteristics, such as expression of Twist, and ALDH1 are detectable in some CTCs of metastatic breast cancer patients. Expression of EMT markers is what allows cells to evade apoptosis, migrate to distant sites and develop resistance to therapies (Kasimir-Bauer 2009). The property of heterogeneity seen in primary tumours seems to apply to CTCs as well and has very important implications with respect to treatment alterations over the course of the disease. The other side of this argument however is that if breast cancer is heterogeneous, how useful is information derived from a single CTC? Essentially, this question is not too different from one that is asked of small biopsy samples taken from one area of a heterogeneous primary tumour. How representative is a biopsy of the true systemic cancer? Discrepant HER2 amplification found between primary tumours, metastatic tumours, and CTCs raise further questions about the current protocols used in diagnosis and treatment

decisions. New technologies like deep sequencing might enable us to delve into the true heterogeneity between single cancer cells. It will also allow us to study cancer cell line models, and determine how similar single cells from cancer cell lines really are, are they different when grown as tumours in vivo and do they produce genetically different tumours? Molecular profiling studies will help researchers to investigate this issue further.
Overall, we are not yet at a point where we can clearly list the biological or molecular characteristics that define a CTC. We first need to standardize methods of analyses of CTCs to advance its utility in the clinic. There is a substantial amount of research interest in this field, and newer, better technologies enter the market steadily. It is entirely possible that different technologies are currently detecting different subsets of the CTC population (Nelson, 2010). What role does time of sampling, amount of sample drawn or individual patient characteristics have on the quality of the assay, are a few of the questions that will need to be tackled. More studies need to be developed around CTCs present in early breast cancer, and their presence and usefulness should be correlated with clinicopathological data, and gene expression data derived from currently recommended breast cancer prognostic markers and tests, for eg: Mammaprint and Oncotype Dx (Krishnamurthy et al., 2010). CTCs have the potential of being established as a reliable prognostic or diagnostic biomarker for early breast cancer, progression to metastasis, response to treatment, and development of anti-metastatic drugs. Given the current interest and impetus in CTC research and technology, this milestone could be reached in the near future.

6. Acknowledgement

We acknowledge the Canadian Breast Cancer Foundation, Ontario Region, for funding the research project grants of SJD and the doctoral fellowship of NK, to work on the characterization of the early molecular events and circulating tumour cells associated with breast cancer metastasis. We would like to thank Dr. Mark Clemons, Dr. Philippe Bedard, Dr. Robert Buckman and Manoj Mathew for their contribution in recruiting and consenting patients to our study. Our deep gratitude goes out to all of the patients at the Princess Margaret Hospital, Toronto, that have, and continue to enthusiastically provide us with blood samples for our study. We would also like to acknowledge all of the members of Susan Done's laboratory for their encouragement and supportive conversations that were essential in the development of our project.

7. References

Aktas, B. et al., 2009. Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Research: BCR*, 11(4), p.R46.

Ashworth, T.R., 1869. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. *Australian Medical Journal*, 14, pp146-147.

Bidard, F.-C. et al., 2010. Single circulating tumor cell detection and overall survival in nonmetastatic breast cancer. *Annals of Oncology: Official Journal of the European Society for Medical Oncology / ESMO*, 21(4), pp.729-733.

Bidard, F.-C. et al., 2008. Prognosis of women with stage IV breast cancer depends on detection of circulating tumor cells rather than disseminated tumor cells. *Annals of*

Oncology: Official Journal of the European Society for Medical Oncology / ESMO, 19(3), pp.496-500.

Braun, S. et al., 2005. A pooled analysis of bone marrow micrometastasis in breast cancer. *The New England Journal of Medicine*, 353(8), pp.793-802.

Chiang, A.C. & Massagué, J., 2008. Molecular basis of metastasis. *The New England Journal of Medicine*, 359(26), pp.2814-2823.

Cristofanilli, M. et al., 2007. Circulating tumor cells in metastatic breast cancer: biologic staging beyond tumor burden. *Clinical Breast Cancer*, 7(6), pp.471-479.

Cristofanilli, M. et al., 2004. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *The New England Journal of Medicine*, 351(8), pp.781-791.

Cristofanilli, M. et al., 2005. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 23(7), pp.1420-1430.

Fehm, T. et al., 2009. Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. *Breast Cancer Research: BCR*, 11(4), p.R59.

Fehm, T. et al., 2010. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. *Breast Cancer Research and Treatment*, 124(2), pp.403-412.

Fidler, I.J., 2003. The pathogenesis of cancer metastasis: the "seed and soil" hypothesis revisited. *Nature Reviews. Cancer*, 3(6), pp.453-458.

Flores, L.M. et al., 2010. Improving the yield of circulating tumour cells facilitates molecular characterization and recognition of discordant HER2 amplification in breast cancer. *British Journal of Cancer*, 102(10), pp.1495-1502.

Gerges, N., Rak, J. & Jabado, N., 2010. New technologies for the detection of circulating tumour cells. *British Medical Bulletin*, 94, pp.49-64.

Gertler, R. et al., 2003. Detection of circulating tumor cells in blood using an optimized density gradient centrifugation. *Recent Results in Cancer Research. Fortschritte Der Krebsforschung. Progrès Dans Les Recherches Sur Le Cancer*, 162, pp.149-155.

Gijs, A.M., 2004. Magnetic bead handling on-chip: new opportunities for analytical applications. *Microfluid Nanofluid*. 1.pp 22-40.

Gradilone, A. et al., 2011. Circulating tumor cells (CTCs) in metastatic breast cancer (MBC): prognosis, drug resistance and phenotypic characterization. *Annals of Oncology: Official Journal of the European Society for Medical Oncology / ESMO*, 22(1), pp.86-92.

Graves, H. & Czerniecki, B.J., 2011. Circulating tumor cells in breast cancer patients: an evolving role in patient prognosis and disease progression. *Pathology Research International*, 2011, p.621090.

Hard, R.C., Jr, Miller, W.G. & Romagnoli, G., 1989. A background-free assay for cells forming antibody to glucose oxidase coupled with an enzyme-linked immunosorbent assay (ELISA) to quantitate antibody secretion. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*, 37(6), pp.909-912.

Harris, L. et al., 2007. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 25(33), pp.5287-5312.

Hüsemann, Y. et al., 2008. Systemic spread is an early step in breast cancer. *Cancer Cell*, 13(1), pp.58-68.

Iakovlev, V.V. et al., 2007. Quantitative detection of circulating epithelial cells by Q-RT-PCR. *Breast Cancer Research and Treatment*, 107(1), pp.145-154.

Ignatiadis, M. et al., 2011. HER2-positive circulating tumor cells in breast cancer. *PloS One*, 6(1), p.e15624.

Kasimir-Bauer, S., 2009. Circulating tumor cells as markers for cancer risk assessment and treatment monitoring. *Molecular Diagnosis & Therapy*, 13(4), pp.209-215.

Kraan, J. et al., 2011. External quality assurance of circulating tumor cell enumeration using the CellSearch(®) system: a feasibility study. *Cytometry. Part B, Clinical Cytometry*, 80(2), pp.112-118.

Krishnamurthy, Savitri et al., 2010. Detection of minimal residual disease in blood and bone marrow in early stage breast cancer. *Cancer*, 116(14), pp.3330-3337.

Liu, M.C. et al., 2009. Circulating tumor cells: a useful predictor of treatment efficacy in metastatic breast cancer. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 27(31), pp.5153-5159.

Marrinucci, D. et al., 2007. Case study of the morphologic variation of circulating tumor cells☆. *Human Pathology*, 38(3), pp.514-519.

Nagrath, S. et al., 2007. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*, 450(7173), pp.1235-1239.

Nelson, N.J., 2010. Circulating tumor cells: will they be clinically useful? *Journal of the National Cancer Institute*, 102(3), pp.146-148.

Pantel, K., Brakenhoff, R.H. & Brandt, B., 2008. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nature Reviews. Cancer*, 8(5), pp.329-340.

Perou, C.M et al., 1999. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proceedings of the National Academy of Sciences of the United States of America*, 96(16), pp.9212-9217.

Perou, C.M. et al., 2000. Molecular portraits of human breast tumours. *Nature*, 406(6797), pp.747-752.

Pinzani, P. et al., 2006. Isolation by size of epithelial tumor cells in peripheral blood of patients with breast cancer: correlation with real-time reverse transcriptase–polymerase chain reaction results and feasibility of molecular analysis by laser microdissection. *Human Pathology*, 37(6), pp.711-718.

Przybytkowski, E. et al. 2011. The use of ultra-dense array CGH analysis for the discovery of micro-copy number alterations and gene fusions in the cancer genome. *BMC Medical Genomics*, 4(16).

Riethdorf, S. et al., 2007. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clinical*

Cancer Research: An Official Journal of the American Association for Cancer Research, 13(3), pp.920-928.

Ring, A.E. et al., 2005. Detection of circulating epithelial cells in the blood of patients with breast cancer: comparison of three techniques. *British Journal of Cancer*, 92(5), pp.906-912.

Sandri, M.T. et al., 2010. Changes in circulating tumor cell detection in patients with localized breast cancer before and after surgery. *Annals of Surgical Oncology*, 17(6), pp.1539-1545.

Schardt, J.A. et al., 2005. Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. *Cancer Cell*, 8(3), pp.227-239.

Schmidt-Kittler, O. et al., 2003. From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. *Proceedings of the National Academy of Sciences of the United States of America*, 100(13), pp.7737-7742.

Shadeo, A. et al., 2006. Comprehensive copy number profiles of breast cancer cell model genomes. *Breast Cancer Research*. 8(1),R9.

Sieuwerts, A.M. et al., 2008. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. *Breast Cancer Research and Treatment*, 118(3), pp.455-468.

Smirnov, D.A. et al., 2005. Global gene expression profiling of circulating tumor cells. *Cancer Research*, 65(12), pp.4993-4997.

Sørlie, T. et al., 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America*, 98(19), pp.10869-10874.

Sørlie, T. et al., 2006. Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms. *BMC Genomics*, 7, p.127.

Stott, S.L. et al., 2010. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proceedings of the National Academy of Sciences of the United States of America*, 107(43), pp.18392-18397.

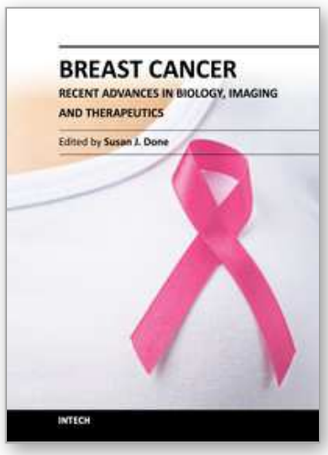
Talasaz, A.H. et al., 2009. Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. *Proceedings of the National Academy of Sciences of the United States of America*, 106(10), pp.3970-3975.

Van der Auwera, I. et al., 2010. Circulating tumour cell detection: a direct comparison between the CellSearch System, the AdnaTest and CK-19/mammaglobin RT-PCR in patients with metastatic breast cancer. *British Journal of Cancer*, 102(2), pp.276-284.

van de Vijver, M.J. et al., 2002. A gene-expression signature as a predictor of survival in breast cancer. *The New England Journal of Medicine*, 347(25), pp.1999-2009.

Weigelt, B., Peterse, J.L. & van 't Veer, L.J., 2005. Breast cancer metastasis: markers and models. *Nature Reviews. Cancer*, 5(8), pp.591-602.

Wikman, H., Vessella, R. & Pantel, K., 2008. Cancer micrometastasis and tumour dormancy. *APMIS: Acta Pathologica, Microbiologica, Et Immunologica Scandinavica*, 116(7-8), pp.754-770.



Breast Cancer - Recent Advances in Biology, Imaging and Therapeutics

Edited by Dr. Susan Done

ISBN 978-953-307-730-7
Hard cover, 428 pages
Publisher InTech
Published online 14, December, 2011
Published in print edition December, 2011

In recent years it has become clear that breast cancer is not a single disease but rather that the term encompasses a number of molecularly distinct tumors arising from the epithelial cells of the breast. There is an urgent need to better understand these distinct subtypes and develop tailored diagnostic approaches and treatments appropriate to each. This book considers breast cancer from many novel and exciting perspectives. New insights into the basic biology of breast cancer are discussed together with high throughput approaches to molecular profiling. Innovative strategies for diagnosis and imaging are presented as well as emerging perspectives on breast cancer treatment. Each of the topics in this volume is addressed by respected experts in their fields and it is hoped that readers will be stimulated and challenged by the contents.

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen