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Short Communication

The relevance of RT-PCR detection of disseminated tumour cells is hampered by the expression of markers regarded as tumour-specific in activated lymphocytes

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(SCCA)

ABSTRACT

Marker genes, commonly used to detect circulating tumour cells in RT-PCR-based tests: squamous-cell carcinoma antigen, epidermal growth factor receptor, mammaglobin, small breast epithelial mucin, but not carbonic anhydrase 9, were shown to be expressed in normal, mitogen-stimulated peripheral blood mononuclear cells (PBMNC). Thus, considering the inflammatory reactions often accompanying cancer development, to reduce false-positive results of the metastatic tumour cell tests, molecular markers should be validated not against normal peripheral blood, but against activated lymphoid cells, such as *in vitro* mitogen-stimulated PBMNC.

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1. Introduction

The early detection of disseminated disease in asymptomatic cancer patients may help to predict recurrences and to select

more effective treatment regimens. To detect circulating tumour cells (CTC) in cancer patients, high sensitivity reverse transcription-polymerase chain reaction (RT-PCR)-based assays have been developed.¹ However, molecular markers

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used in these assays, the so-called cancer cell markers, present controversial specificity, and false-positive findings of RT-PCR assays are frequent. In effect, the last decade's constant attempts to apply RT-PCR for micrometastasis detection have so far failed to produce a commonly accepted, routinely applied diagnostic method.

The difficulties in developing reliable CTC detection tests may lie in the common, but mistaken assumption that markers of high specificity for CTC are those not detected in normal blood, and thus their specificity is evaluated against their expression in the peripheral blood of healthy volunteers. Yet accumulating evidence indicates that normal tissue is not an adequate control for assessing the specificity of molecular markers used for CTC detection. Already the early stage cancer was shown to affect gene expression patterns in peripheral blood cells.² The symptoms of ongoing inflammatory reactions, such as raised erythrocyte sedimentation rate, elevated concentrations of circulating C-reactive protein, high levels of numerous cytokines and increased percentages of activated T lymphocytes (e.g. CD4+HLA-DR+) are often exhibited by the peripheral blood of cancer patients.^{3–9} Many of those parameters correlate with disease progression and prognosis,^{3–5,7,8} and the systemic inflammatory response has been shown to be a strong independent prognostic factor in patients with advanced cancer.^{10,11}

Considering the above, activated cells are the likely component of the peripheral blood, bone marrow or regional lymph nodes of cancer patients.

Therefore, to verify the specificity of cancer cell detection, we addressed the question as to whether some commonly used molecular markers are inducible in normal peripheral blood mononuclear cells (PBMNC). The following markers were selected for RT-PCR analysis: squamous-cell carcinoma antigen (SCCA), epidermal growth factor receptor (EGFR), mammaglobin (hMAM), small breast epithelial mucin (SBEM) and carbonic anhydrase 9 (CA9).

2. Materials and methods

2.1. Isolation and culture of PBMNC and spleen lymphocytes

PBMNC from three healthy donors (samples 1, 2, 3) were obtained by standard Ficoll-Uroplene gradient centrifugation. For activation, PBMNC were cultured for 12 h and for 6 days in RPMI-1640 medium with L-glutamine (Gibco), supplemented with 10% heat-inactivated FCS (Gibco), and 50 µg/ml of gentamycin (Sigma), with the addition of phytohemagglutinin (PHA 1 µg/ml, Wellcome).

Spleen cells of three patients with immune thrombocytopenic purpura, obtained by mechanical dissection of spleens after therapeutic splenectomy, were centrifuged on Ficoll-Uroplene gradient. Mononuclear cells were cultured for 2 days in RPMI-1640 medium containing L-glutamine (Gibco), supplemented with 50 µg/ml of gentamycin and with human (to avoid stimulation by xenogenic antigens) heat-inactivated AB serum (10%). Following the culture, lymphocyte enrichment was performed by Nycodenz (Nycomed) gradient centrifugation.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was isolated from approximately 5×10^7 cells of each sample according to Chomczynski and Sacchi.¹² Two µg of the total RNA were reverse transcribed with random hexamer primers, in the final volume of 20 µl, using SuperScript (Invitrogen), according to the manufacturer's instructions. The quality of cDNA preparations was controlled by the PCR of DNA polymerase β (*pol* β) that is a single copy gene (containing no known pseudogenes) expressed in all cell types. Its transcript is rather stable in the cell cycle, and characterised by low-level expression, in contrast to house-keeping gene products commonly used as cDNA controls, such as β -actin and GAPDH. PCR for *pol* β was performed with 0.2 µl of cDNA as follows: 40 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, preceded by 4 min at 95 °C and followed by 7 min at 72 °C. Specific oligonucleotide primers, sense GAT-ATTTTGGGGACTTTGAAA and antisense CTCTTCTGAAAC-TGCCACAG produced 129bp DNA fragment. Subsequently, 2 µl of cDNA was examined for SCCA, EGFR, hMAM, SBEM and CA9 markers by PCR. Specific primers and PCR conditions used were described previously.^{13–17} PCR was performed in a 25 µl mix using Taq Fast Start polymerase (Roche Diagnostics GmbH). The mixture (0.8 µl) after the first round of PCR served as a template for the second round of PCR. For each PCR, a set of control samples included one positive control, and two negative controls (a sample with no template and a sample with the placental DNA). As positive RT-PCR controls, the following human cell lines were used: MCF7 for SCCA, MCF7 and HeLa for EGFR; MCF7, HeLa and A431 for hMAM; MCF7 for SBEM; and A431 and HeLa for CA9.

3. Results and discussion

All the control cDNAs obtained from cell lines were RT-PCR positive for the respective markers. PCR controls with the placental DNA template were negative for all markers tested. The latter control excluded the putative pseudogene detection.

The representative results are shown in Fig. 1. We have demonstrated here that while normal PBMNC (donors 1 and 2) were RT-PCR negative for SCCA, EGFR, hMAM, SBEM and CA9, PHA-stimulated PBMNC, in both early (12 h following stimulation, samples 1S and 2S) and late (6 days following stimulation, sample 3S) phase of stimulation, were positive for SCCA, EGFR, hMAM, and SBEM, but not for CA9. This implies that any condition involving activated cells may contribute to false-positive RT-PCR results. In this respect, of the markers examined, CA9 seems to present potentially the best value for RT-PCR-based CTC detection. However, because of its trace expression in sample 1, its activation-related transcription requires further investigation.

In addition, unstimulated, pooled lymphocytes obtained from spleens of three patients with immune thrombocytopenic purpura expressed SCCA, EGFR and SBEM but not hMAM and CA9 transcripts (Fig. 1).

While the number of publications on the molecular tumour markers continues to grow, our results support and

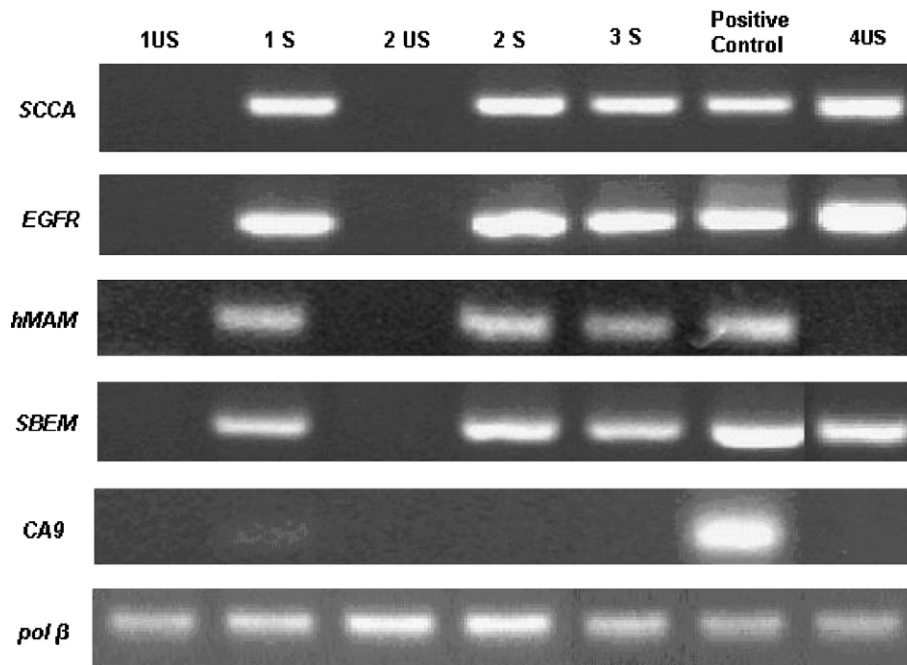


Fig. 1 – One-step RT-PCR for small breast epithelial mucin (SBEM) transcript detection and nested RT-PCR for squamous-cell carcinoma antigen (SCCA), epidermal growth factor receptor (EGFR), mammaglobin (hMAM) and carbonic anhydrase 9 (CA9) transcripts' detection in unstimulated (1US and 2US) and PHA-stimulated (12 h – 1S and 2S, and 6 days – 3S) PBMNC, and in unstimulated pooled lymphocytes obtained from spleens of three patients with immune thrombocytopenic purpura (4US). RT-PCR for each sample was repeated five times except for SCCA that was repeated seven times. *pol β*: PCR product of polymerase β - internal control of cDNA preparation.

extend those of other authors, emphasising the problem of biological factors influencing RT-PCR-based CTC assays. Jung and colleagues¹⁸ have shown that the expression of CK19 and CEA in lymphatic cells is inducible upon cytokine stimulation and found these markers to be expressed in over 50% of bone marrow specimens from patients with chronic inflammatory diseases. Similarly, Krüger and colleagues¹⁹ have demonstrated hMAM expression to be inducible by cytokines in bone marrow and peripheral stem cells from patients without epithelial cancer. Ballestrero and colleagues,²⁰ contrary to the above, have not found hMAM expression in normal peripheral blood and bone marrow cells following cytokine exposition, but have shown maspin expression to be inducible, and identified the transcripts of both markers in normal peripheral blood leukocytes following aphaeretic procedures. Polyclonal mitogen stimulation applied in our study have shown hMAM to be inducible in PBMNC.

Our data provide an explanation for the origin of the so-called 'illegitimate' expression of epithelial cell genes in lymphoid cells. The false-positive results of CTC detection assays may actually result from the presence of activated lymphoid cells due to inflammatory reactions known to accompany cancer development.²¹

In conclusion, in clinical practice, an issue of transcription activation of different markers in inflammatory conditions, underestimated so far, has to be considered as an important factor interfering with RT-PCR assessment of tumour dissemination. RT-PCR, especially nested RT-PCR tests, are so sensitive that we are able to detect one cancer cell within millions of back-

ground cells, but if low-specificity markers are employed, the detected cell might equally be an activated lymphocyte. If so, such assays are of limited prognostic value. Therefore, validating the specificity of molecular markers for RT-PCR-based detection of disseminated tumour cells is a critical step and is dependent on appropriate controls. We emphasise that these controls should consider the inflammation-induced expression of marker genes. The marker expression in non-malignant inflammatory diseases would be informative and this will be an interesting issue for further basic science studies, but it represents an unrealistic approach while validating new molecular markers for metastatic cancer cell detection, as each new marker would require studies of peripheral blood, bone marrow, and nodules of numerous untreated patients. Instead, a simple model of lymphoid cell activation, such as the one shown in this paper, i.e. *in vitro* polyclonally-stimulated normal peripheral blood cells, could serve for testing the specificity of new markers.

Conflict of interest statement

None declared.

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