Reverse transcriptase-polymerase chain reaction analysis of cytokeratin 19 expression in the peripheral blood mononuclear cells of normal female blood donors

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Abstract

Background—Early detection of haematogenous dissemination of epithelial tumours afforded by the analysis of epithelial antigen expression in the peripheral blood mononuclear fraction (PBMN) and bone marrow may confer a worse prognosis to patients with carcinoma. Cytokeratin 19 is a protein normally expressed by epithelial cells including normal and malignant mammary cells. Previous studies have demonstrated that analysis of cytokeratin 19 expression by the reverse transcriptase-polymerase chain reaction (RT-PCR) can detect one epithelial cell in as many as 10^2–10^3 haematopoietic cells. Despite its sensitivity concern has been voiced recently about the specificity of this technique owing to the detection of cytokeratin 19 expression in the PBMN of normal volunteers and the bone marrow of patients with haematological malignancies.

Aims—To assess the sensitivity and specificity of RT-PCR detection of cytokeratin 19 in PBMN of normal female blood donors.

Methods—Blood was taken from 52 normal female blood donors and PBMN separated through Fycol gradient centrifugation. Cytokeratin 19 was measured using a two step nested RT-PCR assay.

Results—No amplification was found in the first step for any of the samples studied, whereas in the second step amplification was observed in 10 of the 52 samples. Both steps could detect one MCF-7 cell (the cytokeratin 19 positive control) in 10^5 CEM (cytokeratin 19 negative control) cells.

Conclusions—As both PCR steps are sensitive to the 10^4 level, performing only the first amplification step may decrease the non-specificity of this method. Further studies are needed to define the specificity and sensitivity of this technique in blood and bone marrow specimens of women with breast cancer.


Detection of epithelial antigen expression in the blood and bone marrow of patients with carcinoma offers the opportunity for early detection of the haematogenous spread of these tumours. The prognostic implications of these findings are under evaluation but preliminary evidence suggests a worse outcome for patients who are found to harbour bone marrow micrometastasis in the context of otherwise clinically limited disease.

Cytokeratin 19 is an intermediate filament protein expressed by normal and malignant mammary cells in addition to other epithelial cells and derived malignancies, such as cancer of the gall bladder and prostate. Haematopoietic cells, however, normally do not express this antigen. Therefore, the detection of cytokeratin 19 expression in the blood and bone marrow of patients with epithelial tumours has been interpreted as a manifestation of the haematogenous dissemination of these tumours.

RT-PCR is a very sensitive technique for the detection of cells expressing a particular gene product. In fact, several authors have described the detection of cytokeratin 19 expressing cells in the blood and bone marrow of patients with breast carcinomas with a sensitivity of 10^4–6.

Recently, concerns about the specificity of RT-PCR detection of cytokeratin 19 expression have been voiced, based on the detection of the expression of this epithelial antigen in the peripheral blood mononuclear fraction (PBMN) and bone marrow of normal controls and patients with haematological malignancies. In an attempt to further elucidate this question, we undertook the analysis of cytokeratin 19 expression by the PBMN cells of 52 normal female blood donors.

Materials and methods

SAMPLES

Blood was obtained through a standard venesection technique from 52 normal female blood donors. The samples used for this study comprised 20 ml of whole blood collected at the time of venesection for blood donation according to the Fundação Hemocentro de São Paulo Blood Bank's protocol. The PBMN cell fraction was separated through Fycol gradient centrifugation as described previously.
denaturation at 94°C for 50 seconds, and primer annealing and chain extension at 72°C for two minutes. Aliquots of 10 μl of the PCR products from each round were electrophoresed on 2% agarose gels and stained with ethidium bromide for further direct visualisation. The primers yielded products of 1069 base pairs in the first round and 745 base pairs in the second round of the PCR technique. RNA integrity and adequate cDNA synthesis was confirmed by RT-PCR, using β₂ microglobulin as an internal control. All reactions were repeated three times.

**Results**

**CYTOKERATIN 19 RT-PCR DILUTION ASSAYS**

The breast cancer cell line MCF-7 was used as a positive control for cytokeratin 19 expression and the lymphoblastoid CEM cell line was used as a negative control. MCF-7 cells were mixed with CEM cells at different proportions for this experiment. Cytokeratin 19 mRNA expression was studied in different dilutions of MCF-7 cells in CEM cell suspensions, ranging from 1:10–1:10⁶ MCF-7 to CEM cells, using the first and second PCR steps outlined in the methods section. We found that both steps could detect one MCF-7 cell in 10⁶ cells (data not shown).

**RT-PCR STUDY OF CYTOKERATIN 19 EXPRESSION IN FEMALE BLOOD DONORS**

We studied the PBMN cell fraction of 52 normal female blood donors using the two step cytokeratin 19 RT-PCR detection protocol for mRNA outlined above. Interestingly, although both PCR steps showed similar sensitivities (to the 10⁻⁵ level) in the aforementioned MCF-7 dilution experiments, cytokeratin 19 mRNA transcripts could not be detected in any of the normal samples in the first step, yielding a specificity of 100%. In the second step, however, in 10 of 52 samples, cytokeratin 19 mRNA transcripts were detected, yielding a specificity of 77% (fig 1).

**Discussion**

Because of its sensitivity, RT-PCR amplification of cytokeratin 19 mRNA is a useful technique for studying minimal breast cancer involvement of blood and bone marrow. The prognostic implications of RT-PCR detected cytokeratin 19 expressing cells in the blood and bone marrow of patients with breast cancer, however, are still under investigation. One of the main reasons for the current scepticism about the value of this technique is the recent RT-PCR amplification of cytokeratin 19 mRNA from the blood of 20% of normal controls and in a patient with chronic myeloid leukaemia. The possibility that haematopoietic cells illegitimately synthesise cytokeratin 19 transcripts has been suggested to explain these findings.

Another explanation for the observed non-specificity could be the incorporation of a second step into the PCR technique. In fact, when we studied both steps in terms of their sensitivity in dilutional assays, we found that both of them could detect one MCF-7 cell in 10⁶ CEM.
RT-PCR analysis of cytokeratin 19 in normal female blood donors

...cells. However, the second step was positive in ~36% of normal controls, whereas the first step of the technique did not detect cytokeratin 19 transcripts in any of the controls studied. In the papers by Data et al. and Krismann et al. it was impossible to tell at which step amplification of cytokeratin 19 transcripts occurred, either in the positive patients or in the false positive controls. It is possible, therefore, that the potential increase in sensitivity provided by a second PCR step may occur at the expense of a lower specificity of the whole assay. In our study, the first step of this technique achieved a degree of sensitivity similar to that described in the literature, with 100% specificity, therefore, the need for a second step may have to be re-evaluated.

Our results were based on a breast cancer cell line (MCF-7) and PBMN cells from normal adult female blood donors. Therefore, in order to extrapolate these results to other types of tumour and patients, further studies need to be carried out using different tumour cell lines and other normal control cell populations. Furthermore, additional studies are needed to confirm our findings and to assess the value of the first step of this technique for the detection of cytokeratin 19 transcripts in the blood and bone marrow of patients with breast cancer.


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