

# Detection of Tumour Cells in the Peripheral Blood of Patients with Breast Cancer. Development of a New Sensitive and Specific Immunomolecular Assay

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Malignant cells in the peripheral blood of patients with solid tumours are of considerable importance for the prognosis and therapeutic correlation. Their detection however is difficult due to lack of sensitivity, specificity and technical problems in standardisation.

In this original article we show a new sensitive method overcoming the hitherto known difficulties by combining traditional antibody-techniques with a RT-PCR. Due to this method 2 tumour cells within 5 ml of peripheral blood can be detected in spiking experiments.

**Key Words:** Tumour cells, Peripheral blood, Immunomolecular test, RT-PCR

Malignant cells in the peripheral blood of patients suffering from solid tumours have been described since 1955 (1,2). They can be detected by monoclonal antibodies (3-5) and other means such as morphology with new methods such as Laser Scanning Microscopy (6). Riethmueller (7,8) and others (9) have focused on the need for newer methods in immunocancerology and have shown the difficulties for the interpretation of cancer cells found in the peripheral blood of tumour bearers. Hence, we have tried to combine 2 different approaches such as traditional antibody techniques with molecular assays (10,11), to overcome the difficulties one system alone can cause.

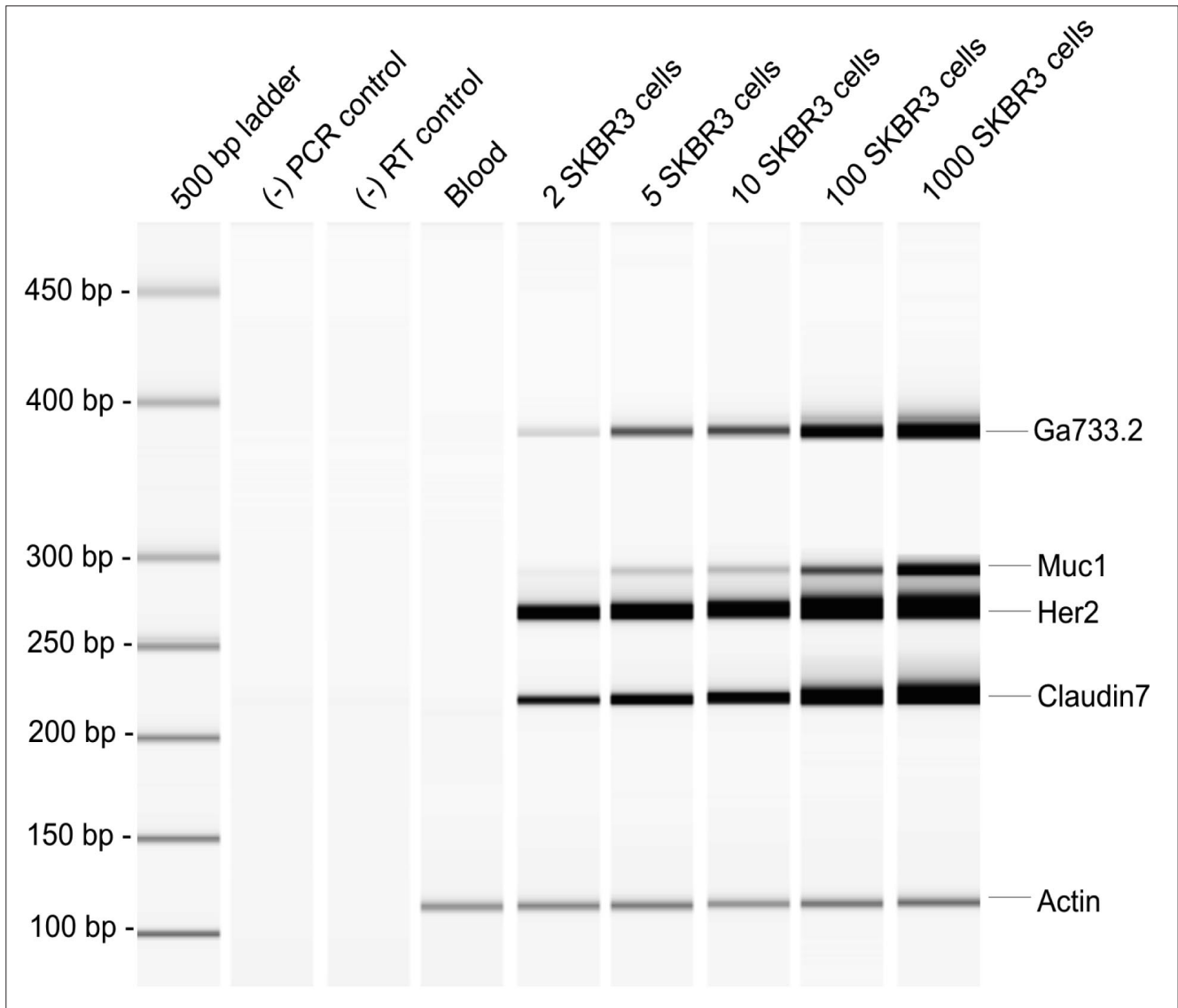
## Materials and Methods

Human breast cancer cells from tissue cancers (Sk Br3) were kindly provided by AdnaGen and used in a serial dilution in our laboratory. Two cells from this dilution in a Petri dish were chosen and picked up with an Eppendorf pipette under the inverted microscope. These 2 breast cancer cells were added to 5 ml of peripheral EDTA-blood from normal healthy volunteers for standardisation of the spiking experiments (Dilution experiments ranging from 100 to 1 cells were

carried out to find the detection level of our assay). Further processing was carried out within the following 4 hours.

*Tumour cell enrichment.* These 5 ml of spiked blood were mixed with 100 µl of an antibody cocktail fixed on immunomagnetic beads containing 3 different monoclonals namely ber-ep 4 (DAKO, Germany), Muc 1 (BD, USA), GP 1.4 (Neomarkers, USA), for an epithelial membrane marker, for a mucin marker and a glycoprotein marker respectively. These antibodies were titrated, fixed on immunomagnetic beads and used as a ready for use mixture commercialised as "AdnaTest BreastCancerSelect" by AdnaGen. Incubation was followed by a washing step in PBS (tube rotator, 20 rpm, 2 hours, room temperature). After incubation the cells were washed with PBS and lysed by adding a washing/lysing buffer (DynaL, Norway). The supernatant was recovered and the mRNA recovered by a magnetic separation.

*mRNA isolation.* For the mRNA isolation the mRNA Direct Micro Kit (DynaL, Norway) was used. 20 µl of pre-treated Dynabeads were incubated with 200 µl of the lysate (10 minutes, tube rotator, 20 rpm). After 2 more washings in PBS the mRNA-bead mix-



**Fig. 1 -** Results of the spiking experiment with SkBR 3 Cells: 2, 5, 10, 100 and 1000 tumour cells were added to 5 ml EDTA - blood form healthy controls and the existence of Ga 733.2, Mucin 1, Her 2 and Claudin 7 genes assayed. Actin served as house keeping gene, for the negative controls see text. The migration of the PCR products was compared with a ladder of standard products with known base pairs. One clearly recognised the sensibility of the test by the net 3 tumour bands in the 2-cell experiment, increasing to 4 tumour bands from 5 cells onwards.

ture was diluted in 29.5 µl of distilled water, incubated for 5 minutes at 50°C, centrifuged at 640 rpm and chilled down immediately on ice (2 minutes).

**RT - PCR.** Total mRNA/bead mixture (29.5 µl) was reversed transcribed in 0.5 µl RNase inhibitor (40 U/µl, Promega, Germany), 4 µl RT buffer, 4 µl dNTPs and 2 µl Sensiscript reverse transcriptase (Qiagen, Germany). Reverse transcription was performed in a one step reaction (60 minutes at 37°C, 5 minutes at

93°C). Thereafter the mixture was chilled down on ice and stored at - 20°C.

**"Tumour specific PCR".** For the analysis of the tumour associated mRNAs a multiplex PCR was performed. Primer selection was optimised and the reagents as "AdnaTest BreastCancerSelect" used. In brief, this primer-mixture consists of 5 specific primer pairs for the amplification of 4 tumour markers and one house keeping gene. The tumour markers are: Ga

733.2, Ca 15.3, Her 2, Claudin 7. As house keeping gene, beta actin was used in our experiments. PCR-reactions were performed in a final volume of 50  $\mu$ l PCR mixture containing 8  $\mu$ l cDNA, 3.4  $\mu$ l primer-mixture (AdnaGen), 25  $\mu$ l Hot Star Taq Master Mix (Qiagen) and 13.6  $\mu$ l water.

PCRs were performed as follows: Pre-denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 94°C, annealing at 60°C for 1 minute, extension at 72°C for 1 minute and a final extension step at 72°C for 10 minutes.

Controls consisting of "unspiked material" were run in parallel with each experiment to exclude contaminations and false positives. mRNA and cDNA were replaced by water in the reverse transcription and polymerase chain reaction experiments.

Visualisation of the data was carried out in an Agilent Bioanalyzer electrophoresis or gel stained with ethidium bromide. The reaction was considered positive in the case of visualisation of at least 1 tumour marker and the beta actin band. All included controls (cDNA and PCR) had to be negative.

## Results

All results are convincing and a typical analysis is shown in Fig.1. Briefly, characterised within the frame of the ladder on the left with the molecular weights from 25 to 400 base pairs, we show the negative controls and the results of the spiking experiments with 2, 5, 10, 100 and 1000 cells. The interpretation is semi-quantitative only leading to an approximate impression of the tumour cell number existing in peripheral blood (Fig.1). Results of the spiking experiment with SkBR 3 cells: 2, 5, 10, 100 and 1000 tumour cells were added to 5 ml EDTA-blood from healthy controls and the existence of Ga 733.2, Mucin 1, Her 2 and Claudin 7 genes assayed. Actin served as house keeping gene, for the negative controls (see text). The migration of the PCR products was compared with a ladder of standard products with known base pairs. One clearly recognised the analytical sensitivity of the test by the net 3 tumour bands in the 2-cell experiment, increasing to 4 tumour bands from 5 cells onwards. Blood without spiked tumour cells revealed no tumour associated bands but the internal control actin only confirming a high degree of specificity. Thus, spiked tumour cells were detected in blood with high sensitivity and specificity using a combination of different antibodies for tumour cell selection with different markers in a multiplex RT-PCR.

## Discussion

Tumour immunology has been of considerable interest in modern medicine above all in western countries (12). However, the methods used were based on morphology and immunohistochemistry (13), while the development of molecular methods (7,14) were successful in biopsies. Even the progress made by biconal antibodies and the combination of fine needle biopsies with blood analysis (15,16) could not answer the question regarding the forbidden antigen expression causing nonspecificity of the results.

For this reason and to bypass this phenomenon of tumour biology we have built up a new assay combining traditional immunology using a monoclonal antibody cocktail with molecular biology employing a RT-PCR. For the cocktail, 3 monoclonals have been chosen specifically for mucin, membrane epithelial antigen and a glycoprotein antigen. This combination should be relevant for the majority of breast cancer cells. Even this selection would not resolve the problem of forbidden antigen expression. Hence, we have added an additional step of molecular selection for Claudin, Ga733.2, Muc 1 and Her 2 genes. These transcripts should not occur in the blood stream being derivatives from epithelial or neoplastic elements only (17). The Claudin 7 gene codes for the respective protein associated with tight junction proteins (18). An overexpression seems to be relevant for breast cancer development in the mouse model. The mucin gene, coding for the MUC 1 protein, occurs on the surface of membrane cells and seems to be correlated with the aggressivity of some of the mammary tumours (19). Clinicians are more familiar with the Mucin antigen under the name of CA 15.3 antigen. The Ga 733.2 is coding for a typical epithelial marker used by us for the characterisation of forbidden (tumour) cells in the peripheral blood of patients. It has been known as EGP 40 (epithelial growth protein) or EpCAM (epithelial adhesion molecule), being a typical pan-epithelial marker (20). The Her 2 gene being responsible for coding the HER 2 proteins occurs in about 35 % of all breast cancers over-expressed by either amplification or increased transcription (21). It seems to be an interesting marker offering also new hopes for the strategy of immune intervention by monoclonal anti HER 2. Tim Allen Mersh (22) as early as in 1981 and other distinguished researchers have shown the influence of tumour cells in blood, lymphatics and bone marrow on the prognosis of patients suffering from various types of malignancies. Relevant tests are urgently needed to overcome the problems of sensitivity, specificity, for-

bidden and "allowed" antigen expression of tumour cells in the peripheral blood, since diagnostic and even therapeutic consequences will depend on reliable tests.

**Acknowledgements:** This work was supported by the Wissenschaftsfonds Wissenschaft and Forschung Land Steiermark.

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Received: April 10, 2004

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