SPANDIDOS PUBLICATIONS

Detection of isolated tumour cells in the blood and bone marrow of patients with gastric cancer by combined sorting, isolation and determination of MAGE-1, -2 mRNA expression

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Abstract. The detection of isolated (circulating or disseminated) tumour cells (ITC) in patients with cancer requires very sensitive methods, as such cells are very rare. In the present study, the method that combines the negative isolation of $CD45^-$ leukocytes from the blood and bone marrow of patients with gastric cancer by flow cytometry, followed by the positive isolation of single cytokeratin-positive (CK^+) cells by a Laser Capture Microdissection System for the determination of MAGE-1, -2 mRNA expression was used to detect ITC. This study shows that this method is highly sensitive as it allows to determine ß-actin-mRNA expression in a single CK^+ cell. Using ≥5 CK^+ cells as a cut-off level, the MAGE-1 mRNA expression was detected in 100% of CK^+ cells in the peripheral blood and in 75% of bone marrow samples of patients with gastric cancer. The MAGE-2 mRNA expression was observed in 40 and 58% of samples, respectively. Furthermore, an analysis of primary tumours and locoreginal lymph nodes with respect to the mRNA expression of the two genes showed that MAGE-1 mRNA expression was detected in 88% of the primary tumours and in 67% of the lymph node samples, whereas the MAGE-2 mRNA expression was observed in 72 and 67% of the cases, respectively. Thus, the method described here allows the precise and sensitive determination of tumour-associated gene expression in single ITC present in the blood and bone marrow of patients with gastric cancer.

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Introduction

In many types of cancer, isolated (circulating or disseminated) tumour cells (ITC) in the blood and bone marrow are present (1-5). The detection frequency of these cells, in the blood and bone marrow, varies substantially not only in different types, but in the same type of cancer (6). Since these cells are very rare (app. 1 per 10^5 to 1 per 10^7 leukocytes), highly sensitive methods of detection are required.

There have been many attempts to develop techniques, which could be reliably used for the detection of ITC. These included immunocytochemistry with the use of antibodies for different tumour markers, although they are not tumour-specific (7). In conjunction with the antibody staining techniques, reverse transcriptase polymerase chain reaction (RT-PCR) was introduced as a powerful method for further analysis of potentially micrometastasizing tumour cells (8-12). This highly sensitive technique was designed for the detection of mRNA of presumptive tumour markers (13-17). Although this technique seemed ideal for detecting malignant cells, it frequently led to contradictory results to those obtained by immunocytochemistry. One of the many examples of tumour cell markers used are cytokeratins (CK), which are expressed by the epithelial, not necessarily malignant cells (18-22). The CK^+ cells are usually not present either in peripheral blood or bone marrow of normal subjects, though occasionally are detected (6). Data obtained by applying antibody staining and RT-PCR techniques showed contradictory results (13,18). This contradiction questioned the method of choice, however, the results seemed promising. The main downfall of this approach has been the false positive results either in immunocytochemistry or RT-PCR. Although, the former may not be as sensitive as RT-PCR, the cellular source of the mRNA signal cannot be established without it. Furthermore, the false positive mRNA signals may derive from other (non-tumour) cells, e.g. PSA mRNA expressed by blood monocytes (23) or squamous cell carcinoma antigen and epidermal growth factor receptor mRNAs on activated lymphocytes which are present in some cancer patients (24), while false negative signals may arise from the diluting out of specific mRNA by coextracted RNA from other cells.

With the introduction of the Laser Capture Microdissection System (LCM), a new possibility for ITC detection emerged. The LCM system, which is composed of a fluorescent microscope coupled with a laser module, enables the identification and selection of cells of interest by catapulting them out into a test tube lid with a laser impulse (25). By taking advantage of this system, we designed the method that concentrates, identifies CK^+ cells and allows their isolation for further analysis at the molecular level.

Materials and methods

Determination of MAGE-1 and MAGE-2 mRNA expression in tumour cell lines by real-time, nested PCR. Two tumour cell lines, HPC-4 (human pancreatic adenocarcinoma) and DeTa (colorectal adenocarcinoma) were used (26,27). The cell lines were cultured by bi-weekly passages in RPMI-1640 medium with 5% foetal calf serum and 25 μg/ml gentamycin. All cell lines were regularly tested for *Mycoplasma sp*. contamination according to the procedure described by Ziegler-Heitbrock and Burger (28). The total RNA (tRNA) was extracted from $2x10^6$ cells of each tumour cell line by the single-step isolation method using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The first-strand cDNA was obtained from the tRNA samples (2 μg) with M-MLV reverse transcriptase (Sigma, St. Louis, MO) and anchored oligo-dT (Sigma) primer as specified by the manufacturer's protocol. The cDNA was then used for nested PCR. For the first round of PCR, 20 μl reactions, containing 5 μl of cDNA, 2 μl of 10X PCR buffer, 1.2 μl of 25 mM $MgCl_2$, 0.5 μM of each of the outer MAGE-1 and MAGE-2 primer and 2 units of *Taq* DNA polymerase (Sigma) were run in a thermocycler (MJ Research, Watertown, MA). The following cycle profile was used: denaturation at 94˚C for 6 min, annealing at 60˚C for 30 sec and extension at 72˚C for 2 min for the first cycle; denaturation at 93˚C for 40 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 20 sec for 14 cycles; denaturation at 93˚C for 40 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec for 50 cycles; final extension at 72˚C for 2 min. The outer and inner primer pairs for MAGE-1 and MAGE-2 are listed in Table I. For the second round of PCR, 5 μl of each sample from the first reaction was transferred into glass capillaries, where real-time PCR, with the inner primer pair for MAGE-1 and MAGE-2, was performed using the LightCycler thermocycler (Roche Diagnostics, Mannheim, Germany). The 10 μl reaction mix for each sample composed of 0.5 μM of each of the inner primer, 1.6 μl of 25 mM $MgCl_2$ and 1 μl of FastStart DNA SYBR-Green I Reaction Mix (Roche Diagnostics). The cycle profile for this PCR run was as follows: initial denaturation at 94˚C for 10 min, then denaturation at 93˚C for 40 sec, annealing at 58˚C for 30 sec and elongation at 72˚C for 30 sec for 30 cycles, followed by the final extension at 72˚C for 2 min. To verify the amplified product, a melting curve analysis using LightCycler software was performed for each sample. As a negative control, the human urothelial cell line (HCV-29) was used.

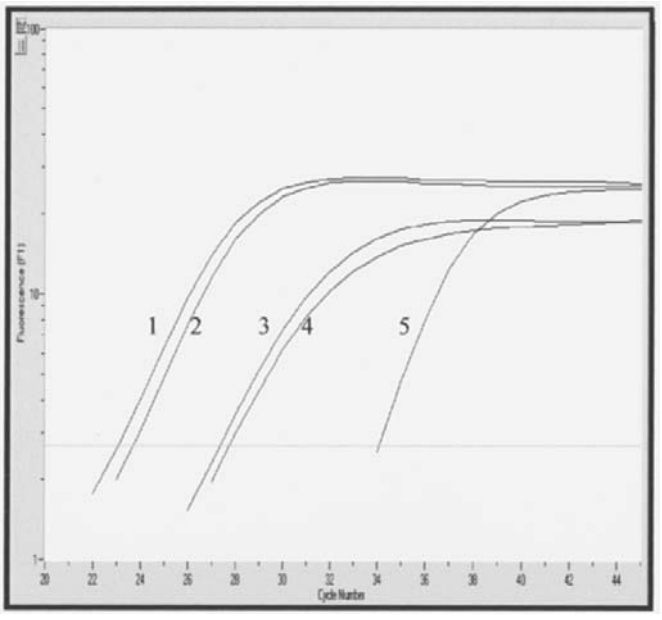

Figure 1. MAGE-1 and MAGE-2 mRNA expression in two tumour cell lines by real-time, nested PCR. 1, MAGE-1 (HPC-4); 2, MAGE-1 (DeTa); 3, MAGE-2 (HPC-4); 4, MAGE-2 (DeTa); 5, negative control.

Determination of the ß-actin mRNA threshold by real-time PCR. Different numbers of HPC-4 tumour cells, from 1 to 500,000, were sorted by flow cytometry (FACS Vantage SE, BD Biosciences Immunocytometry Systems, San Jose, CA) into separate test tubes. The PALM RNA isolation kit (P.A.L.M., Bernried, Germany), designed for tRNA isolation from a small number of cells, was used for the extraction of tRNA. The obtained cDNA was then used in a real-time PCR reaction for the mRNA detection of the housekeeping gene, ß-actin, using the above protocol. The ß-actin primer sequences were as follows: sense 5'-GGATGCAGAAGGA GATCACTG-3', antisense 5'-CGATCCACACGGAGTAC TTG-3'. The cycle profile for the ß-actin real-time PCR run was as follows: initial denaturation at 94˚C for 10 min, then denaturation at 95˚C for 10 sec, followed by annealing at

Table I. The primer sequences for MAGE-1 and MAGE-2.

	MAGE-1	MAGE-2
Outer sense	5'-GTA-GAG-TTC-GGC-CGA-AGG-AAC-3'	5'-CAT-TGA-AGG-AGA-AGA-TCT-GCC-T-3';
Outer antisense	5'-CAG-GAG-CTG-GGC-AAT-GAA-GAC-3'	5'-GAG-TAG-AAG-AGG-AAG-AAG-CGG-T-3'
Inner sense	5'-TAG-AGT-TCG-GCC-GAA-GGA-AC-3'	5'-CAT-TGA-AGG-AGA-AGA-TCT-GCC-T-3'
Inner antisense	5'-CTG-GGC-AAT-GAA-GAC-CCA-CA-3'	5'-CAG-GCT-TGC-AGT-GCT-GAC-TC-3'

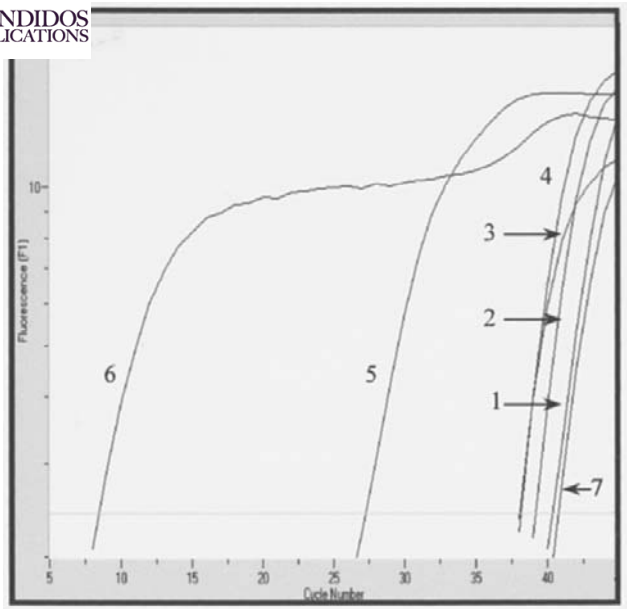

Figure 2. ß-actin mRNA expression in sorted HPC-4 cancer cells. 1, 1 cell; 2, 10 cells; 3, 100 cells; 4, 1000 cells; 5, 100,000 cells; 6, 500,000 cells; 7, negative control.

64˚C for 40 cycles. A melting curve analysis was performed for each sample.

Expression of MAGE-1, -2 mRNA in tumour (HPC-4) cells admixed to normal blood. In a pilot study, HPC-4 tumour cells were added to the blood from healthy donors in the proportion of 1 per $1x10^5$ leukocytes. Erythrocytes were lysed with BD FACS Lysing Solution (BD Biosciences, San Jose, CA) and leukocytes were washed with ice-cold PBS. The smears of cells on PEN membrane-covered microscope slides (P.A.L.M.) were fixed with 80% ethanol for 1 min, as previously described (25). Cells were stained with phycoerythrin (PE) conjugated anti-cytokeratin (A45/B-B3) monoclonal antibody (mAb, Micromet, Munich, Germany), which detects common epitopes of cytokeratin 8, 18 and 19. The slides were then analysed under the IX71 inverted fluorescent microscope (Olympus, Tokyo, Japan) and 1, 5, 10, 20 and 100 CK^+ cells were laser cut and catapulted out into separate test tube lids by using the LCM System (P.A.L.M.). Then, tRNA was isolated from these cells using the highly-sensitive PALM RNA isolation kit.

MAGE-1, -2 mRNA expression in the primary tumour and locoreginal lymph nodes of patients with gastric cancer. Snap-frozen microtome sections of the primary tumours (n=32) and lymph nodes (n=27) from the resection specimens of gastric cancer were placed on the PEN membrane-covered microscope slides (P.A.L.M.). The slides were then stained with hematoxylin-eosin to determine the presence of cancer cells under a light microscope based on their morphology and 50 to 100 from parallel slides were laser cut and isolated using the LCM system. Then, tRNA was isolated and MAGE-1, -2 mRNA expression was examined in the same manner as described above.

MAGE-1, -2 mRNA expression in CK^+ cells isolated from the peripheral blood and bone marrow of patients with gastric cancer. The whole blood and bone marrow samples from patients with gastric cancer were lysed with BD FACS Lysing Solution, washed in ice-cold PBS, then labelled with PE conjugated anti-CD45 mAb (Dako, Glostrup, Denmark) and negatively sorted for $CD45^-$ cells by flow cytometry (FACS Vantage) as previously described (29). The smears of $CD45^-$ cell population on PEN membrane-covered microscope slides (P.A.L.M.) were then fixed with 80% ethanol and incubated with PE-labelled A45/B-B3 mAb. At least 5 CK^+ cells were isolated using the LCM system and tRNA was isolated. The RT reaction was performed as described above, though instead of M-MLV, the Enhanced M-MLV reverse transcriptase (Sigma) was used. The synthesized cDNA was then used in real-time, nested PCR to check MAGE-1, -2 mRNA expression using the same protocol as described above.

Results

Determination of MAGE-1 and MAGE-2 mRNA expression in tumour cell lines by real-time, nested PCR. Initial experiments indicated that the tumour cell lines, HPC-4 and DeTa, expressed similar amounts of mRNA for MAGE-1 and -2 (Fig. 1). Furthermore, no mRNA expression was observed for either gene in the non-malignant cell line, HCV-29 (data not shown). In order to establish the sensitivity of ß-actin mRNA detection, different numbers of HPC-4 cells were used for real-time PCR. The data suggested that the mRNA detection threshold was a single cell, from which a positive real-time PCR signal was obtained (Fig. 2). An increase in the number of cells was proportional to the increase in the mRNA ß-actin expression. A melting curve analysis using the LightCycler software showed that the signals were obtained from the actual products and not primer dimers (data not shown).

MAGE-1, -2 mRNA expression in the HPC-4 cells isolated from the peripheral blood using the Laser Capture Micro-dissection System (LCM). In the model studies, HPC-4 cells were admixed to the blood from normal donors, stained with

Table II. mRNA expression of MAGE-1 and MAGE-2 in primary tumour and lymph node samples of patients with gastric cancer.

mRNA	Primary tumour (n=32)		Lymph nodes (n=27)	
	Number of positive	%	Number of positive	%
MAGE-1	28	88	18	67
MAGE-2	23	72	18	67

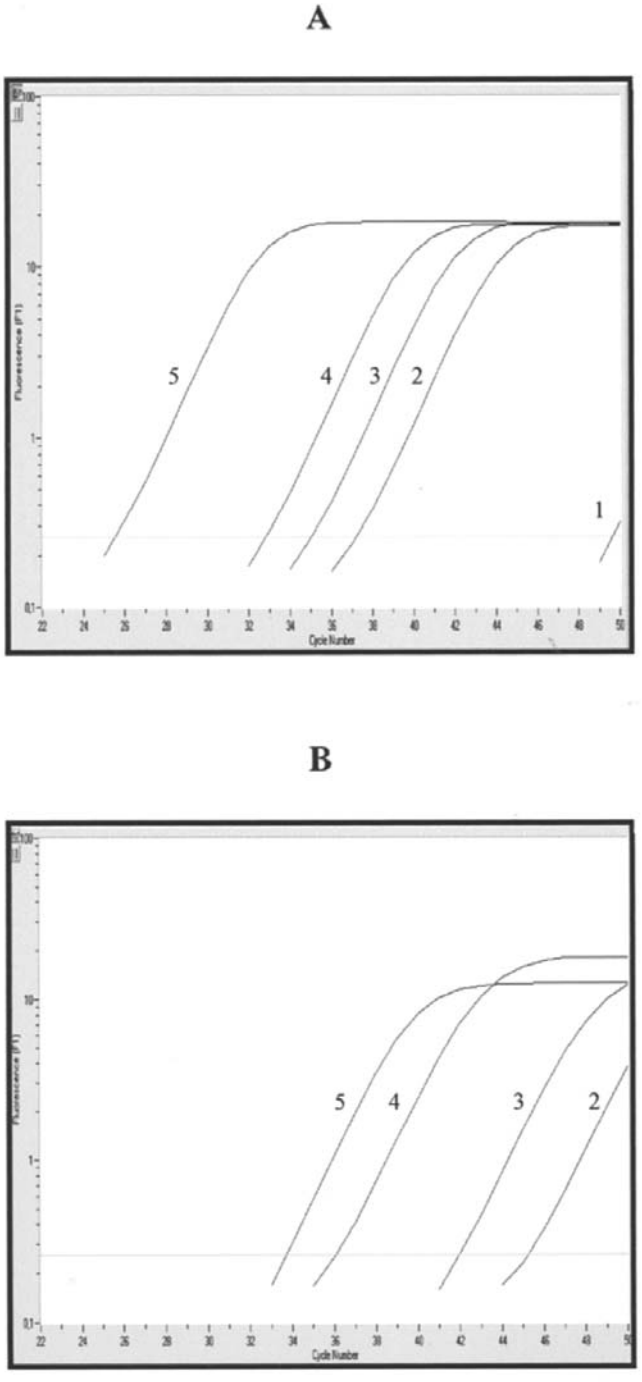

Figure 3. (A) mRNA expression for MAGE-1 in HPC-4 cells isolated from the blood cells as detected by real-time PCR (LightCycler). 1, 1 cell; 2, 5 cells; 3, 10 cells; 4, 20 cells; 5, 100 cells; 6, negative control (out of range). (B) mRNA expression for MAGE-2 in HPC-4 cells isolated from the blood cells as detected by real-time PCR (LightCycler). 1, 1 cell (out of range); 2, 5 cells; 3, 10 cells; 4, 20 cells; 5, 100 cells; 6, negative control (out of range).

PE-labelled anti-CK mAb, and then 1, 5, 10, 20 and 100 CK^+ cells were isolated using the LCM system. Preliminary experiments showed that ethanol fixing and PEN membrane-covered microscope slides substantially reduced the mRNA loss in comparison to standard microscope slides and formalin fixation. Additionally, ethanol fixation was shorter, did not alter the cell morphology and had no negative effect on the CK-staining. Therefore, it was chosen for further studies. Membrane-covered microscope slides enabled to cut around chosen cells and catapult them out intact into the test tube lid. Furthermore, in order to increase the sensitivity of the RT reaction, the Enhanced M-MLV reverse transcriptase was used. The mRNA expression for MAGE-1 and -2 was detected (Fig. 3A and B). The sample where tRNA was isolated from 100 HPC-4 cells showed the strongest mRNA signal for both of the tested genes, however, a positive signal was already detected in the sample containing five HPC-4 cells.

MAGE-1, -2 mRNA expression in the primary tumour and locoreginal lymph nodes of gastric cancer patients. Cancer cells were isolated from resected primary tumour specimens (n=32) and locoregional lymph nodes (n=27). Expression of MAGE-1 mRNA in primary tumour samples was observed in 28 (88%) and MAGE-2 in 23 (72%) cases (Table II). The respective positive cases of the lymph node samples for MAGE-1 and MAGE-2 were 18 (67%) (Table II).

MAGE-1, -2 mRNA expression in CK^+ cells isolated from the peripheral blood/bone marrow samples from patients with gastric cancer. The CK^+ cells were identified in 17 smears of $CD45^-$ cells isolated from 5 peripheral blood and 12 bone marrow samples. Table III shows that 5 out of 5 (100%) cases, where CK^+ cells were isolated by LCM from peripheral blood, expressed mRNA for MAGE-1. The mRNA coexpression for MAGE-2 was detected twice (40%). In the bone marrow (Table III), MAGE-1 mRNA was observed in 9 out of 12 (75%) cases and MAGE-2 mRNA in 7 samples (58%). Transcription of the two genes occurred in 5 cases (42%). In two cases (17%) mRNA for MAGE-2 only was detected. Altogether, 16 out of 17 CK^+ samples showed positive expression for either gene.

Discussion

Our previous studies indicated that the application of FACS sorting of $CD45^-$ cells increases the sensitivity of detection of ITC as defined by the presence of CK (29). Since not all circulating CK^+ cells are necessarily tumour cells, the present study was undertaken to prove that these cells are most likely

Table III. Expression of MAGE-1 and MAGE-2 mRNA in CK^+ cells isolated from peripheral blood and bone marrow samples of patients with gastric cancer.

mRNA	Peripheral blood (n=5)		Bone marrow (n=12)	
	Number of positive	%	Number of positive	%
MAGE-1	5	100	9	75
MAGE-2	2	40	7	58

ls as judged by the isolation of single CK+ cells and tion of MAGE-1, -2 mRNA in them. This also allows the determination of the cellular source of tumour marker-mRNA, as false positive mRNA signals may be a problem in the evaluation of ITC detection in cancer patients (6). Up to now, the procedures using RT-PCR for the detection of disseminated tumour cells were based on tRNA isolation from the whole blood or bone marrow samples (4,5,11,19). The false positive signals may be due to the illegitimate transcription of several tumour-associated or epithelial-specific genes, which was observed in the haematopoietic cells in the bone marrow of a control subject group (30). On the other hand, RNA from normal cells can dilute out the actual mRNA signal specific for the tumour cells, thus leading to false negative results (14).

We also observed the presence of MAGE-1, -2 mRNA in plasma of some gastric cancer patients (unpublished observation), which is likely associated with circulating tumour-derived microvesicles (31). Here, through the application of three different techniques, we have established the protocol that is designed for the specific selection of single tumour cells in order to study the expression of tumour-associated genes in them. This technique included sorting out of CD45+ cells from the blood/bone marrow, isolation of single CK+ cells from the CD45- population by LCM, the extraction of tRNA from them, RT and nested, real-time PCR. The data suggest that this technique is highly sensitive since in the model study we were able to obtain the mRNA signal for ß-actin from a single cell. However, it should be pointed out, that the single-cell mRNA signal detected was of a housekeeping-gene origin. These types of genes are constitutively expressed in a cell, which makes their mRNAs more 'abundant' in a cell as compared to the inducible genes. In the case of the MAGE-1, -2 mRNA expression, which is inducible, a positive signal was detected in tRNA isolated from five HPC-4 cells, which set up the detection threshold for further studies.

As previously reported, disseminated gastrointestinal cancer cells do not express the CD45 molecule (29). Using anti-CD45 mAb and flow cytometry, we have negatively sorted out CD45- cell population from the peripheral blood/bone marrow samples of patients with gastric cancer. This negative sorting enabled us to remove all leukocytes (CD45+), that allowed enrichment of the presumptive tumour cells. In the model study, cancer (HPC-4) cells admixed to normal blood were used for the determination of specificity and sensitivity of the method. Following lysis of erythrocytes, the cells were ethanol-fixed on PEN membrane-covered microscope slides and were stained with anti-CK mAb. It was found that ethanol fixing is much more efficient as compared to formalin fixing, which is in keepings with the results obtained by others (25). Ethanol fixing is a much shorter procedure, which reduces the sample exposure time to ribonucleases, thus preventing mRNA loss. Furthermore, the cells were undamaged, as seen under the fluorescent microscope and the fixing did not have any negative effects on anti-CK mAb staining. To further prevent the mRNA loss, membrane-covered microscope slides were used. LCM isolation of the selected cells from the standard microscope slides requires a much stronger laser impulse causing the destruction of the cell structure, which may result in mRNA loss.

To ascertain that MAGE-1, -2 mRNA in ITC was derived from cancer cells, RNA was isolated from primary tumours and locoreginal lymph nodes. The mRNA for MAGE-1 and MAGE-2 was observed in the majority (88 and 72%, respectively) of gastric cancer samples. This is in accordance with other observations indicating the expression of MAGE-1, -2 mRNA in ~40% and MAGE-1 in 59.9% of the gastric cancer specimens (32,33). The higher rate of detection found in this study may be due to the use of isolated cancer cells as a source of RNA. In the case of the lymph nodes, 67% samples expressed mRNA for the two of the genes.

The CK staining is commonly used for the detection of ITC (7,21). Out of the 17 CK+ samples (peripheral blood and bone marrow samples), 94.1% showed the mRNA expression for either MAGE-1 or -2, or both pointing to the fact that these cells are indeed cancer cells and that this method is highly sensitive. It should be noted that the total number of the viewed CD45- specimens was over 300, which only confirms that finding a potential tumour cell is very difficult and elusive.

The data suggest that the designed method used for selection and identification of ITC is highly sensitive. Its main attribute is the concentration of potential tumour cells (by elimination of blood and haematopoietic CD45+ cells) into a small sample volume, which can be easily transferred onto a microscope slide and stained for tumour markers. The LCM system enables to identify and catapult a cell of interest for other downstream molecular studies. This feature, coupled with a highly sensitive real-time nested PCR, further helps to identify a tumour cell with an even higher probability, though the deficient expression of the marker gene in micrometastatic tumour cells (30) remains a problem. It should be kept in mind, that the detection of molecular tumour markers alone without the proper identification of their cellular origin may not necessarily indicate their malignant nature as several tumour-associated antigens may be expressed by activated lymphocytes (24). Thus, enrichment, identification and isolation of CK+ cells and the determination of tumour-associated gene expression makes this method more reliable than the use of molecular methods alone applied to the whole blood or bone marrow samples.

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