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ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · APRIL 2005

Impact Factor: 5.09 · DOI: 10.1002/ijc.20698 · Source: PubMed

Int. J. Cancer: **114,** 291–298 (2005)

Real-time quantitative PCR determination of urokinase-type plasminogen activator receptor (uPAR) expression of isolated micrometastatic cells from bone marrow of breast cancer patients

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Disseminated tumor cells (DTC) in bone marrow are independently related to poor outcome in patients with breast cancer. Phenotypic characterization of DTC may be useful to improve evaluation of the metastasizing potential of DTC and also to more accurately target aggressive tumor cells. DTC were screened in bone marrow aspirates from breast cancer patients by immunocytochemistry with an anticytokeratin (anti-CK) antibody (A45B/B3). Because the cell permeabilization and fixation required for intracellular CK staining is deleterious for mRNA, we used microaspiration to isolate single tumor cells stained with a monoclonal antibody directed against a membrane epitope, epithelial cell adhesion molecule (EpCAM), in CK-positive cases. Urokinase-type plasminogen activator receptor (uPAR) was quantified by real-time quantitative RT-PCR. The SKBR3 human breast cancer cell line was used to calibrate RT-PCR. A linear relationship was observed between the cycle threshold (Ct) of uPAR and 18S gene expression and SKBR3 cells spiked (1, 3, 7, 10 and 20) in control patient bone marrow. EpCAM-positive cells were aspirated in 21 out of 25 bone marrow specimens from breast cancer patients with CK-positive cells and uPAR mRNA expression was determined in 16 cases. A high level of uPAR mRNA in DTC was detected in 8 out of 16 patients (50%) and was associated with a more aggressive primary tumor phenotype (estrogen receptor [ER]-negative, progesterone receptor [PR]-negative or HER2-positive) ($p = 0.01$). We demonstrated that real-time quantitative RT-PCR was reliably adapted to phenotype analysis of isolated micrometastatic cells. A larger study would be useful to confirm the importance of uPAR to define higher risk subgroups of breast cancer patients with micrometastatic disease.

Key words: breast cancer; micrometastasis; urokinase-type plasminogen activator receptor; real-time PCR

Dissemination of single tumor cells to the bone marrow is a common event in cancer. Anticytokeratin (anti-CK) antibodies are the most specific and sensitive probes for the detection of individual epithelial tumor cells in mesenchymal organs.[1] The clinical significance of CK-positive cells in the bone marrow of breast cancer patients is still a matter of debate, but recent studies have shown that the presence of these cells is independently related to poor outcome in localized disease.[2–4] Little is known about the nature of disseminated tumor cells (DTC) and most of the phenotyping analyses of these cells have been based on double staining techniques.[5] More recently, a method for comparative genomic hybridization (CGH) analysis applicable to single cells has been described.[6,7] Single DTC are an extremely rare event compared to normal cells in bone marrow. A method is therefore needed to isolate viable single cells in order to obtain good quality mRNA and allow accurate quantitative gene expression analysis. The plasminogen activation system plays an essential role in fibrinolysis and extracellular matrix degradation, including cancer invasion, metastasis and angiogenesis. Its components are expressed in tumor and normal cells.[8,9] Urokinase type plasminogen activator receptor (uPAR) is a glycosyl-phosphatidylinositol–anchored glycoprotein localized on the outer layer of the plasma membrane of cells.[10,11] It binds to its specific ligands such as urokinase type plasminogen activator (uPA) and the precursor to uPA (pro-uPA). uPAR does not possess a transmembrane domain, but adaptor molecules able to couple the binding of uPA to uPAR to transmembrane signaling have been proposed, including integrins β1 and β2.[12,13] uPAR activation ultimately leads to degradation of the extracellular matrix and facilitates cellular movement for tumor cells, which appears to be necessary for diverse functions including local invasion and metastasis of tumor cells.[9] Phenotypic characterization of the protease activator expression of disseminated carcinoma cells may therefore define the invasive potential of these cells. In breast cancer, elevated uPAR protein in primary tumors is associated with poor prognosis due to an increased rate of metastasis relapse.[8,14,15] Hemsen *et al.*[15] recently analyzed uPAR expression in primary tumor cells, tumor-surrounded fibroblasts, lymph node and (for 37 of them) micrometastatic cells, in bone marrow of 93 patients with breast carcinoma at diagnosis. They showed variable level of uPAR expression by immunohistochemistry (IHC) in 90% of tumor cells and 73% of intratumoral fibroblasts. Only the fraction of uPAR positive in tumor cells was positively correlated with tumor cells in bone marrow. uPAR expression has also been described as a pejorative prognostic factor in endometrial cancer,[16–19] breast cancer[17–19] and gastric cancer,[20–22] although contradictory results have been reported for ovarian cancer.[23] uPAR may be a marker to define a critical subpopulation of DTC.

We present here a study of feasibility analysis of uPAR expression on disseminated breast cancer cells detected in bone marrow by real-time RT-PCR on single isolated cells. Because cell permeabilization and fixation, which are required for intracellular CK staining, affect the yield of intact mRNA, we chose to identify and isolate tumor cells from the bone marrow of breast cancer patients with a high-affinity monoclonal antibody (BerEP4) directed against the epithelial cell adhesion molecule (EpCAM). This cell surface protein is expressed in simple epithelial tissues and most neoplasms derived from these tissues.[24]

Patients and methods

Patients

From December 1999 to July 2000, in a prospective trial of the feasibility and evaluation of the prognostic value of micrometastatic disease in the bone marrow of breast cancer patients underway at the Institut Curie, 136 patients were screened, including 59 patients with stage IV disease. CK-positive cells were detected in 65 patients by immunocytochemistry on routine cytospins. Ep-

Grant sponsor: "Programme Incitatif et Coopératif", Institut Curie, Paris, France.
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Received 5 March 2004; Accepted after revision 6 August 2004
DOI 10.1002/ijc.20698
Published online 12 November 2004 in Wiley InterScience (www.interscience.wiley.com).

CAM staining and the microaspiration procedure were performed within 24 hr of sampling in 25 of these 65 patients selected for the presence of CK-positive cells. Cells were put in a culture medium for 24 hr. Only 21 samples could be isolated on the basis of EpCam-positive staining on viable cells. Clinicopathologic factors and staging were classified according to the TNM classification of malignant tumors of the International Union Against Cancer (UICC):[25] 12 stage IV, 2 stage III, 1 stage II and 1 stage I. Samples were obtained before chemotherapy (neoadjuvant/adjuvant or for metastatic disease) from bone marrow aspiration performed under local anesthesia from the posterior iliac crest (4–5 ml per sample). Informed consent was obtained from all patients. The characteristics of the 16 patients are shown in Table I. The median age was 60 years (range: 36–75 years). Median follow-up was 40 months.

Human breast cancer cell line

A well-characterized human breast cancer cell line, the SKBR3 cell line established from the pleural effusion of a hormone-independent human breast cancers (estrogen receptor [ER]-negative and progesterone receptor [PR]-negative), was used.[26] Cells were maintained in 75-cm^3 flasks in DMEM (Gibco BRL, Paisley, United Kingdom) supplemented with 10% FCS (Dutscher, Brumath, France), and 2 mM glutamine (Sigma-Aldrich, Saint Quentin, France), at 37°C in a 5% CO_2 air humidified incubator.

Bone marrow preparation

The procedure for bone marrow preparation has previously been described.[27] A total of 3–7 ml of bone marrow aspirate were collected on EDTA (Vacutainer; Becton Dickinson, LePont de Claix, France), processed under laminar flow and separated by Ficoll/Hypaque density centrifugation (Sigma-Aldrich, Saint Quentin, France; density, 1.077 gm/ml) in Leucosep tubes (Merck, Strasbourg, France) (830 gm, 15 min, 20°C). The mononuclear cell (MNC) layer was harvested, washed and resuspended in PBS solution + 0.1% BSA.

Immunocytochemic staining

For IHC, the MNC layer was resuspended in PBS solution + 0.1% BSA at 1×10^6/ml, cytocentrifuged onto polylysine-coated slides (Hettich Universal 16A cytocentrifuge, Tuttlingen, Germany). The pancytokeratin (pan-CK) monoclonal antibody A45-B/B3 (Micromet, Munich, Germany), which recognizes several CK epitopes, CK 8, CK 18 and CK 19, was applied for cell detection after fixation with 4% paraformaldehyde. Endogenous alkaline phosphatase was blocked and the slides were incubated with the primary antibody A45 B/B3 (2 μg/ml). Immune complexes were revealed by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique (Dako, Trappes, France) and stained with fuchsin solution (New Fuchsin; Sigma-Aldrich, Saint Quentin, France). Cells were counterstained with Mayer hematoxylin (Sigma-Aldrich, Saint Quentin, France). A total of 3×10^6 mononuclear cells (3 slides) were evaluated for each patient. Negative controls were performed on an equivalent number of cells with a mouse monoclonal anti-fluorescin isothiocynate (FITC)-immunoglobulin G1(IgG1) (1/1,250) (Sigma-Aldrich Immuno Chemicals, Saint Quentin, France). Detection of CK+ cells on slides was performed by digital microscopy with the ACIS (ChromaVision Medical Systems, San Juan Capistrano, CA, USA).

In order to confirm that the EpCAM-positive cells were also CK-positive, we performed double immunolabeling according to the technique described by Van der Loos *et al*.[28] EpCAM antibody was revealed by an anti-mouse IgG labeled with the fluorochrome Alexa488 (green). For anti-CK labeling, A45B/B3 antibody was applied and revealed by a polyclonal anti-FITC antibody, and staining was performed by an anti-rabbit antibody labeled with fluorochrome Alexa594 (red) (Molecular Probe, Leiden, Netherlands) (excitation wavelength 594 nm). Fluorescence microscopy was used to detect the cells (Leica, DMIRB, Rueil Malmaison, France).

Cell suspension staining and micromanipulation

For microaspiration, viable MNC were suspended in PBS solution + 0.1% BSA at a density of $2–3 \times 10^6$ cells/ml. The cell suspension was incubated with the EpCAM primary antibody BerEP4 (2 μg/ml) (Dako, Trappes, France) for 1 hr at room temperature in the presence of 5% decomplemented AB serum to prevent nonspecific binding. The cells were then washed twice in PBS solution + 0.1% BSA. EpCAM-positive cells were incubated with the secondary mouse anti-FITC monoclonal antibody (1/50) (DAKO) for 1 hr. Cells were again washed twice and suspended in PBS solution + 0.1% BSA. A total of $2–5 \times 10^5$ cells, in a volume of 200 μl, were deposited onto each slide at a density such that enough space was left between individual cells for subsequent isolation. All manipulations were performed in suspension in order to avoid cell death. Cell screening was performed using indirect immunofluorescence and phase contrast with a DMIRB inverted microscope (Leica DMIRB). Positive cells were isolated with a micromanipulator (Nanoject) and placed on a fresh drop of PBS solution + 0.1% BSA then examined to ensure that no contaminating cells were coisolated and that isolated cells were stained for EpCAM in fluorescence (Fig. 1). RNAse-free paraffin oil was used for aspiration with the micromanipulator. The cells were then reaspirated and picked into PCR tubes placed on ice. All isolated single cells from 1 patient were placed in a same tube. Cells were immediately suspended in RNA plus® kit (QBiogene, Illkirch, France) and frozen at −80°C.

RNA extraction, cDNA synthesis and real-time PCR amplification

Total RNA was extracted from all cells according to the manufacturer's instructions including overnight precipitation. Glycogen (20 ng) (Boehringer Mannheim, Mannheim, Germany) was added as RNA carrier to optimize the extraction efficiency. The RNA samples were then stored in RNAse-free distilled water at −80°C before analysis. Reverse transcription of RNA was performed in a final volume of 20 μl containing 200 μM of each nucleotide triphosphate, 6.7 mM $MgCl_2$, 5 Units of RNAse inhibitor (Promega, Lyon, France), 5 μM random Hexamer (Pharmacia, Uppsala, Sweden) and 200 Units of Mo-MuLV reverse transcriptase (Life Technologies, Eragny, France) and 14 μl of the extracted RNA or H_2O was added. The samples were incubated at 65°C for 5 min, then 42°C for 30 min followed by cooling at 5°C for 5 min.

PCR conditions

PCR amplification was performed in the presence of specific target, doubly labeled fluorogenic probes (Taqman™ probe, Applied Biosystems, Foster City, CA) allowing automated quantification of the amplified products in real-time with the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Primers and probes were chosen with the assistance of the Primer Express computer program (PE, Applied Biosystems). We con-

TABLE I – PATIENT CHARACTERISTICS ($N = 16$)

$n = 16$	Total	Low level uPAR $n = 8$	High level uPAR $n = 8$	p
UICC clinical stage				0.50
I	1	1	0	
II	1	1	0	
III	2	1	1	
IV	12	5	7	
Histology				0.44
Ductal	10	4	6	
Lobular	3	2	1	
Others[1]	3	2	1	
Hormone receptor				
ER and/or PR+	13	8	5	
ER and PR negative	3	0	3	0.054
HER2[2]				
Positive	6	1	5	
Negative	9	7	2	0.02

[1]One apocrine, one undifferentiated and one tubular.– [2]Missing in 1 patient.

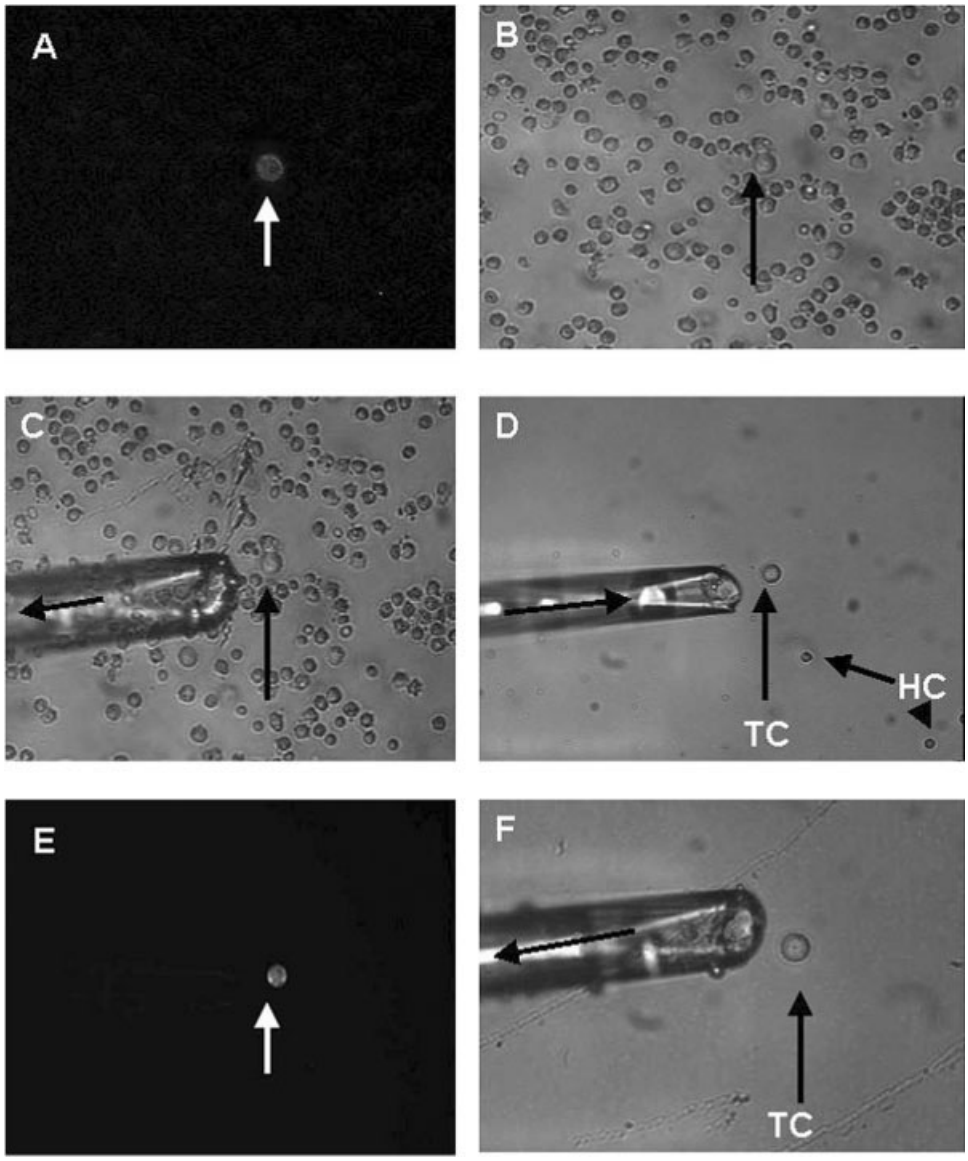

FIGURE 1 – The step by step description of epithelial cell isolation by microaspiration. *(a)* Indirect immunofluorescence screening of a cell strongly stained by BerEP4 antibody at 100×. *(b)* Microscopic localization of the immunostained cell. *(c)* Aspiration of the cell (and possibly adjacent hematopoietic cells) with micropipette device. *(d)* Transfer of the aspirate to a fresh slide covered with PBS and microscopic isolation of the epithelial tumor cell (TC) from the hematopoietic cells (HC). *(e)* Verification of the immunofluorescence staining of the isolated cell. *(f)* Reaspiration of the single BerEP4 positive cell then transferred into the PCR tube placed on ice.

ducted BLASTN searches against dbEST and nr (www.ncbi.nlm.nih.gov) in order to confirm the gene specificity of the chosen nucleotide sequences.

uPAR forward and reverse primers in the 5′ and 3′ directions were GCCCAATCCTGGAGCTTGA (exon 5/6) and TCCCCTTGCAGCTGTAACACT (exon 6), respectively. The Taqman probe was AATCTGCCGCAGAATGGCCGCCA (exon 6) carrying a 5′ FAM reporter label and a 3′ TAMRA quencher group. Transcripts of RNA 18S (Applied Biosystems, Foster City, CA, USA) were also quantified as endogenous RNA of reference genes to normalize each sample.

PCR reactions were performed using the Core Reagent Kit, as previously described.[29] All primers, probes and reagent for real-time PCR were purchased from Applied Biosystems (Warrington, UK). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, a incubation step at 50°C with UNG, then 40 cycles at 95°C for 15 sec and an annealing temperature of 60°C for uPAR and 65°C for RNA 18S. Each experiment was performed in 2 independent runs. PCR products were then migrated on 2% agarose gel in order to confirm the presence of a single band with the expected size (62 bp).

To prevent carryover of contaminating DNA, the reaction was carried out in the presence of deoxyuridine Tri phosphate (dUTP). Two nontemplate controls were included in each amplification run. Accurate quantification was achieved by the generation of calibration curves by serial dilutions of SKBR3 human breast cancer cell line cDNA in order to quantify the PCR efficiency for each run. Quantitative values were obtained from the threshold cycle (Ct) number at which the increase in fluorescent signal associated with an exponential increase of PCR products was detected (i.e., 10-fold the standard deviation of background noise signal, using Applied Biosystems analysis software). ΔCt values of the sample were determined by subtracting the average of duplicate Ct values of the target gene from the average of duplicate Ct values of the reference gene. The relative gene expression level was also normalized relative to a positive calibrator, consisting of 1 of the samples of the calibration curve (SKBR3 dilutions) of the assay. Results were expressed as "N target" and determined as follows:

$$\text{N target} = 2^{(\Delta\text{Ct sample} - \Delta\text{Ct calibrator})}$$

In parallel, serial dilutions of SKBR3 spiked cells in normal bone marrow samples (10 to 1 cell) were performed to assess the linearity of the assay).

Results

Identification and isolation of micrometastatic cells

In a series of 25 bone marrow specimens from patients with advanced breast cancer, selected for their positivity to anti-CK A45 B/B3 antibody, we were able to aspirate EpCAM-positive cells in the bone marrow of 21 breast cancer patients. The median number of cells identified by immunocytochemistry with A45 B/B3 on the fixed slides of these 21 patients was 6 cells for 1×10^6 cells (range: 0.3–1,000). The step-by-step description of epithelial cell isolation is shown in Figure 1. Viable cells were first screened by indirect immunofluorescence using EpCAM antibody. Only strongly stained cells were identified and localized (Fig. 1*a* and *b*). The identified cells were then aspirated with the micromanipulator under optical microscopic control (Fig. 1*c*). A few adjacent hematopoietic cells could be aspirated in the same manipulation. Aspirated cells were then transferred to a fresh slide covered with PBS. Epithelial tumor cells were isolated from the hematopoietic cells (Fig. 1*d*) by their immunofluorescence staining (Fig. 1*e*). Epithelial cells were then reaspired 1-by-1 and picked into the PCR tube placed on ice (Fig. 1*f*). A total of 1–25 EpCAM-positive cells were obtained from each patient (median: 3 cells). Similarly, SKBR3 cells were spiked in control patient bone marrow and were processed according to the same protocol as for breast cancer patient bone marrow samples (Fig. 2).

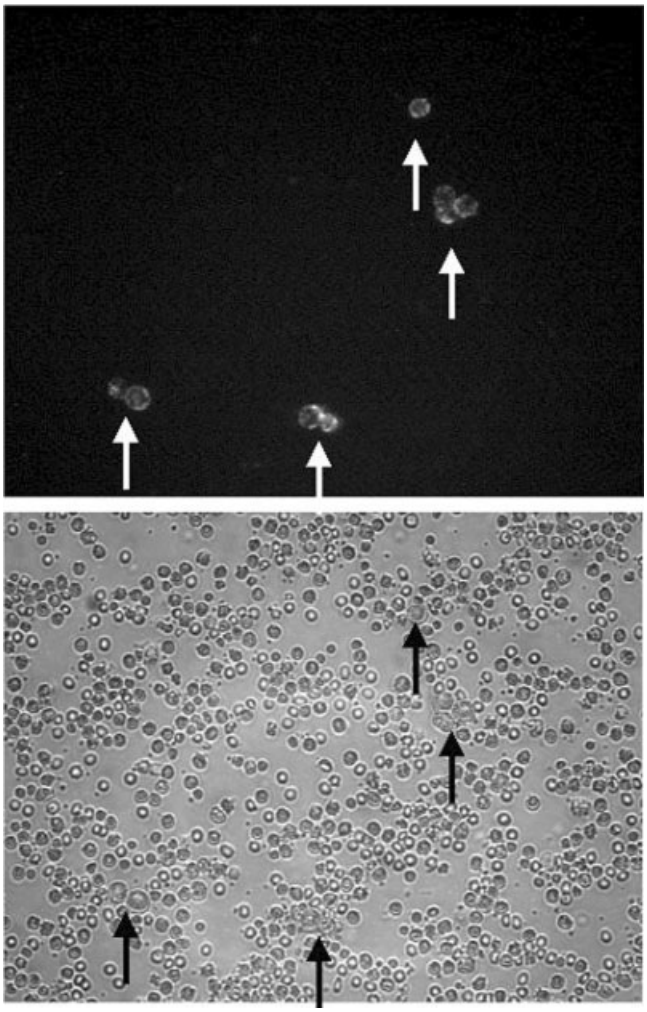

FIGURE 2 – SKBR 3 cells spiked in bone marrow of a control patient, stained with BerEP4 antibody. At 100×, indirect immunofluorescence (up) and in visible light (down).

For 5 patients, we performed double staining with EpCAM and A45 B/B3 antibodies to demonstrate that cells strongly stained with EpCAM were also CK-positive cells, as illustrated in Figure 3.

Quantification of gene expression

uPAR mRNA expression could be quantified in 16 human specimens. Tumor characteristics and results are presented in Table I. As previously described,[29] we were able to accurately amplify the target mRNA of 1 single SKBR3 cell. The relative uPAR gene expression level was expressed by subtracting the Ct values of the target gene from the Ct values of the reference gene (RNA 18S as described in Material and methods). It was also normalized relative to a positive calibrator, consisting of 1 of the samples of the calibration curve (SKBR3 dilutions) of the assay. As shown in Figure 4, a linear relationship was observed between the cycle threshold (Ct) of uPAR and 18S gene expression and SKBR3 cells spiked (1, 3, 7, 10 and 20) in control patient bone marrow microaspirated under the same conditions as for BerEP4-positive cells detected in breast cancer patients ($r = 0.71$, $p < 0.001$ and $r = 0.74$, $p < 0.001$ for uPAR and RNA 18S, respectively).

The distribution of uPAR mRNA relative values ranged from 0 to 26 (median: 7.53). The median value was chosen to classify uPAR mRNA values into 2 groups: low level (from 0 to 5.60) and high level (from 10.70 to >19). Results are shown in Table I and Figure 5

We did not observe any correlation between the number of CK-positive cells in bone marrow aspirate and the uPAR level in micrometastatic cells ($p = 0.54$).

HER2 status of the primary tumor and micrometastatic cells was determined by immunohistochemistry (+++ or ++ and fluorescence *in situ* hybridization (FISH) positive) as previously described[30] and was available for all but 1 of the patients. HER2 overexpression (+++) was detected in 6 of the 15 samples (40%). A total of 5 out of 6 patients with overexpressed HER2 had a high level of uPAR in their micrometastatic cells ($p = 0.02$). All 3 cases with the highest level of uPAR in micrometastatic cells showed HER2 overexpression in the primary tumor. A total of 3 of the 16 patients who were negative for both ER- and PR-negative had detectable or high levels of uPAR in their micrometastatic cells. These 3 patients also overexpressed HER2.

The number of patients enrolled in this study was not sufficient to draw any conclusions regarding prognosis.

Discussion

Several studies on patients with primary breast cancer have demonstrated that the presence of CK-positive or Muc-1-positive immunostained cells in bone marrow is associated with an unfavorable prognosis[2–4,31,32] and a recent pooled analysis on more than 4,000 patients has confirmed its independent prognosis value.[33] However not all patients detected with micrometastatic cells in bone marrow will relapse, then the phenotypic characterization of these cells could improve the prognostic evaluation of the patients. In this study, we developed a quantitative RT-PCR assay to measure the level of uPAR mRNA expression in different specimens after isolation of micrometastatic cells from bone marrow of breast cancer patients. This represents the 1st report of the feasibility of phenotyping isolated viable breast micrometastatic cells by RT-PCR.

The detection of cells based on the use of the EpCAM antibody is a critical step for isolation of DTC, as weakly stained EpCAM cells have been described in the bone marrow of healthy donors.[34] We aspirated cells from cases stained with anti-CK antibody, then only strongly labeled EpCAM-positive cells were selected. Klein *et al.*[34] have also demonstrated the neoplastic nature of some EpCAM-positive cells but not all, using CGH that revealed genomic aberrations. To confirm the specificity of labeling, we performed anti-EpCAM and anti-CK double staining in a subgroup

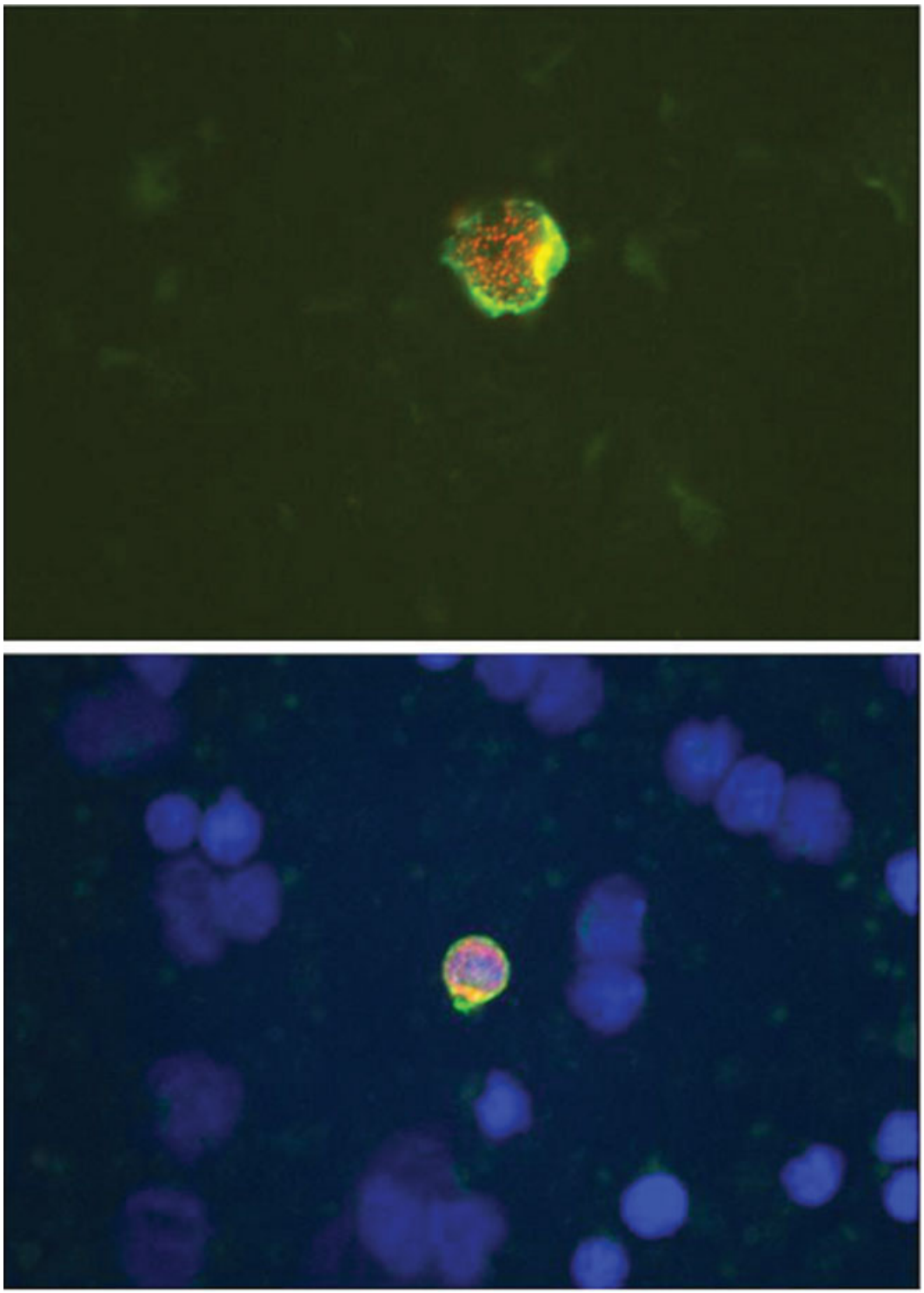

FIGURE 3 – Double staining immunofluorescence of 1 patient's (patient number 306) tumor cells: anti-CK stains red in the cytoplasm and EpCAM stains green on the membrane.

of our patients. Note that 1 uPAR-negative patient (patient number 397) was clearly EpCAM- and anti-CK-positive.

The double immunostaining procedure has been used to study the phenotype of DTC.[35] The expression of EpCAM is relatively heterogeneous in disseminated mammary carcinoma cells. Coexpression of CK and EpCAM in these cells has been described in 74% of the cases in a double immunostaining study.[5] Few studies using staining for CK and uPAR in either blood or bone marrow samples have been previously described in breast cancer patients.[36–39]

For mRNA detection, we used a recently developed quantitative RT-PCR method (Taqman) based on real-time analysis of PCR amplification. This assay is highly sensitive and allows accurate quantification of mRNA expression, as previously demonstrated.[29]

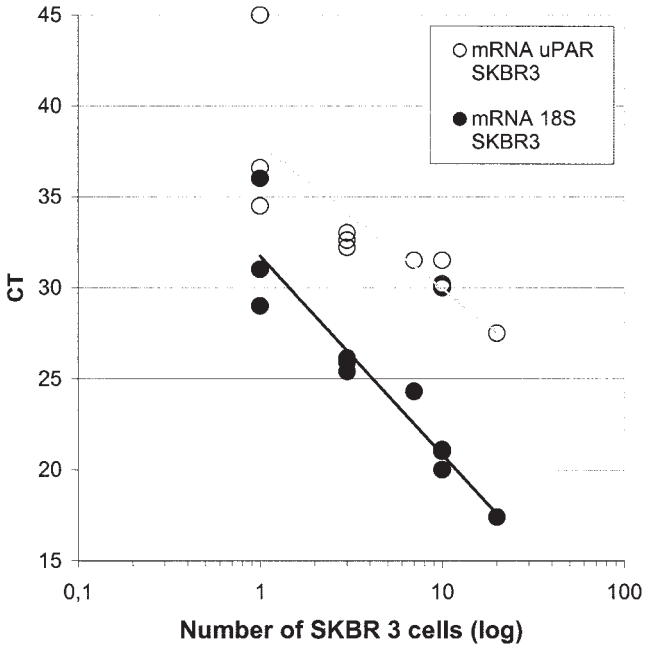

FIGURE 4 – Cycle threshold (Ct) for mRNA of uPAR and 18S gene expression, in 1, 3, 7, 10 and 20 SKBR3 cells spiked in control patient bone marrow and microaspirated under the same conditions as for BerEP4-positive cells detected in breast cancer patients. A linear relationship was found between the mRNA level detected by real-time RT-PCR and the number of cells.

The quality of RNA was a critical step; this may explain the fact that only 16 samples were assayed, due to RNA degradation or because there was not enough material. The real-time PCR method had several advantages over other RT-PCR-based quantitative and biochemical methods. It does not require post-PCR sample handling, thereby avoiding problems related to carryover, which has considerable importance in this field. The target gene is normalized with an endogenous reference gene (18S RNA) to compensate for sample-to-sample variations of mRNA levels. The rationale for using 18S RNA, which is highly expressed RNA, is to provide a more accurate measurement of a limited material. Ideally, another reference gene with lower expression could be used but this was not possible due to the low amount of available RNA. To compensate for any inter-PCR variation between the runs, calibration of the expression of the target gene was carried out. A standard curve for a calibrator (SKBR3 human breast cancer cell line expressing detectable level of uPAR) was analyzed in each experiment under the same conditions as those used for unknown samples and was used to calculate PCR efficiency in each run. Similar PCR efficiencies were observed in each PCR run.

In our study, high levels of uPAR mRNA in micrometastatic cells were associated with a more aggressive primary tumor phenotype (ER-negative, PR-negative and HER2-positive). Furthermore, despite the limited number of cases, we observed a nonsignificant trend toward a poorer prognosis in patients whose bone marrow micrometastatic cells expressed high levels of uPAR (data not shown). High levels of uPAR expression in the primary tumor, mainly studied by immunohistochemistry, has been associated with poorer prognosis in gastric, colon, breast and endometrial cancers.[16,21,37,40–42] Hemsen *et al.*[15] observed a significant positive correlation between uPAR-positive cells measured by IHC and the expression of estrogen receptor in primary breast tumors (67% of the ER-positive tumors coexpressed uPAR on more than 50% of their tumor cells). However, they observed high uPAR expression in only 33% of ER-negative cells. Togel *et al.*,[37] in a more limited study including 15 bone marrow samples from breast cancer patients, showed that 10 cases were positive for uPAR (66%). This analysis was performed by double immunostaining (anti-uPAR and anti-CK antibodies) after immunomagnetic preenrichment using the human epithelial antigen. The prognostic value of these results was not evaluated in their study, but the authors reported that uPAR expression on cancer cells from leukapheresis was reduced in comparison with bone marrow cells. They suggest a possible less aggressive nature of these cells compared with cells found in bone marrow.

In other solid tumors, Foca *et al.*[43] demonstrated that the level of uPAR mRNA, analyzed by Northern blot, was correlated with the invasive potential of 34 endometrial cancers. Memarzadeh *et al.*[16] analyzed uPAR expression by immunohistochemistry in 65 primary endometrial tumors and showed that high uPAR expression in primary tumors was positively correlated with the grade of the disease and also positively correlated with recurrence and mortality rates in patients with adenocarcinoma of the endometrium. Heiss *et al.*[20] were the first to demonstrate uPAR expression analyzed by IHC in the bone marrow of patients with gastric carcinoma as an independent prognostic factor for disease-free and overall survival. They recently confirmed their results on a series of 156 patients.[21]

uPAR may be one of the parameters involved in the promotion of micrometastasis. It induces plasminogen-dependent proteolysis of the extracellular matrix components, and is therefore involved in invasion, cell extravasation, migration and metastasis. uPAR is overexpressed only in tumor cells and not in normal mammary cells.[44] It is expressed by several cell types in tumors, including epithelial cells, but also endothelial cells, macrophages[45,46] and tumor-surrounding fibroblasts.[15] But only the fraction of uPAR-positive primary tumor cells, but not fibroblasts, was positively correlated with the presence of tumor cells in bone marrow. uPAR expressed by tumor-associated macrophages may also have a direct role in angiogenesis by mediating endothelial cell migration and differentiation. Interestingly, the recent work by Aguirre-Ghiso[47,48] on human breast, prostate, melanoma and fibrosarcoma cell lines demonstrates that the level of active phospho-ERK and the ERK/p38 activity ratio are predictive of the behavior of the cell lines: either proliferation or tumor growth arrest (dormancy). The high ERK/p38 ratio was induced by high uPAR expression, which activated $\alpha 5\beta 1$-integrin and epidermal growth factor. It was then hypothesized that uPAR could act as a switch between proliferation and dormancy of minimal residual tumor cells.[21]

Preliminary results of transcriptome analysis of individual tumor cells isolated from bone marrow of cancer patients have recently been reported. This study included 1 breast tumor sample. We can expect, in the future, that larger studies would greatly improve the detection of genes differentially expressed by single DTC and bone marrow, providing novel markers for minimal residual disease detection.[7]

Furthermore, phenotypic analysis of isolated tumor cells could provide characteristics differentiating the high and low metastatic potential of these cells, which would allow the development of accurate prognostic parameters and therapeutic applications, such as targeting uPAR by specific antibodies.[49]

In conclusion, we have developed and optimized a sensitive method for phenotype analysis of isolated micrometastatic cells from the bone marrow of breast cancer patients. This analysis was mainly performed on stage IV breast cancer patients and we demonstrated that real-time quantitative RT-PCR was reliably adapted to analyze rare cells. Larger studies will be useful to definitely confirm the importance of uPAR as surrogate marker of aggressiveness in micrometastatic disease, allowing identification of subgroups of patients at higher risk. Many methods are now also under development to target uPAR for the purposes of diagnosis, imaging or treatment of solid tumors.

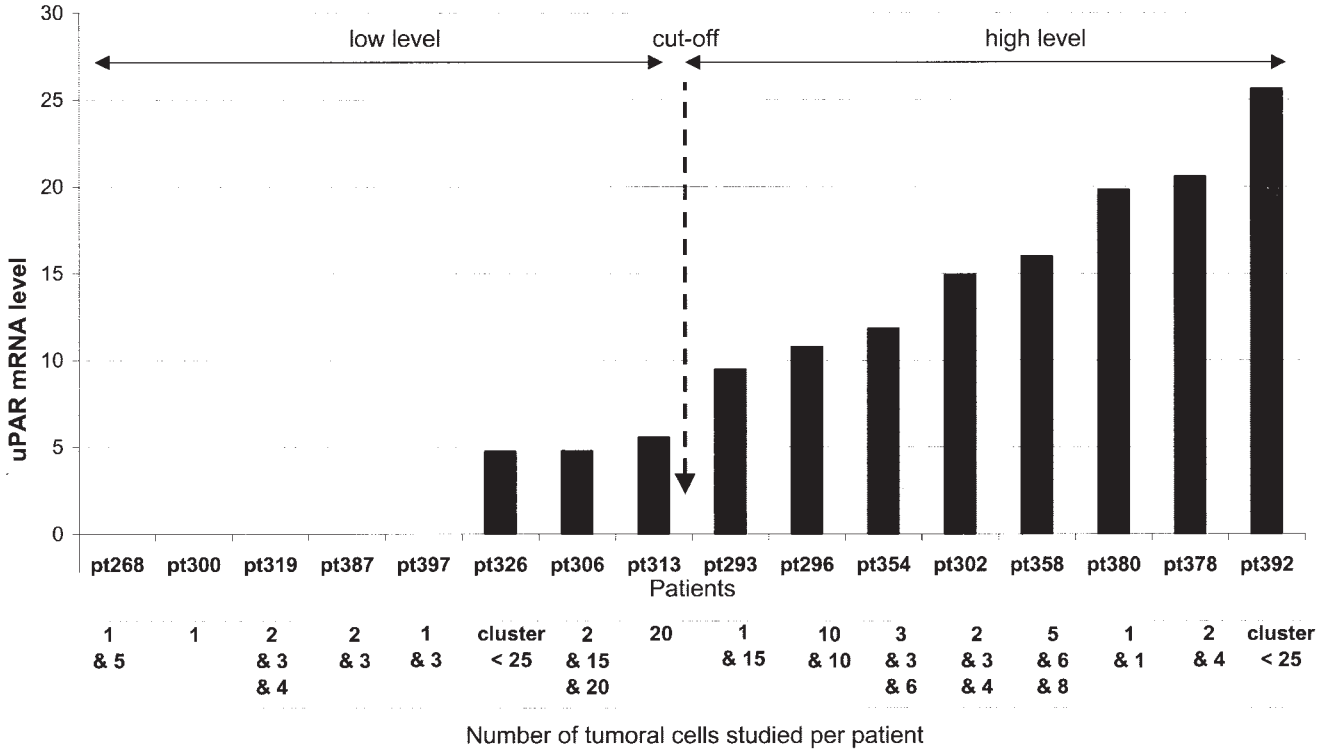

FIGURE 5 – Level of uPAR expression compared to mRNA 18S level on isolated micrometastatic cells in 16 patients (difference between Ct for uPAR mRNA and Ct for 18S mRNA) × 1,000/($2^{\Delta\Delta Ct}$ uPAR/18S). A total of 5 patients were completely negative, 3 weakly positive (and considered to be negative for comparison between 2 groups of 8 patients), 5 were positive and 3 were strongly positive. The number of cells assayed for each patient in separated experiments is indicated under the patient's identification number. The calculated mRNA level is the mean of assays, normalized to the 18S RNA.

Acknowledgements

We thank N. Brünner, Royal Veterinary and Agriculture University, Copenhagen and D.K. and L.H. Pedersen, Biotechnological Institute, Kogle Allé, Hoersholm; we thank D.K. for advice on uPAR assays. We thank Mrs C. Barbaroux for her technical assistance and P. Mandé for manuscript preparation.

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