TITLE: **DETECTION OF CIRCULATING TUMOR CELLS IN THE PERIPHERAL BLOOD OF SOLID TUMOR PATIENTS**

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1. BACKGROUND

A considerable body of evidence indicates that tumor cells are shed from a primary tumor mass at the earliest stages of malignant progression. Some of these cells will travel via the Peripheral Blood (PB) to sites anatomically distant from the primary tumor and form metastases. As individual disseminated tumor cells present in low numbers, they can be occult to standard methods of investigation. However, these Circulating Tumor Cells (CTCs) are understood to be a source of eventual lethal metastases, the major cause of treatment failure in cancer patients. Detection of these cells is a promising method for both diagnosis and clinical management of cancer patients.

Many studies have concluded that the presence of CTCs provides important prognostic information predictive of disease-free and overall survival in various malignancies. By contrast, other reports have found no statistically significant relationship between CTC detection and prognosis. This discrepancy is most likely because even in metastatic patients, the frequency of CTCs is extremely low (estimated to be in the range of 1-10 CTCs/ml of PB). While existing technology can identify these CTCs and correlate them with disease, no method has sufficient sensitivity to reliably measure a statistically significant number of cells, especially at early stages of the disease where treatment can be most effective. We hypothesize that the issue of varying conclusions regarding the prognostic significance of CTCs may be attributed to the technical difficulties associated with the reliable detection of such rare events.

Several CTC detection methods have been explored and described in the literature. Each of these methods relies on either a difference in protein expression or in genetic composition between the tumor cells and the normal hematopoietic cells. At present, cell-based detection with Immuncytochemistry (ICC) and molecular detection with RT-PCR are considered to be the gold-standard methods of CTC detection. However, cell-based

detection is constrained by the absence of a means of detection that is both efficient and robust. Consequently, ICC approaches require additional, typically complicated and almost certainly cell-damaging methods of enrichment to achieve adequate speed and sensitivity. RT-PCR-based amplification of transcripts present in cancer cells, but not in surrounding non-neoplastic cells, is increasingly applied to sensitively detect CTCs in clinical samples. Theoretically, the RT-PCR-based amplification of tumor/tissue -specific mRNA sequences would be an ideal method of CTC detection, but the lack of morphological confirmation of the malignant cytologic features of the cells in question, the difficulties associated with quantitation of low transcript levels, and illegitimate transcription of the respective markers significantly limit the use of these assays. In summary, each of the existing technologies has critical limitations for CTC detection, characterization and quantification. In order to implement screening for CTCs into current risk classification systems and treatment protocols and to determine the true clinical significance of CTCs the evident technical difficulties, however, have to be solved.

2. AIMS

A. General aims

The main goal of our work was to develop and improve approaches for CTC detection in the PB of cancer patients and to use this technology in patient studies to investigate the prevalence and characteristics of CTCs. The aims were to build methods that provide improved sensitivity and specificity of CTC detection while meets the requirements of a practical clinical CTC assay. Both cell-based and molecular methods of CTC detection were investigated for areas of improvement. In addition, steps of pre-analytical and analytical procedures were dissected in order to identify potential sources of inaccuracy and irreprocubility. The developed instrumentations and preparation methods were used to investigate the prevalence of various rare cells, such as CTCs and Cytomegalovirus (CMV)-infected

leukocytes. Furthermore, together with four different European laboratories a pilot study was conducted to assess the variation and inconsistencies of CTC detection and to formulate the basis of a standardized, quantitative CTC detection protocol.

B. Specific Aims

The subject of this thesis is based on five different studies:

Specific aims of Study #1

1. To assess the effect of various sample preparation procedure on the real-time quantitative RT-PCR (qRT-PCR)-based detection of CTCs.
2. To assess the effect of various sample preparation procedure on the real-time qRT-PCR-based quantification of CTCs.

Specific aims of Study #2

1. Define the features and boundaries of conventional and real-time qRT-PCR detection.
2. Compare the capabilities of conventional PCR controls and Crossing Point (CP) values to identify false negativity.
3. To validate real-time qPCR detection at its most sensitive level.

Specific aims of Study #3

1. To test reproducibility of CTC detection between different laboratories using a standardized real-time qRT-PCR-based protocol
2. To investigate the influence of sample processing, RNA isolation, cDNA synthesis and PCR amplification in different laboratories.
3. To compare qualitative and quantitative results of real-time qRT-PCR-based detection between laboratories.
4. To test the feasibility of standardization of pre-analytical and analytical methods across laboratories.

Specific aims of Study #4

1. To analyze PB samples from Small Cell Lung Cancer (SCLC) patients for the presence of CTCs with the Rare Event Imaging System (REIS).
2. To characterize CTCs with dual-marker immunofluorescence technique.

Specific aims of Study #5

1. To evaluate the feasibility of combining automated microscopy with classical image processing and machine learning to detect fluorescently labeled rare cells.
2. To attempt to improve the performance of rare event image cytometry through the incorporation of artificial intelligence methods for data classification.
3. To test the accuracy of this novel detection methodology on CMV-infected cells in isolated PB leukocytes.

3. METHODS

In Studies#1-3 we used a newly developed real-time, hybridization probe format, quantitative RT-PCR assay (LightCycler-CK20 Quantification Kit, Roche Diagnostics) for the reproducible and quantitative investigation of the putative gastrointestinal epithelia-specific marker, Cytokeratin (CK) 20, in a series of healthy donor blood samples with added colorectal cancer cells (HT29). We added, per ml of PB, 1,000, 100, 10, 1, and 0 HT29 colorectal carcinoma cells by dilution and micromanipulation. In Study#2 we prepared, by serial dilution, sample series comprising 10,000, 1,000, 100, 10, and 1 plasmid DNA copy for CK20, and two different housekeeping genes (PBGD, and B2M) and 20,000, 2,000, 200, 20, and 2 pg of SK29-Mel-1 (melanoma cell line) RNA. Three different enrichment procedures were employed in these studies; Density Gradient (DG) separation (Histopaque-1077, Sigma), and two distinctive Immunomagnetic Enrichment (IME) techniques (Epithelial Enrich, Dynal Biotech, and CellSearch Epithelial Cell Enrichment, Immunicon). In Studies#4-5 we used our automated microscope system, the REIS, for the detection and analysis of cancer cells in PB of cancer patients and for the sensitive and specific detection of CMV-infected cells. The REIS consists of an automated epifluorescence microscope (Nikon Eclipse 1000, Nikon) with a computer-controlled stage (Ludl), automated objective turret, filter cube changer and shutter, as well as a high resolution black-and-white cooled CCD-camera (Sensicam,

Cooke) and a personal computer. For rare event detection, the REIS make use of proprietary software allowing for fast and highly sensitive fluorescent color detection, along with the capability for the analysis of a variety of morphometric features. After ammonium chloride-mediated erythrocyte lysis, PB samples from SCLC patients and healthy controls were labeled with a dual marker indirect immunofluorescence procedure targeting CKs, the intermediate filaments of epithelial cells and disialoganglioside GD2, the predominant ganglioside expressed on the surface of SCLC cells. For the detection of CMV-infected cells PB samples from immunocompromised patients and healthy controls were labeled by a cocktail of antibodies targeting the CMV lower matrix phosphoprotein (pp65), an early antigen in virus replication that is abundantly present in most antigen positive cells. In Study#5 the REIS detected the fluorescently labeled cells with a two-step procedure. The first step consisted of applying previously discovered finding parameters to acquired microscope images to differentiate cells and cell-like objects from obvious artifacts. In the second step an in-depth analysis of digitally measured cytomorphometric features of potential positive cells was perfomed via Decision Tree (DT) analysis. The objects were then classified according to the readout (true- or false-positive) of the DT analysis.

4. RESULTS

From the results of Study#1 we concluded that sample preparation appeared to have little effect on the lower limit of detection with all preparations allowing sensitive detection of 1 tumor cell/ml. Nevertheless, from the results of this study, it appears for the detection of low cell numbers, analysis of triplicate preparations are required to approach a 100% probability of detection. To assess the effects of sample preparation on PCR quantification, we restricted our analysis to the 1,000 and 100 tumor cells/ml samples to avoid complications arising from poor quantitative reproducibility at low copy numbers. Analysis of these samples demonstrated two distinct

quantitative results. For DG enriched and non-enriched samples, the relative CK20 ratio decreased with decreasing cell number, suggesting that results from these preparations may be useful for indicating tumor cell load. In contrast, the relative CK20 ratio for the IME preparations remained constant for the same series at the same level as the calibrator. As the added cells targeted by IME were identical with the calibrator cell RNA, these results suggest that relative quantification of IME preparations may be useful for providing an indication of tumor cell identity. An important finding from Study#1 is that, rather than sample preparation, the statistical chance of capturing low cell and/or copy numbers may be a more significant determinant of assay sensitivity. Whether these problems are causes of cell loss during preparation or inconsistent target-copy capture were examined in Study#2 using replicates of various amounts of DNA and RNA samples. In agreement with previous findings, our results showed inconsistent detection and reduced CP reproducibility for the detection of samples at low concentrations. Moreover, our results also demonstrated a loss of linearity between CP values and concentration at low levels for which, to our knowledge, experimental evidence has not been provided for previously. Results suggested that the onset of poor CP reproducibility, loss of linearity between CP values and concentration and inconsistent detection each coincided within specific ranges of CP values. Thus we hypothesized that CP values may be more accurate controls of analysis reliability than conventional PCR controls due to their quantitative nature and direct relationship to specific gene levels. Based on this we devised a model in which the different features of unreliability could be mapped into different “zones” of PCR reliability with CP values as boundaries for each zone. We devised a second model for using CP values to assess the adequacy of a sample to provide a reliable analysis of a specific target gene, particularly when the target gene was likely to be inconsistently or not detected. Testing of these models in our sample series demonstrated their validity. Based on the insights that we gained from Study#1 and #2 we designed and

performed an inter-laboratory study (Study#3), to assess the influence of pre-analytics, like blood-sample processing, RNA-isolation, and cDNA synthesis, on the variation of RT-PCR-based detection of CTCs in the PB. Four different European laboratories participated in this study using the same standardized protocol for RNA isolation and quantitative RT-PCR. Using this approach fairly comparable data between different laboratories, showing CK20 detection without failure in spiked samples even at low frequencies of tumor cells and increasing CK20 quantitative signals with increasing numbers of spiked tumor cells, were obtained. However, more variation was introduced by the blood sample preparation method and in the spiked PB samples series there was no clear relationship between the number of spiked tumor cells and the normalized CK20 ratio. Although in three of seven runs, the detected CK20 expression levels in the samples with 100 tumor cells/ml were elevated compared with the samples with 10 tumor cells/ml, and the expression levels in the samples with 10 tumor cells/ml were elevated compared with the samples with 1 tumor cell/ml. Results from Study#4 show that REIS-assisted microscopy is a sensitive and highly reproducible method for the detection, enumeration, and characterization of CTCs in PB samples. When the REIS system was tested on PB samples from SCLC patients single cells and clusters of cells labeled with CK and GD2 were successfully recovered. We found that almost equal proportions of limited stage and extensive stage SCLC patients (35 vs. 37%, respectively) were tested positive for CTCs in the PB. However, with advanced stage of the disease GD2-positive cells were more frequently recovered (10% vs. 20% of the limited- and extensive -stage disease patients, respectively). During this study we observed that in order to detect all prospective tumor cells, including heterogeneously and/or weakly stained individual tumor cells and cell clusters the finding parameter set of the REIS had to set wide open. This high level of sensitivity leads to the concomitant pick up of fluorescent signals from debris, not originating from truly positive cells. To improve the performance

of the REIS and to reduce the number of raw objects in Study#5 we tested a novel approach for the detection of immunofluorescently labeled CMV-infected cells that combines classical image processing with cost sensitive DT analysis of digitally measured cellular features. The data from this study showed that DT analysis can substantially reduce the number of false-positive detections (64.11%) that were otherwise indistinguishable by classical image processing methods. We showed that this novel method of rare cell identification decreases false-positive detections without considerably diminishing the sensitivity of the assay (94.3% with 10-fold cross-validation, 91.64% with external validation).

5. CONCLUSIONS

In our investigations we intended to improve novel molecular- and cell -based methods of CTC detection as well as to increase our knowledge on CTCs. Our first study (Study#1) suggested that sample preparation may play a role in determining assay specificity and relative PCR quantification concentrations. Assay sensitivity at low cell numbers, however, appeared to be primarily determined by the probability of tumor cell and/or target gene capture. We speculate that the inconsistency of detection at these cell numbers and lack of replicates used by most studies may have made a significant contribution to the controversial results published to date. The lack of reproducibility and adequate guidelines for the standardization of novel molecular CTC assays continues to be a major obstacle to their clinical application. In our second study (Study#2) we have used a selected sample series to demonstrate the concept that CP values may be more accurate controls of analysis reliability than conventional PCR controls. The observations of this study formed the basis of a set of recommendations to improve the reliability of low-level PCR detection and quantitation. Through the application of the procedures and models described in this study, a more intuitive selection of samples and their results and potentially more accurate assessment of biological processes may

be obtained. The value of these models was further validated in our third inter-laboratory study (Study#3). In this study quantitative PCR has been shown to be a powerful tool to evaluate both qualitative and quantitative differences in the detection of CTCs between laboratories. However, even if standardized protocols were used for all of the steps from sample collection to PCR analysis, the total variation in results increased with the number of pre-analytical steps. This study was the first concerted initiative to standardize real-time qRT-PCR methods for tumor cell detection across laboratories. We concluded that comparable results between laboratories may be possible, but a high level of standardization was required, especially in the field of pre-analytics. The results of this pilot study stress the need for further comparative collaborative investigations of the pre-analytical procedures currently used. In subsequent studies we focused our efforts on cell-based methods of CTC detection. In Study#4 we described the development, testing and improvement of a fluorescence-based automated microscope system (the REIS) that offers a sensitive and reliable assessment of rare cells. We showed that the REIS approach presents a superior alternative to manual microscopic detection and provides an opportunity of high-content cellular analysis of fluorescently labeled rare cells. We demonstrated the methodological power of the new automated rare event analyzer using model systems and clinical samples from SCLC patients. The approach presented herein may enable an improved method for screening of PB samples for CTCs and for obtaining novel information about disease staging and about risk evaluation in cancer patients. However, based on the findings of this study we also realized the shortcomings of this generation of REIS system, the major one being the tedious manual review of the often enormously large image gallery set of collected objects of prospective positive cells. Therefore, our next study (Study#5) was addressing the development of a novel method, compatible with REIS analysis, for recognition of rare cells. To our knowledge, this study represents the first attempt to improve the accuracy of rare event image cytometry through the

implementation of artificial intelligence methodology. Results suggest that cost-sensitive DT analysis of digitally measured cellular features vastly improves the performance of rare event image cytometry. The possibility for screening more cells with an automated system may lead to earlier diagnosis and more accurate therapy monitoring of infectious and cancerous diseases. The earlier and more accurate determination of rare cells can potentially improve prognostication and through the modification of therapy improve the morbidity and survival of these diseases.

In summary, the lack of reliable and standardized method to detect CTCs is a clear barrier to the clinical implementation of CTC detection. As a result, there is an important need to continue further studies that involve improved and standardized techniques. We hope that our studies provided additional insights into the molecular- and cell- based methods of CTC and rare cell detection and this additional knowledge will be translated into clinically useful tests in the near future.

6. SUMMARY

A considerable body of evidence indicates that tumor cells are shed from a primary tumor mass at the earliest stages of malignant progression. Some of these cells will travel via the peripheral blood to sites anatomically distant from the primary tumor and form metastases. As individual disseminated tumor cells present in low numbers, they can be occult to standard methods of investigation. However, these circulating tumor cells (CTCs) are understood to be a source of eventual lethal metastases, the major cause of treatment failure in cancer patients.

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likely because even in metastatic patients, the frequency of CTCs is extremely low (estimated to be in the range of 1-10 CTCs/ml of PB). We hypothesize that the issue of varying conclusions regarding the prognostic significance of CTCs may be attributed to the technical difficulties associated with the reliable detection of such rare events.

The main goal of our work was to develop and improve approaches for CTC detection in the PB of cancer patients and to use this technology in patient studies to investigate the prevalence and characteristics of CTCs. The aims were to build methods that provide improved sensitivity and specificity of CTC detection while meets the requirements of a practical clinical CTC assay. Both cell-based and molecular methods of CTC detection were investigated for areas of improvement. In addition, steps of pre-analytical and analytical procedures were dissected in order to identify potential sources of inaccuracy and irreprocubility. The developed instrumentations and preparation methods were used to investigate the prevalence of various rare cells, such as CTCs and Cytomegalovirus (CMV)-infected leukocytes. Furthermore, together with four different European laboratories a pilot study was conducted to assess the variation and inconsistencies of CTC detection and to formulate the basis of a standardized, quantitative CTC detection protocol.

Results from our investigations provided novel insights into the molecular and cell -based detection and quantitation of rare cells. Through the application of the procedures and models described in this thesis a potentially more accurate assessment of low level signals may be obtained. This would facilitate the earlier diagnosis and more accurate therapy monitoring of cancerous diseases.

7. PUBLICATIONS AND ABSTRACTS

A. Publications related to this thesis

1. Kraeft SK, Ladanyi A, Galiger K, Herlitz A, Sher AC, Bergsrud DE, Even G, Brunelle S, Harris L, Salgia R, Dahl T, Kesterson J, Chen LB. Reliable and sensitive identification of occult tumor cells using the improved rare event imaging system. Clin Cancer Res. 2004 May 1;10(9):3020-8.
IF: 5.623

2. Ladanyi A, Sher AC, Herlitz A, Bergsrud DE, Kraeft SK, Kepros J, McDaid G, Ferguson D, Landry ML, Chen LB. Automated detection of immunofluorescently labeled cytomegalovirus-infected cells in isolated peripheral blood leukocytes using decision tree analysis. Cytometry A. 2004 Apr;58(2):147-56.
IF: 1.061

3. Soong R, Ladanyi A. Improved indicators for assessing the reliability of detection and quantification by kinetic PCR. Clin Chem. 2003 Jun;49(6 Pt 1):973-6.
IF: 5.538

4. Ma PC, Blaszkowsky L, Bharti A, Ladanyi A, Kraeft SK, Bruno A, Skarin AT, Chen LB, Salgia R. Circulating tumor cells and serum tumor biomarkers in small cell lung cancer. Anticancer Res. 2003 Jan-Feb;23(1A):49-62.
IF: 1.347

5. Vlems FA, Ladanyi A, Gertler R, Rosenberg R, Diepstra JH, Roder C, Nekarda H, Molnar B, Tulassay Z, van Muijen GN, Vogel I. Reliability of quantitative reverse-transcriptase-PCR-based detection of tumour cells in the blood between different laboratories using a standardised protocol. Eur J Cancer. 2003 Feb;39(3):388-96.
IF: 3.694

6. Molnar B, Ladanyi A, Tanko L, Sreter L, Tulassay Z. Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. Clin Cancer Res. 2001 Dec;7(12):4080-5.
IF: 5.076

7. Ladanyi A, Soong R, Tabiti K, Molnar B, Tulassay Z. Quantitative reverse transcription-PCR comparison of tumor cell enrichment methods. Clin Chem. 2001 Oct;47(10):1860-3.
IF: 4.371

B. Abstracts related to this thesis

1. Ladanyi A, Sher A, Kraeft SK, Herlitz A, Bergsrud D, Jafri N, Salgia R, Chen LB. Detection of Occult Tumor Cells in the Peripheral Blood of Small Cell Lung Cancer Patients with the Rare Event Imaging System. 2003: American Society of Clinical Oncology meeting, Chicago, IL, USA, Oral Presentation

2. Molnar B, Vlems FA, Ladanyi A, Gertler R, Rosenberg R, Diepstra HS, Röder C, Nekarda H, Tulassay Z, Muijen van GNP, Vogel I. Reliability of quantitative RT-PCR-based detection of tumor cells in blood between different laboratories using a standardized protocol. 2003: Digestive Disease Week meeting of the American Gastroenterological Association, Orlando, FL, USA

3. Ladanyi A, Lechner S, Ballhorn W, Molnar B, Tulassay Z, Kullmann F. Molecular Comparison of Circulating Tumor Cells and Primary Tumors with cDNA Chip Analysis and Differential Display in Colorectal Cancer Patients: A Feasibility Study. 2002: Digestive Disease Week meeting of the American Gastroenterological Association, San Francico, CA, USA, Poster of Distinction Award

4. Molnar B, Floro L, Ruzsovics A, Ladanyi A, Tulassay Z. Quantitative CK20 RT-PCR is in correlation with progression and CA19-9, but not with CEA and CA 72-A immunoassays in the follow-up of Dukes D stage colorectal cancer patients under chemotherapy. 2002: Digestive Disease Week meeting of the American Gastroenterological Association, San Francisco, CA, USA

5. Soong R, Ladanyi A. Detecting low-level expression reliably by real-time RT-PCR. 2002: American Association of Cancer Research meeting, San Francisco, CA, USA

6. Ladanyi A, Kraeft SK, Brunelle S, Even G, Herlitz A, Harris L, Salgia R, Chen LB. Rare Event Imaging System: Identification and Characterization of Occult Solid Tumor Cells 2002: American Association of Cancer Research meeting, San Francisco, CA, USA, Late Breaking Abstract

7. Kraeft SK, Ladanyi A, Brunelle S, Even G, Helritz A, Harris L, Salgia R, Dahl T, Chen LB. Rare Event Imaging System: Identification and Characterization of Occult Tumor Cells. 2002: 2nd National Cancer Institute-EORTC International Meeting on Cancer Diagnostics, Washington DC, USA, Oral Presentation

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C. Other publications

1. Marrinucci D, Bethel K, Bruce RH, Curry DN, Hsieh B, Humphrey M, Krivacic RT, Kroener J, Kroener L, Ladanyi A, Lazarus NH, Nieva J, Kuhn P. Case study of the morphologic variation of circulating tumor cells. Hum Pathol. 2007 Mar;38(3):514-9.
IF: 2.550

2. Ladanyi A, Sipos F, Szoke D, Galamb O, Molnar B, Tulassay Z. Laser microdissection in translational and clinical research. Cytometry A. 2006 Sep 1;69(9):947-60.
IF: 2.115

3. Hsieh HB, Marrinucci D, Bethel K, Curry DN, Humphrey M, Krivacic RT, Kroener J, Kroener L, Ladanyi A, Lazarus N, Kuhn P, Bruce RH, Nieva J. High speed detection of circulating tumor cells. Biosens Bioelectron. 2006 Apr 15;21(10):1893-9.
IF: 3.463

4. Krivacic RT, Ladanyi A, Curry DN, Hsieh HB, Kuhn P, Bergsrud DE, Kepros JF, Barbera T, Ho MY, Chen LB, Lerner RA, Bruce RH. A rare-cell detector for cancer. Proc Natl Acad Sci U S A. 2004 Jul 20;101(29):10501-4.
IF: 10.452

D. Other abstracts

1. Nieva J, Ladanyi A, Bethel K, Curry DN, Hsieh B, Krivacic RT, Kroener J, Kroener L, Kuhn P, Lazarus N, Marrinucci D, Bruce RH. Fast and sensitive detection of occult tumor cells in peripheral blood. 2005: American Association of Cancer Research meeting, Anaheim, CA, USA

2. Seiwert TY, Tretiakova M, Patrick MC, Khaleque M, Husain NA, Ladanyi A, Chen LB, Bharti A, Salgia R. Heat shock protein (HSP) overexpression in lung cancer and potential as a therapeutic target. 2005: American Association of Cancer Research meeting, Anaheim, CA, USA

3. Curry DN, Ladanyi A, Krivacic RT, Hsieh HB, Kuhn P, Ho MY, Chen LB, Bruce RH. Detection of Occult Tumor Cells with Fiber Array Scanning Technology (FAST) at Ultra-high Scan Rates. 2004: AACR-NCI-EORTC International Conference on "Molecular Targets and Cancer Therapeutics", Brussels, Belgium

4. Curry DN, Krivacic RT, Hsieh HB, Ladanyi A, Bergsrud DE, Ho MY, Chen LB, Kuhn P, Bruce RH. High-Speed Detection of Occult Tumor Cells in Peripheral Blood. 2004: Proceed. 26th Ann Intl Conf of IEEE Eng Med Bio Sci