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 10^5–10^7 haematopoetic 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 10^6 CEM (cytokeratin 19 negative control) cells.**

***Conclusions*—As both PCR steps are sensitive to the 10^{-6} 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.**

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Keywords: cytokeratin 19; reverse transcriptase-polymerase chain reaction; breast cancer

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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.[1 2]

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.[3–5] Haematopoietic cells, however, normally do not express this antigen.[6 7] 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.[6]

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^{-6}.[6-8]

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.[6 9] 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.[10]

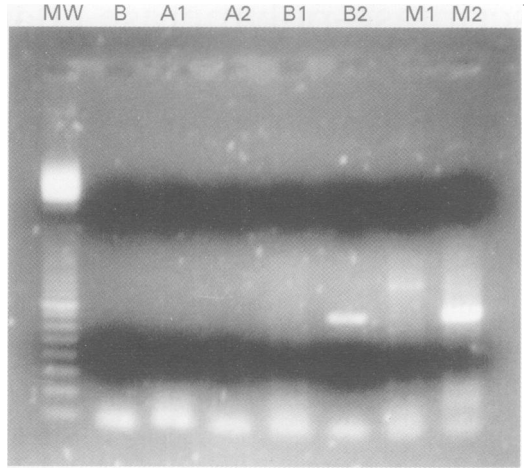

Figure 1 Two step RT-PCR amplification of cytokeratin 19 mRNA in the peripheral blood mononuclear fraction (PBMN) of two normal female blood donors illustrating two representative cases (A and B). MW, molecular weight; B, blank; A1, B1, first step of PCR amplification of PBMN from normal female blood donors A and B, respectively; A2, B2, second step of PCR amplification of the same two blood donors; M1, M2, first and second PCR amplification steps, respectively, of the positive control MCF-7 cell line. Note that B2 is positive only in the second round of amplification.

CELL LINES

MCF-7 and CEM cell lines were obtained from America Type Culture Collection. Cells were maintained at 37°C, 5% CO_2 in RPMI containing 10% fetal bovine serum, 1% penicillin, and streptomycin (Gibco BRL; Gaithersburg, Maryland, USA).

ISOLATION OF RNA AND CDNA SYNTHESIS BY RT-PCR

Total RNA from 10^6 cells was extracted by the monophasic solution of phenol and guanidine isothiocyanate method.[11] The integrity of RNA molecules was monitored on a formaldehyde 1% agarose minigel. One microgram of total RNA was reverse transcribed with Superscript II RNase H-reverse transcriptase (Life Technologies Inc, Gaithersburg, Maryland, USA) using the random primer extension method as recommended by the manufacturer. The cDNA was diluted 1:5 in distilled water and stored at −70°C until further use.

POLYMERASE CHAIN REACTION

Ten microlitres of cDNA was used for first round PCR using 20 pmol of each primer[12]: 5'- AAGCTAACCATGCAGAACCTCACGACCGC-3' and 5'- TTATTGGCAGGTCAGGAGAAGAGCC-3' added to 100 mM dNTP, 5 µl PCR buffer, and 1 U Taq polymerase (Cenbiot, Rio Grande do Sul State, Brazil), in a total volume of 50 µl. Thirty five cycles were performed consisting of 50 seconds denaturation at 94°C, annealing and extension for one minute and 30 seconds at 72°C. For the second round of amplification 5 µl of the previous reaction was added to 20 pmol of nested primers: 5'-TCCCGCGACTACAGCCACTACTACACGGACC-3' and 5'-CGCGACTTGACTTGATGTCCATGGCCGCTGGTTAC-3', 200 mM dNTP, 1.5 mM $MgCl_2$, 5 µl PCR buffer, 1 U Taq polymerase and distilled water up to 50 µl. PCR conditions were 35 cycles of 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 β_2 microglobulin as an internal control.[13] 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 1:10–1:10^6 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 10^6 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^{-6} 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[9] and in a patient with chronic myeloid leukaemia.[6] The possibility that haematopoitic cells illegitimately synthesise cytokeratin 19 transcripts[6 9] 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 10^6 CEM

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*[6] and Krismann *et al*[9] 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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