Development of a Novel Tissue-Specific Method to Detect Cytokeratin 20-Positive Circulating Tumor Cells in Metastatic Colorectal Cancer

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Abstract: Introduction: Although many studies have shown the vast potential of circulating tumor cells (CTCs) detection in cancer diagnosis and prognosis, our understanding of their clinical significance is still far from complete. A major obstacle arises from the lack of well-established tumor or tissue-specific markers to detect CTCs by immunocytochemical staining after immunomagnetic enrichment (IE). Methods: We have established the utility of cytokeratin 20 (CK20), a gastrointestinal tract specific marker, for the specific detection and identification of colorectal cancer (CRC) CTCs. This breakthrough was successfully validated in spike-in experiments using CRC cell line models followed by a pilot study which recruited 32 metastatic CRC patients, 25 benign colorectal diseases patients and 27 normal subjects. Results: CK20-positive CTCs were detected in 90% metastatic CRC patients but not in benign colorectal diseases patients and normal subjects using this refined assay. Conclusions: These impressive results have laid the foundation for further development of CK20-positive CTCs as a promising marker in diagnosis, prognostication and treatment monitoring of metastatic CRC.

Keywords: Tissue-specific; cytokeratin 20-positive; circulating tumor cells; metastatic; colorectal cancer

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1. Introduction

Tumors are classified into 2 major categories: benign and malignant. One main difference between benign and malignant tumors is their tendency to metastasize and recur[1]. Before tumor cells metastasize to their target organ, they may circulate in the bloodstream for a short period of time[1]. Therefore, identification of circulating tumor cells (CTCs) can be used to detect malignancy, predict metastasis, evaluate prognosis, assist in the management of cancer patients and monitor for recurrence and metastasis after primary therapy[1]. Two approaches are often used to detect CTCs: 1) molecular-based method to detect a target mRNA expression and 2) cytometric method to isolate and enumerate individual cells[2]. The advantages of the molecular-based method are that it is more sensitive and rapid than the cytometric method[2]. However, it is often criticized for failing to discriminate mRNA expression between CTCs and that from other cell types such as leukocytes and non-tumor epithelial cells present in the patient blood, severely compromising assay specificity[2]. Moreover, the expression of the target mRNA in the cells does not correlate with the actual number of CTCs[3]. On the other hand, cytometric methods currently represent the standard approach for the identification of CTCs because they enable both the morphological examination of malignant phenotype such as nucleus/cytoplasmic atypia (which is impossible with molecular-based methods because cells are lysed to extract RNA) and further characterization on a single-cell level that can help to confirm the malignant nature and invasive potential of the CTCs[4]. On the other hand, weaknesses of cytometric methods include cell loss...
during the various steps in the immunomagnetic enrichment (IE) procedures, technical variations during manual immunocytochemical staining (IS) and failure of general epithelial markers like broad spectrum cytokeratin (CK) and BerEP4 to provide information on the primary tumor origin of the CTCs which is important for appropriate adjuvant and therapeutic treatment of the patients\textsuperscript{[2,4,6-8]}. Despite recent studies having addressed the clinical impact of detecting CTCs in various kinds of cancers, conflicting results continue to emerge which are the results of non-standardized protocols, different reagent kits used, non-specific epithelial markers detected (Table 1). However, the researchers have made an important consensus that cytopathological examination of CTCs after IE with further characterization for their malignant potential, represents a promising approach that may develop into a routine diagnostic test in the future\textsuperscript{[2,4,6-8]}. 

Colorectal cancer (CRC) has the highest incidence of all cancers in Hong Kong (Hong Kong Cancer Registry, 2014). The five-year overall survival rate for CRC decreases with advancing disease stage. The survival rate is 95% for stage I, 87% for stage II, 55% for stage III, and < 5% for stage IV\textsuperscript{[9]}. Surgery is the standard treatment for patients with stage I and II CRC, whereas patients with stage IIb (large volume, invasion of nearby organs) and III (nodal involvement) CRC are at a greater risk of recurrence and often need post-operative adjuvant chemotherapy (additional radiotherapy for rectal cancer). Moreover, survival rate improves in those patients with metastatic CRC who respond to systemic chemotherapy, and selected patients with local bowel recurrence or resectable liver metastasis (or, to lesser extent, lung metastasis) can enjoy long-term disease-free survival after surgery. Therefore, early detection of disease recurrence and metastasis is essential for improving survival.

CK20 is a low-molecular-weight cytokeratin that shows restricted expression in the GI epithelium, urothelium, and Merkel cell\textsuperscript{[10]}, and this profile is maintained in malignant tumours of these cells. In surgical pathology, CK20 protein is expressed in 90-95% of CRC cases and therefore CK20 mRNA is considered a useful marker in the detection of CRC in the blood using reverse-transcriptase PCR (RT-PCR) technique\textsuperscript{[11,12,13]}. However, conflicting results are found (Table 1) which are mainly due to the following factors: a) the transcription of CK20 gene in haematopoietic cells and benign epithelial cells from GI tract, therefore the transcription level of CK20 gene does not necessarily reflect the amount of tumor cells in the blood sample; b) the lack of standardization of the techniques and protocols across laboratories; c) the lack of standardization in the selection of polymerase chain reaction (PCR) primers, the number of PCR cycles and even interpretation of PCR results.

### Table 1. CTCs detected by molecular technologies or immunocytochemical staining in various cancers

<table>
<thead>
<tr>
<th>Types of Cancer</th>
<th>Methodology/ Marker</th>
<th>Volume of blood (mL.)</th>
<th>Number of patients</th>
<th>Positive results in patients (%)</th>
<th>Conclusions of study</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal</td>
<td>Magnetic cell sorting &amp; staining for pan-CK (CK5, 6, 8, 17, 19)</td>
<td>8</td>
<td>59</td>
<td>32</td>
<td>CK positive cell number is associated with tumour grade.</td>
<td>Bilkenroth U, et al. Int J Cancer 2001;92:577-582.</td>
</tr>
</tbody>
</table>

**Abbreviations:** CK20, cytokeratin 20; CK, cytokeratin; RT-PCR, reverse transcription-polymerase chain reaction
results\textsuperscript{[13,14-15]}. As a consequence, consistent comparison between the studies is difficult, and standardization with automation is urgently needed.

Existing prognostic markers of CRC have limitations and more accurate, convenient markers are needed. The widely used serum marker, carcinoembryonic antigen (CEA) is not specific to CRC and is unreliable for the detection of disease recurrence following surgery\textsuperscript{[16]}. Other promising markers such as thymidylate synthase\textsuperscript{[17]}, vascular endothelial growth factor\textsuperscript{[18]}, loss of heterozygosity at 18q\textsuperscript{[19]} and microsatellite instability\textsuperscript{[20]} may be prognostic or predictive of treatment responses in CRC. However, these assays require a tumor specimen, which is not conveniently obtained or attractive to patients because an invasive procedure is required. Imaging modalities such as positron emission tomography scans, magnetic resonance colonoscopy may be useful, but these tests are expensive and not cost-effective for routine post-operative surveillance. Therefore, a non-invasive, cost-effective and accurate detection method is urgently needed.

2. Methodology

Development of a novel tissue-specific method to detect cytokeratin 20-positive CTCs in metastatic CRC.

The clinical significance of CTCs from patients with tumors is still under debate due to conflicting findings between studies\textsuperscript{[21,22]}. With the rapid technological advancements achieved in the last few years, the detection and analysis of CTCs has become more standardized and reliable\textsuperscript{[23]}. A typical example is CTCs detection and enumeration by the CellSearch System, which has recently been recognized to be capable of providing novel prognostic information that allows a defined stratification of risk of death in metastatic breast cancers\textsuperscript{[7]}. This system uses ferrofluids coated with antibodies against epithelial cell adhesion molecule for epithelial cell capture and antibodies targeting CK8, 18 and 19 for epithelial cell identification\textsuperscript{[2]}. However, the limited anti-CK antibody panel used may account for its low sensitivity and specificity when used in certain cancer types such as liver cancer and nasopharynx cancer. Therefore, we hypothesized that CTC detection and enumeration using a specific marker may reflect the patients’ conditions more accurately. In 2005, we detected successfully an especially high level of circulating CK20 mRNA in 33% CRC patients without apparent evidence of metastasis (pN0 in TNM staging system)\textsuperscript{[24]}. Subsequently, more than half of those patients were found to develop metastasis in 2007. Based on this evidence, we further hypothesized that viable CK20-positive CTCs are present in CRC patients which may account for their high incidence of metastasis. We then began to design an assay to detect CK20-positive CTCs using IE and IS approach which, at that time, only detected general epithelial markers like broad spectrum CK and BerEP4 and were not specific to any cancer or tissue types. In order to solve this problem of non-specific detection, we blocked the mouse anti-BerEP4 antibody that linked to magnetic beads using a polyclonal goat anti-mouse antibody (Figure 1).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Principle to detect CK20 antigens of an epithelial cell after blocking of the magnetic beads linked anti-BerEP4 antibody.}
\end{figure}

3. Results

This concept was implemented in the capture of SW480 cells (a CRC cell line) followed by blocking of the mouse anti-BerEP4 antibody and detection of CK20 antigen by IS with anti-CK20 antibody. The results were very impressive because this refined assay not only prevented the visualization of BerEP4 staining, but also exposed the CK20 antigens for staining by anti-CK20 antibody. The successful blocking of the magnetic beads linked antibody has enabled us to detect other tissue specific antigen like CK20. We continued to examine the sensitivity of this novel assay by spiking various quantities of SW480 cells into blood samples from normal subjects where detection limits were found to range from 100,000 to 100 SW480 cells per 10 mL blood. This breakthrough opened up a new avenue for non-invasive cancer detection and monitoring because it potentially allowed detection of all other specific cancer markers in the CTCs after IE. We extended the application of this assay by detecting and enumerating CK20-positive CTCs in 32 patients with metastatic CRC, 25 patients with benign colorectal diseases and 27 normal subjects. We discovered that there was a broad range in the numbers of CK20-positive CTCs (0-377, median = 45 per 10 mL of blood, sensitivity of detection = 90%) in
patients with metastatic CRC whereas no CK20-positive CTCs was found in patients with benign colorectal diseases and normal subjects (Figure 2, the intra- and inter-assay coefficients of variance were 3% and 6%, respectively).

Figure 2. Number of CK20 circulating epithelial cells in 32 patients with metastatic colorectal cancer, 25 patients with benign colorectal diseases and 27 normal subjects. The median is shown by a black horizontal line.

Moreover, we also compared the number of CK20-positive CTCs detected with that of broad spectrum BerEP4-positive CTCs in 10 metastatic CRC blood specimens and discovered that the number of CK20-positive CTCs detected was lower than that of BerEP4-positive CTCs in each of the same patient specimen (Figure 3). These results indicate that CK20 is more specific than the general epithelial marker BerEP4 because anti-CK20 antibody would mainly detect epithelial cells from GI sites and therefore, CK20-positive CTCs may be a promising marker for detecting and monitoring metastatic CRC. With our expertise in developing circulating tumor markers and competence in IS and interpretation [25-27], our team has laid down a solid foundation for further investigation into the significance of CK20-positive CTCs in metastatic CRC detection.

Figure 3. Comparison of the numbers of CK20 circulating epithelial cells with those of BerEP4 circulating epithelial cells detected in 10 metastatic CRC blood samples.

4. Discussion

4.1 Key issues and problems

Molecular based methods such as reverse transcriptase-polymerase chain reaction (RT-PCR) would destroy the CTCs, making it impossible to count or to analyze them individually. Moreover, the lack of standardized protocols and illegitimate expression of target genes in normal hematopoietic cells limit reproducibility between laboratories and hamper their development into routine molecular tests in diagnostic laboratories.

Current IE methods utilize either broad spectrum anti-CK or anti-BerEP4 antibodies which target general epithelial markers found on numerous epithelial tumor and normal cells. Therefore, specific information on the primary tumor type is not available, hindering both therapeutic decision-making and understanding the micro metastatic process in individual tumor types.

The usual practice of manual IS performed after IE is not standardized, leading to increased variability in CTC detection between laboratories.

CK20 is an epithelial protein expressed in the normal epithelial cells of the colorectal tissue and that this profile is maintained in malignant cells. Therefore, CK20 immunostaining can be detected in both normal and malignant cells of the colorectal tissue.

The prognosis for CRC patients with distant metastasis, lymph node involvement and recurrence is very poor. Early detection of metastasis and relapse is essential to improve their survival. Approximately 40% of CRC patients, without evidence of metastatic disease, subsequently develop recurrent disease in 5 years. This shows that the current staging system alone is insufficient for accurate patient prognostication.

4.2 Elements critical to the solution of the problems

We recently overcame the limitation of only detecting general epithelial cells using immunomagnetic beads coupled with anti-BerEP4 antibodies by blocking detection of those antibodies so that anti-CK20 antibody can be used to demonstrate the gastrointestinal (GI) origin of the BerEP4 positive cells in CRC patients (Figure 1). This advancement revolutionized immunomagnetic CTC detection by allowing the use of antibodies against tissue-specific markers so that an accurate diagnosis of the tumor type can be made. This is critical for therapeutic decision making and also enables us to understand the micromet-
astatic process for that particular tumor type. CK20 is a more specific marker than both CK and BerEP4 to detect CRC in the blood because CK20 is expressed only in cells from the GI tract, urothelium and epidermal Merkel cells. This is in stark contrast to the much broader spectrum of expression for CK and BerEP4. Therefore, CK20-positive CTCs may more accurately reflect the CRC patients’ conditions.

Our team designed a standardized protocol composed of IE and IS using an automatic immunostainer and stringent criteria for morphological analysis. These measures would facilitate reproducible results in the staining process and their objective assessment, which are essential in standardization.

It is now recognized that the identification of occult micrometastatic disease in cancer patients would have important roles in the establishment of prognosis, treatment decisions and monitoring of the efficacy of adjuvant treatment. In the past, micrometastatic detection was mainly focused in the bone marrow. However, aspiration of bone marrow is time consuming and uncomfortable for the patient. Therefore, current research now concentrates on detection of tumor cells in the peripheral blood. Our preliminary results of an elevated number of CK20-positive CTCs in metastatic CRC patients but not in patients with benign colorectal diseases and normal subjects can demonstrate that CK20-positive CTCs may have prognostic significance in CRC patients.

4.3 Potential significance of the results

The use of CK20-positive CTCs as a biomarker in detecting metastatic CRC not only facilitates a more specific diagnosis at the circulation level, but also provides a more accurate prognostic information to the clinicians. Identifying CRC patients who are at particularly high-risk of relapse and metastasis non-invasively so that appropriate adjuvant treatment strategies with intensive protocols can be applied to improve the chance of survival. Our breakthrough in successful blocking detection of the anti-BerEP4 antibody linked to the magnetic beads has opened up a new era in CTCs detection because the tumor origin of CTCs can be detected using their respective specific antibodies. Therefore, in the long run, our work may assist clinicians to make a specific diagnosis, predict prognosis accurately and deliver an effective treatment to the patients with various types of CTCs.

Ethics approval: The study was approved by the Clinical Research Ethics Committee of the Queen Elizabeth Hospital, Hong Kong Special Administrative Region.

References

[14] Dandachi N, Balic M, Stanzer S, Halm M, Resel M,


