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ORIGINAL STUDY

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Analysis of the specificity and selectivity of anti-EpCAM antibodies in breast cancer cell lines

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Abstract: The epithelial cell adhesion molecule (EpCAM) is a membrane glycoprotein that is expressed in most normal human epithelia and overexpressed in most carcinomas. This molecule is responsible for cell-to-cell adhesion and additionally participates in signalling, cell migration, proliferation and differentiation. Therefore, EpCAM has been the target of immunotherapy in clinical trials of several solid tumours. It has been found to play an important role in the detection and isolation of circulating tumour cells (CTCs). The aim of this study was to investigate and compare the specificity and selectivity of different anti-EpCAM antibodies in order to assess their usefulness for CTCs binding. All experiments were performed in six different types of breast cancer cell lines (MCF-7, SkBr-3, T47D, CAMA-1, MDAMB-231, and BT-20) and using three different anti-EpCAM antibodies (EBA-1, AUA-1, and 9C4). Immunofluorescence and Real-Time PCR techniques were applied to analyse the protein and gene expression levels. The experiments revealed that the investigated antibodies differed significantly regarding the specificity of EpCAM antigen binding. The most significant role in targeting CTCs was played by the EBA-1 and 9C4 anti-EpCAM antibodies. They revealed the strongest immunofluorescent signal among other applied antibodies and/or were specific for all examined breast cancer cell lines. The strength and specificity of reaction was dependent not only on the type of antibody, but also on the type of breast cancer cell line. We noted that the diverse sensitivities of reactions depended on the type of applied antibody. We therefore recommend the simultaneous application of different anti-EpCAM antibodies. An appropriate choice of anti-EpCAM antibodies and an evaluation of EpCAM expression in breast cancer appear to be crucial, especially as this antigen is being proposed as a marker for the detection of circulating tumour cells. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 4, 534–541)*

Key words: anti-EpCAM, circulating tumour cells, immunofluorescence, real-time PCR, breast cancer cell lines

Introduction

The epithelial cell adhesion molecule (EpCAM) is a 40kDa monomeric membrane glycoprotein that is expressed in most normal human epithelia [1]. Additionally, EpCAM is overexpressed in the majority of carcinomas and is, therefore, a potential target for the immunotherapy of human solid tumours e.g. colorectal cancer [2] and breast cancer [3]. The molecule has also been named AUA1, ESA, GA733, HEA125, and 323/A3, which describe the same antibody that was raised against this molecule [1, 4]. EpCAM functions not only as a homophilic adhesion protein; it also participates in cell migration, proliferation and differentiation [5–7].

In a number of tumours of epithelial origin, active proliferation of cancer cells is associated with increased *in vivo* EpCAM expression, which is characteristic for neoplastic transformation of tissues that normally reveal no or low EpCAM expression profile, such as squamous epithelium [8, 9]. Moreover, overexpression of EpCAM in breast and ovarian cancer often correlates with a poor prognosis [10, 11]. Osta et al. [5] demonstrated that EpCAM was highly overexpressed in primary and metastatic breast cancer (by as much as 100-fold compared to normal breast tissue). Moreover, silencing of EpCAM gene expression decreases the proliferation, migration and invasive capacity of breast cancer cell lines *in vitro*.

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Circulating tumour cells (CTCs) are detectable in blood samples from the majority of cancer patients, although they are found at very low concentrations of 10^{-6}-10^{-7}/ml. Hence, several different systems have been developed for the isolation and characterisation of CTCs. In most studies, CTCs are defined as being positive for EpCAM and cytokeratins 8, 18 and 19, and negative for CD45. Therefore, some CTC enrichment methods are based on the use of anti-EpCAM antibodies and subsequent detection with anti-cytokeratin antibodies [12–14].

Detection of CTCs in the blood of cancer patients is a non-invasive method that may help in elucidating how these cells spread through the bloodstream, settle down at distant sites, and form metastases. Identification of specific subtypes of circulating tumour cells in peripheral blood of cancer patients can provide information about the biology of metastasis, and can be useful in assessing the invasive potency of individual CTCs. Therefore, it could improve the clinical management of metastatic cancer patients as a diagnostic and prognostic factor [15, 16].

Several dozen antibodies have been raised against the EpCAM antigen to date [17]. They can bind to different domains of the molecule and show low- or high-binding affinity. Therefore, the expression levels of the immunohistochemical EpCAM protein analyses can be distinct because different types of anti-EpCAM antibodies can show differing antigen bindings [1].

Looking at all the recorded data, it appears that EpCAM can play an important role in binding and detecting circulating tumour cells. Hence, the evaluation of EpCAM expression in primary breast cancer is crucial for the application of this antigen as a molecular biomarker. This study aimed to investigate and compare the specificity and selectivity of different anti-EpCAM antibodies in order to assess their ability for CTCs binding. Since the heterogeneity of breast cancer types was also taken under consideration, six different breast cancer cell lines were examined. Additionally, we analysed the differential distribution of the EpCAM protein within cultured breast cancer cells as well as of the expression profiling of EpCAM mRNA.

Material and methods

Cell lines. The following breast cancer cell lines were used in this study: MCF-7, SkBr-3, T47D, CAMA-1, MDAMB-231 and BT-20. Two additional cell lines were used as positive and negative controls. The positive one was EpCAM(+) human colon cancer cell line (LoVo) (all cell lines were obtained courtesy of the Institute of Pathology Charite-Universitatsmedizin Berlin). The latter (negative), was EpCAM(–) mouse embryonic fibroblast cell line (3T3/balb). Fibroblast cells have a mesodermal origin and therefore show no expression of EpCAM.

Cells were routinely cultured in media intended for each type of cell line (MCF-7, L-15, SkBR-3, MDAMB-231 and BT-20 – DMEM, T47D and CAMA-1 — RPMI-1640; Sigma-Aldrich). All media were supplemented with 5% fetal bovine serum (Sigma-Aldrich), L-glutamine (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (penicillin, streptomycin, amphotericin B) (Sigma-Aldrich). Cell lines were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Real-Time PCR technique. Cells were harvested at near-confluence state from three 75cm^2 flasks for each cell line. Cells were detached from the plastic surface by incubation in 0.25% sterile-filtered Trypsin-EDTA solution, pelleted and resuspended in 1ml of TriReagent^O (Sigma-Aldrich). Total cellular RNA was isolated from 3×10^6 cells of each breast cancer cell line using a modified version of the method of Chomczynski and Sacchi [18]. The RNA samples were resuspended in 20 μl of RNase-free water and stored at –80°C. RNA samples were reverse-transcribed (RT) into cDNA (Transcriptor First Stand cDNA synthesis kit, Roche). RQ-PCR was conducted in a real-time PCR detection system (Real-Time PCR 7900HT Fast RT System, Applied Biosystems) using SYBR® Green I as detection dye, and target cDNA was quantified using a relative quantification method. The relative abundance of EpCAM transcript in each sample was standardised by the internal standard of beta-actin (ACTB). For amplification, 2 μl of total cDNA solution was added to 18 μl of Maxima SYBR Green/ROX qPCR Master Mix (2 ×) (Fermentas) and primers (Table 1). One RNA sample of each preparation was processed without the RT-reaction to provide a negative control in subsequent PCR. The relative abundance of mRNA expression of EpCAM in LoVo cell line was used as a positive control. The 3T3 mice fibroblast cell line which does not express EpCAM was used as a negative control of EpCAM amplification. Therefore, all assays of expression patterns of EpCAM in breast cancer cell lines were referred in relation to LoVo cell line.

To quantify the specific genes expressed in the cell lines, the levels of expression of specific mRNAs in each sample were calculated relative to ACTB expression. To ensure the integrity of these results, the additional housekeeping gene Kα-tubulin 1 (Ka1), was used as an internal standard to demonstrate that ACTB mRNAs were not regulated differently in the tested cell lines. Real-Time PCR analysis was performed using the Visual Basic program implemented by Muller et al. [19].

Immunofluorescence technique. Cell lines were detached from the plastic surface of 75 cm^2 cell culture flasks by incubation with 0.25% sterile-filtered Trypsin-EDTA solution and resuspended in an appropriate culture medium. Cells

Table 1. Primer sequences used for Real-Time PCR

Gene	Sequence	Gene ID
EpCAM	fwd 5' GCTGGTGTGTGAACACTGCT 3' rev 5' CCAGGATCCAGATCCAGTTG 3'	NM_002354.2
ACTB	fwd 5' TCTGGCACCACACCTTCTAC 3' rev 5' GATAGCACAGCCTGGATAGC 3'	NM_001101.3
Ka1	fwd 5' TGGAACCCACAGTCATTGATG 3' rev 5' TGATCTCCTTGCCAATGGTGT 3'	NM_006082

Table 2. Mean normalised EpCAM mRNA expression of triplicates for each breast cancer cell line

Description	Mean of triplicates		
	Mean normalized expression	SE of mean normalized expression	SE of mean normalized expression in %
LoVo	3.07E-01	1.23E-02	4.00
3T3	1.72E-05	0.00E+00	0.00
MCF-7	2.67E-01	6.05E-03	2.27
BT-20	2.10E-05	2.76E-06	13.19
CAMA-1	1.50E-01	5.77E-03	3.86
T47D	6.95E-02	3.00E-03	4.32
SkBr-3	3.47E-01	2.98E-03	0.86
MDAMB-231	9.24E-04	5.55E-05	6.01

were counted and placed in groups of 100,000 (10^5), on glass slides by centrifugation in Cytospin (10 minutes, 1,000 rpm). Cells were fixed in 4% paraformaldehyde in PBS for 15 minutes, washed three times in PBS and blocked in 3% bovine serum albumin for 45 minutes. Primary anti-EpCAM antibodies (mouse monoclonal anti-human EBA-1, AUA-1, 9C4, 1:100, 1 hour/RT, Santa Cruz Biotechnology) were used with the respective FITC-labelled secondary anti-mouse antibody (MFP488, goat anti-mouse IgG, 1:500, 1h/RT, MoBiTec). Afterwards, cells were washed three times with PBS and sealed with DAPI medium. Analysis was made under a fluorescence microscope (Zeiss Axio-Imager.Z1). Antigen expression was defined as specific when the staining signal was present on the tumour cell membrane or/and in the cytoplasm. The staining intensity of tumour cells was scored in relation to the staining intensity of control cell lines (-, no staining; +, weak; ++, moderate; or +++, strong intensity).

Results

Real-Time PCR analysis

Using Real-Time PCR, we found an increased expression of EpCAM in the SkBr-3 cell line compared to LoVo. Moreover, we observed decreased EpCAM mRNA levels in MCF-7 (0.87 fold), CAMA-1 (0.49 fold) and T47D (0.23 fold) compared to LoVo. Much lower expressions of EpCAM mRNA in BT-20 and MDAMB-231 breast cancer cell lines, (14,600-fold and 330-fold, respectively), were detected (Table 2; Figure 1).

Immunofluorescence analysis

Immunofluorescent analysis of six human breast cancer cell lines showed that they significantly differed regarding the expression of the EpCAM protein. Differences were also observed in relation to three different antibodies used in the experiment (EBA-1, AUA-1, 9C4) (Table 3). The immunofluorescent analysis of the EpCAM protein expression revealed a similar pattern to the EpCAM mRNA profile when compared to the EBA-1 mAb staining.

A strong positive reaction for EBA-1 was observed in the SkBr-3, CAMA-1 and T47D cell lines (Figures 2A, B, C), and a weak signal was observed in the MCF-7 cell line. The MDAMB-231 and BT-20 cell lines revealed a lack of immunofluorescent signal for the EBA-1 antibody.

For the AUA-1 antibody, a moderate signal was observed only in the SkBr-3 cell line (Figure 3). The

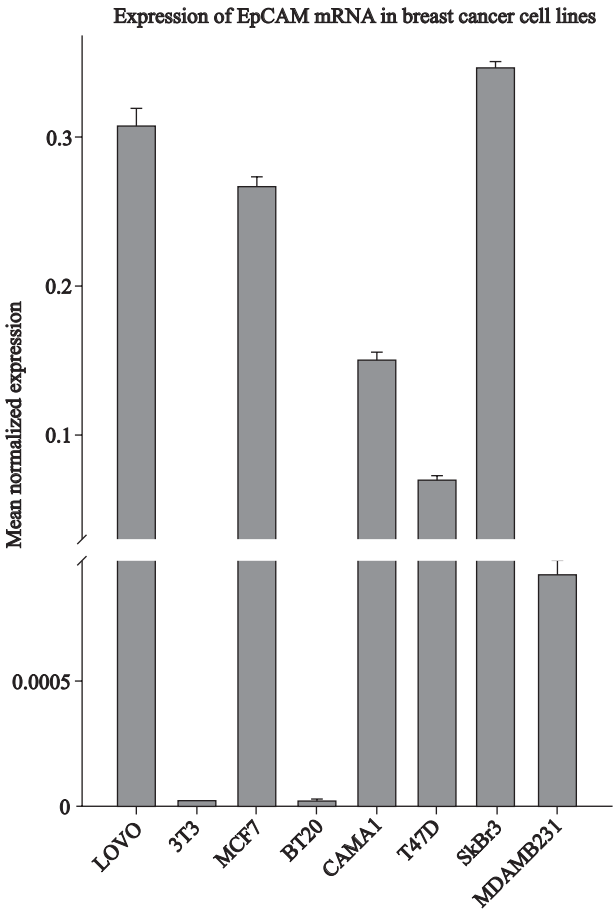

Figure 1. Mean normalised expression of EpCAM mRNA in breast cancer cell lines

Table 3. Results of immunofluorescence analysis of EpCAM protein expression in breast cancer and control cell lines

Cell line	Antibody		
	EBA-1	AUA-1	9C4
MCF-7	+	–	++
SkBr-3	+++	++	++
T47D	+++	–	++
CAMA-1	+++	–	++
MDAMB-231	–	–	++
BT-20	–	–	+++
LoVo	+++	–	+
3T3	–	–	–

Intensity score: – no staining, + weak, ++ moderate, +++ strong

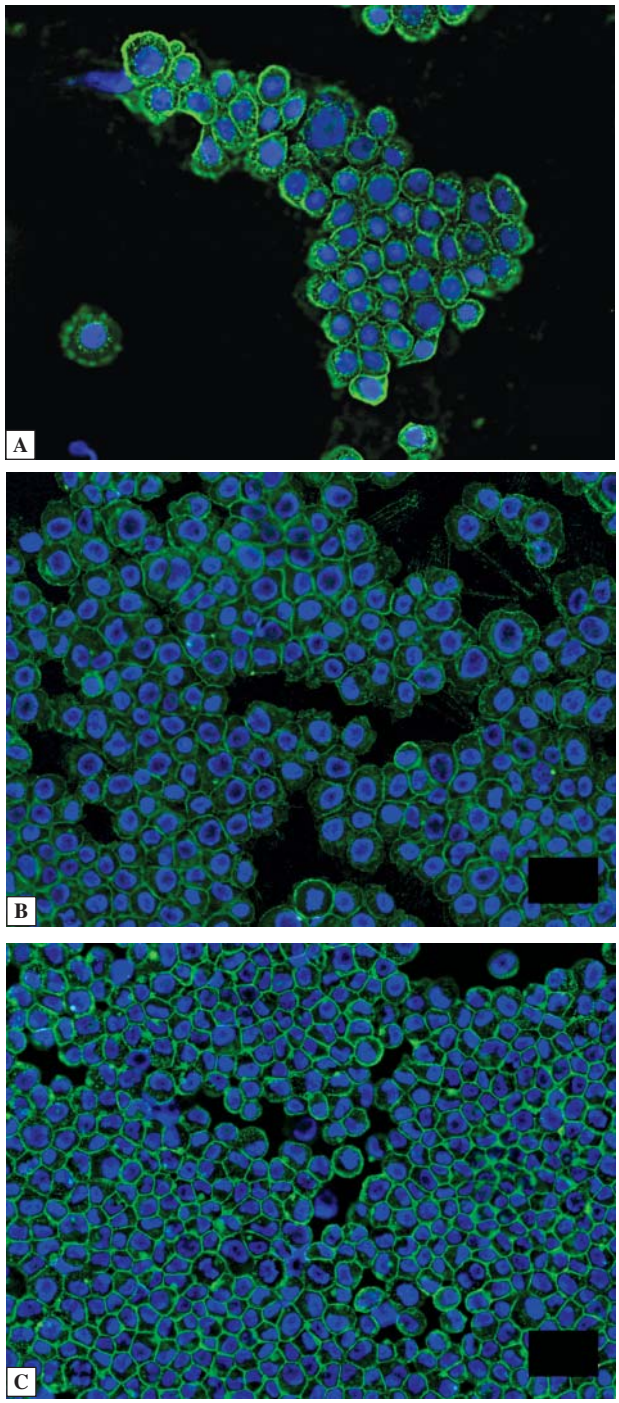

Figure 2. Immunocytochemical localisation of EpCAM with the use of EBA-1 antibody in: **A**) SkBr-3 breast cancer cell line; **B**) CAMA-1 breast cancer cell line; **C**) T47D breast cancer cell line. Strong membranous signal visible. Immunofluorescence technique; FITC — labelling; nuclei counterstained with DAPI. Objective magnification 20 ×

MCF-7, T47D, CAMA-1, MDAMB-231 and BT-20 cell lines were negative for this antibody.

The third antibody used in this assay was 9C4 (Figure 4). All the studied cell lines demonstrated a positive signal with a moderate staining intensity, except for the Bt-20 cell line, in which the signal was strong.

The positive control cell line, LoVo, revealed a strong immunofluorescent signal for EBA-1 (Figure 5A), weak for 9C4 and no signal for AUA-1 antibody. The 3T3 cell line was negative for all examined EpCAM antibodies (Figure 5B).

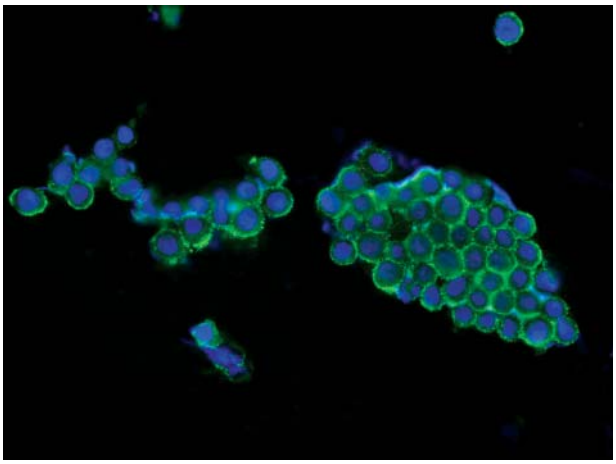

Figure 3. Immunocytochemical localisation of EpCAM with the use of AUA-1 antibody in SkBr-3 breast cancer cell line. Moderate membranous-cytoplasmic signal visible. Immunofluorescence technique; FITC — labelling; nuclei counterstained with DAPI. Objective 20 ×

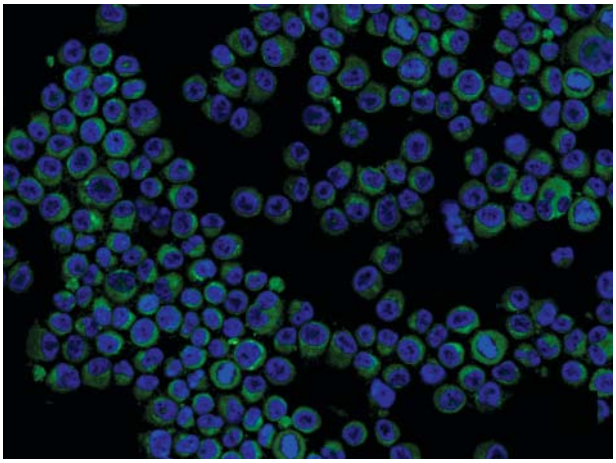

Figure 4. Immunocytochemical localisation of EpCAM with the use of 9C4 antibody in BT-20 breast cancer cell line. Strong cytoplasmic signal visible. Immunofluorescence technique; FITC — labelling; nuclei counterstained with DAPI. Objective 20 ×

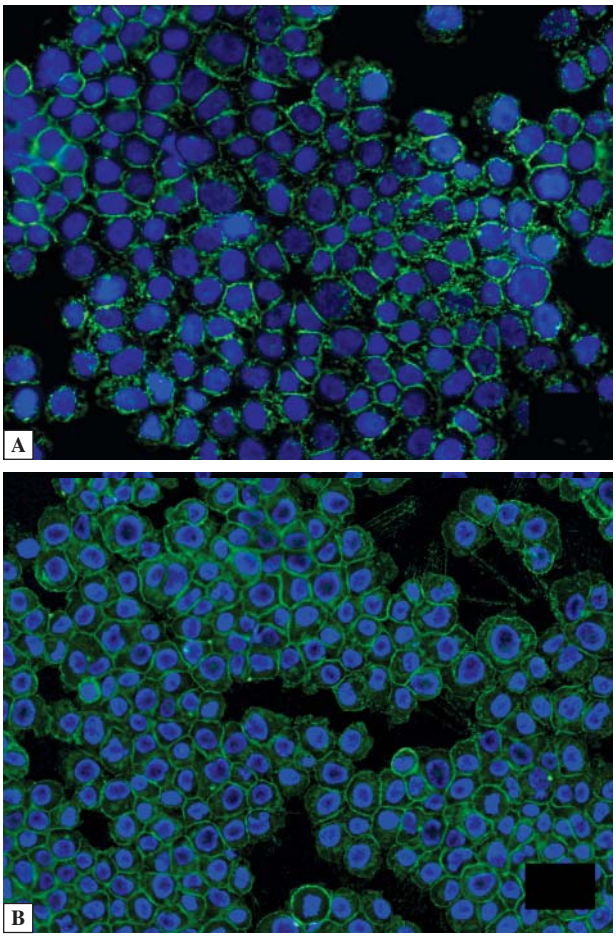

Figure 5. Immunocytochemical localisation of EpCAM with the use of EBA-1 antibody in control cell lines: **A**) LoVo cell line — positive control, **B**) 3T3/balb cell line — negative control. Immunofluorescence technique; FITC — labelling; nuclei counterstained with DAPI. Objective 20 ×

The immunofluorescent study additionally revealed diverse distribution of EpCAM within the breast cancer cells lines, which was also related to the type of applied antibody. Among the EBA-1 positive breast cancer cell lines (SkBr-3, CAMA-1, T47D), strong specific membrane staining was visible (Figures 2A, B, C; 5A). The 9C4 positive cell lines demonstrated mainly cytoplasmic (Figure 4) or/ /and membranous-cytoplasmic (not shown) distribution of the EpCAM protein, depending on the type of cell line.

Discussion

EpCAM is not expressed in non-epithelial tumours (melanomas, sarcomas, lymphomas) but is commonly overexpressed in cancers of epithelial origin. The protein is present in human carcinomas of various origins, including colon, breast, prostate, head and neck, and liver [10, 20–22].

In this study, Real-Time PCR and immunofluorescence analyses of six human breast cancer cell lines revealed that these cell lines differed significantly regarding EpCAM expression. Although more precise methods for cell phenotyping are available (e.g. flow cytometry), in this study immunofluorescence analysis was performed. Tested cell lines were of an adherent type, and therefore the necessary step in their flow cytometry analysis is to detach them from the culture dish surface and separate very carefully. In our study,

cells were detached without separation, and left in a cluster form.

We found differences in EpCAM expression especially in relation to three different anti-EpCAM antibodies used in the experiment (EBA-1, AUA-1, 9C4). The strength and specificity of reaction was dependent not only on the type of antibody, but also on the type of breast cancer cell line. It was shown by Baeuerle et al. that the prognostic value of EpCAM expression depended not only on tumour type, but also on disease stage, tumour microenvironment, and host antitumour immunity [4].

In this study, we found that the EBA-1 antibody revealed the strongest signal, but not for all types of examined cultured cells. Therefore, for immunohistochemical measurement of EpCAM protein expression, applied as a marker of cancer invasiveness, not only the histological subtype of cancer affects the reaction specificity, but also the type of antibody should be taken into account.

Our findings suggest that the best way to obtain the most specific results could be the simultaneous application of different anti-EpCAM antibodies. In the case of the studied breast cancer cell lines, this would be a mixture of EBA-1 and 9C4 antibodies. Additionally, EpCAM immunohistochemistry could be used as a diagnostic factor in the assessment of aberrant tissue morphology.

EpCAM is widely expressed in intensely proliferating cells of the intestitial epithelium [6]. An overexpression of EpCAM has been frequently demonstrated in different types of colorectal carcinomas [23]. Therefore, as a positive control in the experiment, the human colon adenocarcinoma cell line, LoVo, was used. The pattern of EpCAM immunoreactivity within the cell was found to be different in carcinoma cells compared to normal epithelia. In epithelial cells, this molecule is mostly expressed on the basal or basolateral cell membrane, and is located predominantly in the intercellular spaces where epithelial cells form very tight junctions [24]. However, in the present study, the EpCAM molecules were present at all surfaces of cultured breast cancer cells, especially adjacent cells, and showed high immunoreactivity at the cell-cell boundaries. Such overexpression of the EpCAM molecule is regarded as an important mechanism in disrupting cell-to-cell contacts, and thus enabling the cell migration required for the development of metastases. Therefore, the EpCAM might be considered to represent a prometastatic molecule.

The cellular localisation of EpCAM expression can be an important diagnostic factor in differentiating the type of carcinoma. A subcellular redistribution of EpCAM from basolateral localisation in normal tissues to various compartments in cancer cells could also play a role in a differential activation of EpCAM [25].

Similarly to the data of Gostner et al. [26], localisation of EpCAM in the cell was determined by cell culture density. We also found that highly confluent monolayers showed strong membranous EpCAM expression, while separated cells exhibited a much weaker signal. Additionally, differences in the localisation of EpCAM in cultured cells were observed, and were related to the type of applied antibody (i.e. EBA-1 — membranous, AUA-1, 9C4 — membranous and cytoplasmic).

In a large group of primary breast cancers, high levels of EpCAM expression correlated with a larger tumour size and lymph node metastases [27]. In addition, it was also associated with proliferative activity and contributed to neoplastic transformation [1, 8, 9]. According to Gastl et al., the overexpression of EpCAM in breast cancer is clearly associated with poor disease-free and overall survival [28].

Cancer cells detached from the primary tumour site enter the circulatory system, which results in metastases to distant organs e.g. lymph nodes. These cells, known as circulating tumour cells (CTCs), are detectable in blood samples from patients with metastatic cancer. Detection of CTCs in blood is a noninvasive method of sampling and studying tumour cells compared to classic biopsy. Most CTCs enrichment methods are based on the use of EpCAM-specific antibodies and subsequent detection using anti-cytokeratin [13, 14, 29, 30]. EpCAM is a surface marker found only on carcinoma cells. Nevertheless, to minimise the possibility of false-positive or false-negative results, a system of precisely evaluated diagnostic antibodies must be applied (e.g. pan-anti-cytokeratin antibody, a tumour-specific anti-EpCAM antibody).

The results of our study prove that not every type of anti-EpCAM antibody is suitable for targeting cancer cells in different types of breast carcinomas. As mentioned earlier, not only the level of EpCAM expression but also the distribution of this protein within the cell is an important factor in order to detect CTCs. Therefore, a more valuable type of antibody for the isolation of CTCs would be an antibody that is highly specific to the extracellular domain of EpCAM molecule (in this case, EBA-1).

Of all tumour types, CTCs have been most thoroughly investigated in breast cancer [14, 31, 32]. This provided the rationale for our studies on breast cancer cell lines. The detection of CTCs may predict the presence of micrometastases and may provide an earlier indication of disease status than body imaging methods. The detection of CTCs at the time of diag-

nosis may also help predict which patients would benefit most from different therapeutic strategies, e.g. from chemotherapy.

The aim of this study was also to analyse mRNA expression to compare the potential prognostic relevance of EpCAM mRNA and the immunoreactivity of this protein. In the studied breast cancer cell lines, we found an association between the intensity of immunostaining and EpCAM mRNA expression levels, especially with the EBA-1antibody. The results of our study support the results of other investigators who validated EpCAM mRNA expression in untreated node-negative breast cancer patients. They also demonstrated the association between high and low mRNA expression and high and low immunostaining levels, respectively [33]. Therefore, the analysis of the EpCAM mRNA expression can serve as an additional valuable, precise and sensitive method of EpCAM expression evaluation, because mRNA expression is not always followed by respective protein expression. Moreover, measurements of antigens' mRNA cannot provide data on the protein distribution within the cells which is essential for the use of EpCAM antigen for CTCs binding.

In conclusion, the EBA-1 and 9C4 anti-EpCAM antibodies may play a significant role in targeting circulating tumour cells. The former, because it provided the strongest immunofluorescent signal of all the other tested antibodies. The latter, because it was specific for all examined breast cancer cell lines.

However, the diversity of reaction sensitivity depends on the type of applied antibody. Therefore, the simultaneous application of different anti-EpCAM antibodies is recommended. Thus, an appropriate choice of the anti-EpCAM antibodies and evaluation of EpCAM expression in breast cancer appears to be crucial, especially as it is proposed that this antigen be used as a marker to detect circulating tumour cells.

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