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Detection and characterisation of disseminated tumour cells in bone marrow of breast cancer patients by immunostaining of Her-2 and MUC-1 in combination with Thomsen-Friedenreich (CD176)

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Summary. Disseminated tumour cells (DTCs) in the bone marrow derive from many primary tumours, such as breast cancer. Their mere existence hints to present or future metastasis and implicates a worse prognosis for the patient. DTCs may possess different characteristics in comparison to the primary tumour due to events like Epithelial-Mesenchymal-Transition. Therefore these cells might be able to survive chemotherapy and cause relapses of the disease at a later point.

We aimed to detect and further characterise DTCs by an immunostaining approach with three different antigen markers (Her-2, MUC-1 and TF, also known as CD176). For that reason, bone marrow of 41 breast cancer patients was obtained during surgery; DTCs were enriched by density gradient centrifugation and cytospins were prepared. After fixation, immunofluorescent double-stainings were carried out with antibodies against CD176 in combination with HER-2 or MUC-1. Cells co-expressing two antigens were found in all staining combinations (Her-2 and CD176: 46.14%; MUC-1 and CD176: 18.15% of all cases).

Cells that stained for a single antigen only were also found (Her-2: 36.86%; MUC-1: 34.45%; CD176: 29.65% of all cases).

Significant correlations between the stainings of all markers could be shown ($p<0,001$).

In conclusion, Thomsen-Friedenreich Antigen (TF, CD176) is a promising marker in combination with the established marker Her-2 and other markers like MUC-1. These results may serve as a basis for future DTC detection routines and help to individualize medical treatment, reducing side effects and increasing the efficiency of the therapy.

Key words: Imunostaining, Bone marrow, DTCs, Thomsen-Friedenreich-antigen

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Introduction

The occurrence of remote metastasis is the main reason for cancer-related death in patients with epithelial carcinoma, for example breast cancer. For the formation of these metastases single epithelial cells detach and disseminate from the primary tumour, enter the circulation and settle down in a new host organ. In breast cancer, the most frequent sites to harbour metastasis are the liver, lungs, brain and bone (marrow). Tumour residues, specifically in the bone marrow, are called disseminated tumour cells (DTCs) (Wolfle et al., 2006) and have been shown to survive in a dormant state for long time periods (Pantel and Woelfle, 2004; Goss and Chambers, 2010). Once activated, they then turn into an aggressive state and not only build the seed for metastasis formation in the bone marrow, but also spread from there (Chambers et al., 2002). This way of metastatic spread was formerly regarded as one of the late processes during malignant progression, but is now seen as one of the early events in cancer outgrowth (Husemann et al., 2008).

The incidence of DTCs in patients with primary

breast cancer was shown to be correlated with a poorer survival prognosis than for patients without detectable DTCs in their bone marrow (Braun et al., 2005). Furthermore, the presence of DTCs in bone marrow was associated with a higher tumour stage, presence of lymph node metastasis and low hormone receptor expression. Therefore the presence and detection of DTCs could be associated with a prognostic relevance in breast cancer patients. The detection of DTCs in bone marrow (and CTCs in peripheral blood) was already introduced into the international tumour staging systems (Hermanek et al., 1999; Singletary et al., 2003; Harris et al., 2007). Although the prognostic relevance of DTCs and CTCs was widely shown (Fehm et al., 2008; Ross and Slodkowska, 2009; Molloy et al., 2011), their predictive value still remains to be clarified more in detail (Alsamarai et al., 2012).

Currently there are two different detection methodologies available for DTCs and CTCs: nucleic acid based Real-Time PCR techniques and detection of CTCs/DTCs by immunohistochemical staining procedures (Pantel et al., 2008). Due to the small number of CTCs/ DTCs (1 per 10^{6-7} leukocytes (Ghossein et al., 1999)) a common step in both methods is the enrichment of the cell fraction containing CTCs/DTCs prior to detection. Most of these enrichment systems rely on antibody-based enrichments that capture cells expressing the epithelial surface marker EpCAM (Bednarz-Knoll et al., 2011). However it is known that its presence varies significantly in different tumour types, and EpCAM is also heterogeneously expressed in the same tumour (Thurm et al., 2003; Braun et al., 2005). Moreover, disseminated tumour cells often undergo epithelial mesenchymal transition (EMT), in the process of which the expression of epithelial markers, such as EpCAM, becomes down regulated (Bednarz-Knoll et al., 2011). Ultimately EMT-transformed tumour cells would thus escape the above-mentioned enrichment procedure.

Due to these difficulties, we sought to develop a different approach for DTC detection. Here we present an immunohistochemical detection method for DTCs from bone marrow, with an enrichment of the target cell fraction via density gradient centrifugation with subsequent DTC detection based on a combined immunofluorescent staining for the following well-known cancer antigens:

CD176, also known as Thomsen-Friedenreich antigen (TF), is a specific oncofetal carbohydrate epitope (Gal-beta-1-3-GalNAc-alpha-O-Ser/Thr) expressed on the surface of various carcinomas, especially in breast cancer (Schindlbeck et al., 2005a,b). TF mediates endothelium adhesion and the formation of metastases (Schindlbeck et al., 2005a,b). In this study, we co-stained TF with Her-2 and Muc-1.

Her-2 is a well-established and specific tumour marker for breast cancer that can be therapeutically targeted with substances like Trastuzumab and Lapatinib in daily clinical use. Simultaneously, as previously mentioned, it is also one of the most promising targets to detect DTCs. Her-2 is a tyrosine-kinase receptor of the EGF receptor family (Wolff et al., 2007) which also includes HER1 (EGFR), HER3, and HER4. Nowadays it is known that the Her-2 status of the primary tumour does not necessarily correspond to the Her-2 expression of the respective DTCs. This phenomenon is also known as Her-2 switch (Solomayer et al., 2006; Krawczyk et al., 2009). The therapeutic consequences, which are now under investigation within the DETECT III study, are to apply a Her-2 directed treatment with Lapatinib additional to standard therapy with Denosumab (www.detect-studien.de, 2013) in the case of an Her2-negative primary tumour with Her2-positive CTCs.

Mucin 1 (Muc-1) is another widely accepted DTC marker. It is regulated by estrogen receptor alpha and is known to be overexpressed in breast cancer (Zaretsky et al., 2006). Furthermore, it was reviewed to be a target for breast cancer immunotherapy (Yang et al., 2007) and it was proposed for assessment as biomarker for treatment and prognosis of breast cancer (Hanson et al., 2001).

The immunofluorescent staining of the above-described antigens for the detection of cancer cells was already established on blood samples spiked with breast cancer cell line cells (Andergassen et al., 2013) with recovery rates of about 80%. In the present work, we apply the method to bone marrow samples of adjuvant breast cancer patients to further validate it in terms of DTC detection and simultaneous cancer cell characterization. This simple, fast and cost-effective technique could lead to a better understanding of the nature of DTCs. Ultimately, this knowledge could pave the way for better adapted therapeutic strategies in breast cancer patients in the future.

Materials and methods

Bone marrow

Bone marrow aspirates of 41 primary breast cancer patients were collected intraoperatively from left and right anterior superior iliac spines under general anaesthesia after informed consent was obtained (Ethical vote: 148-12). Generally, 5-10 ml of bone marrow was aspirated per patient. Anti-coagulation was guaranteed by the addition of 1ml Heparin.

Data concerning the Her-2 status of the primary tumour were obtained for 27 patients by immunohisto-chemistry (IHC-Test 4B5, Ventana MedicalSystems, S.A., Illkirch, Frankreich) while TF was never analysed in the primary tumour material.

Between 2 and 3 ml of the aspirate were used for the presented experiments. To remove lipids from the samples, 25ml Hank's Salt Solution (Biochrom AG, Berlin) were added to each probe and subsequently centrifuged (170g, 10 min, 9°C); the upper lipid phase was carefully discarded. The remaining bone marrow-containing layer was transferred to a new tube with 8 ml Ficoll Paque Plus® (GE Healthcare, Uppsala) and

carefully layered onto it. After a centrifugation step (1105g, 20 min, RT) the resulting buffy coat was transferred into a fresh tube, washed with PBS (Biochrom AG, Berlin), spun down (535g, 10 min, RT) and resuspended in 5 ml PBS. Then, cells were counted in a haemocytometer using 0.4% Trypan Blue Solution (Sigma, Steinheim) to exclude dead cells from the cell count. Cytospins with $1x10^6$ cells each were prepared by spinning the samples on cover slips (500 rpm, 5 min, RT). The used coverslips (Thermo Scientific; Waltham, MA) were specially treated by the manufacturer so that tissue sections and cells adhere to the glass electrostatically without the need for special coatings. PBS was removed and slides were air-dried overnight.

Staining

The prepared slides were fixed in acetone and air-dried before blocking in blocking solution (5% BSA in PBS) for 15 minutes at room temperature (Bovine Serum Albumine was obtained from Sigma, Steinheim). The incubation in blocking serum is necessary to prevent unspecific binding of the antibodies during staining procedure.

For the TF Her-2 doublestaining, slides were first incubated for 45 minutes at room temperature in DAKO S 3022 medium with antibodies against Thomsen-Friedenreich (TF) (NEMOD-TF2-antibody; IgM K, mouse; NEMOD Biotherapeuticals, Berlin; dilution of 1:50). Slides were washed in PBS twice for 5 minutes. A secondary Cy-2 conjugated antibody (Goat-Anti-Mouse IgM, Jackson Immuno-Research, West Grove) was added in a dilution of 1:200 in DAKO S 3022 medium and again incubated for 30 minutes at room temperature (RT) in the dark. The following steps were carried out in the dark.

After washing with PBS twice for 5 minutes, a secondary blocking step was carried out with Ultra V Block (Labvision TA-060-UB) for 15 minutes. Antibodies against Her-2 (c-erb B-2 Ab-2 mouse IgG; NeoMarkers, Fremont) were applied in a dilution of 1:300. Slides were incubated for 45 minutes at RT. After two further washing steps with PBS (5 minutes each), a secondary Cy3-coupled antibody (Goat-Anti-Mouse IgG, Jackson Immuno Research, West Grove; Dilution: 1:200) was applied and incubated for 30 minutes.

For the staining with TF doublestaining, MUC-1, different antibodies were used: Muc-1 antigen antibody: Mucin 1 (mouse IgG, Santa Cruz Biotechnology, Santa Cruz, dilution: 1:50 in DAKO S 3022) followed by a secondary antibody: Goat-Anti-Mouse IgG-FITC, Santa Cruz Biotechnology, Santa Cruz, dilution: 1:200 with DAKO S 3022). For this staining, the secondary antibody against TF had to be adjusted: CY-3 (Goat-Anti-Mouse IgM, Jackson Immuno-Research, West Grove, dilution: 1:200 with DAKO S 3022).

Positive and negative controls for the staining were included using blood of a healthy donor with or without addition of tumour cells and subsequent staining with the same antibody combinations as used for bone marrow samples (Fig. 1). The staining was thereby shown to be specific to tumour cells.

The used antibodies for TF, Her-2 and Muc-1 were all raised in mouse. To prevent cross reactions, antibodies of different subclasses (IgG, IgM) were applied. Controls were implicated, staining slides with only one of the primary antibodies but both secondary antibodies (Fig. 2) and with both primary but only one of the secondary antibodies to demonstrate specificity of the staining (Fig. 3)

The cell nuclei were counterstained with DAPI Vectashield mounting Medium (Vectashield H-1200) and finally covered with cover slips and sealed with transparent nail polish.

To show individual staining patterns of the antibodies against TF, Her-2 and Muc-1, a triple staining was carried out. Therefore for Her-2, the following antibody was used: Her-2 receptor/Cy5 conjugated (polyclonal, rabbit, Bioss, Woburn). This antibody was also tested for crossreactions with the other antibodies used for the triple staining. No crossreactions were seen.

Microscopy

The evaluation of the slides was performed on a Zeiss Axioskop epifluorescence microscope, including an AxioCam MRm high resolution microscopy camera. A PH2 Plan neofluar 40x/0.75 objective was used.

Pictures of representative areas were taken and colour channels were merged by PaintShop Pro 6.00. (Figs. 4-6). Stained cells were counted manually and the number of DTCs in comparison to surrounding bone marrow cells was calculated.

For additional investigations, a triple immuno-fluorescence staining was carried out and processed with a confocal laser microscope (Leica TCS SP5 II; Leica, Wetzlar, Germany) (Fig. 7).

Statistics

Statistical evaluation was done by SPSS v. 20.0.

Non-parametrical correlations were drawn by Spearman's Rho Test for significance.

Results

Detection of Disseminated Tumour Cells (DTC) by immunofluorescence staining for TF together with Her-2 or Muc-1.

We analysed bone marrow aspirates of 41 breast cancer patients after written consent was obtained. Bone marrow cytospins were stained for combinations of TF with the established DTC markers Her-2 or Muc-1 (Figs. 4-5). Single stainings for each of the antigens were also included (Fig. 2C).

32 out of 41 slides stained positive for either TF or Her-2 or the combination of both, leading to a staining percentage of 78.05%. Similar percentages were found when staining bone marrow samples with TF and Muc-1: 87.5% of all slides were stained by TF in combination with Muc-1. It was tested if these amounts of staining had statistical significance in correlation to a non-specific staining. Spearman's Rho test revealed a statistical significance of staining for both cases ($p<0.001$) (Table 1).

A thorough evaluation of the stainings revealed that 46.14% of the cells stained with TF and Her-2 show co-staining with both antigens, whereas 17.0% are positive for TF only and 36.86% are positive for Her-2 only. The samples with immunofluorescent stains for TF and Muc-1 double stainings were found in 18.15%, Muc-1 single staining was detected in 34.45% and TF in 47.40% of all stained cells (Table 2).

For a control of specificity of the used antibodies TF, Her-2 and Muc-1 were stained simultaneously and staining was analysed by confocal laser scanning microscopy, due to the ability of this sophisticated microscope to display four colour channels at the same time (DAPI was also used as nuclear counterstain). All staining patterns were clearly distinguishable (Fig. 7), meaning that the used antibodies stain different cellular structures.

Negative control using blood of a healthy donor did

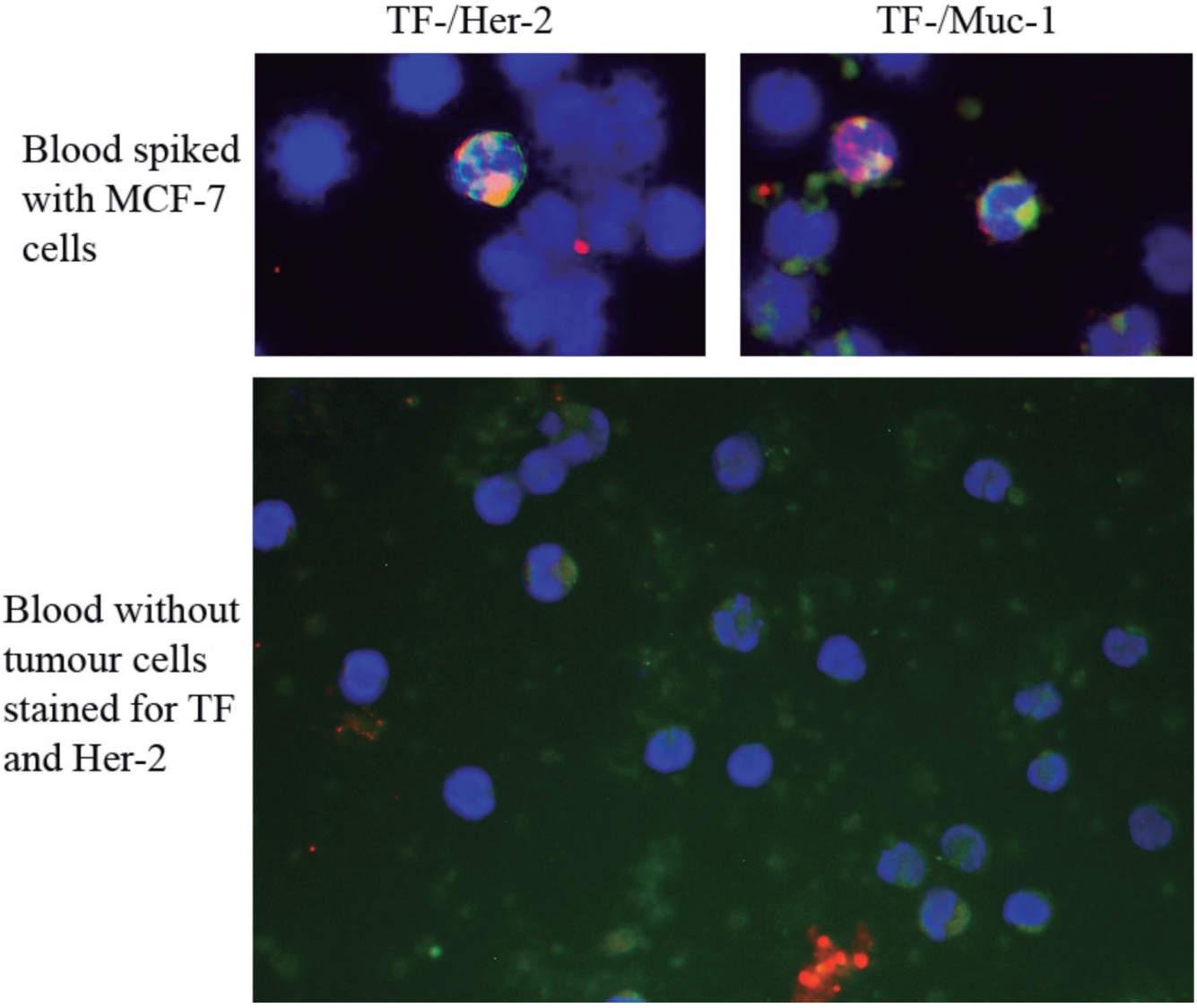

Fig. 1. Control stainings I – Positive and negative control. Blood samples of a healthy donor were spiked with MCF-7 cells (upper row) and stained with the same antibody combinations as bone marrow samples. Blood sample without addition of tumour cells stained for TF and Her-2 (lower row).

not render any staining, but positive control (blood of a healthy donor spiked with MCF-7 cells) showed a tumour cell specific staining (Fig. 1). Cross reaction of the secondary antibodies were ruled out as well by

Table 1. DTC detection frequency.

	TF-Her-2	TF-Muc1
Σ patient samples	41	40
# of patient samples with positive stainings	32	35
% positive stainings	78.05	87.50
Significance	p<0.001	p<0.001

Table 2. Statistics on positive stainings within the different staining subgroups*.

TF/Her-2	TF single staining	Her-2 single staining	TF + Her-2 double staining
% of stained cells	17.00	36.86	46.14
TF/Muc-1	TF single staining	Muc-1 single staining	TF + Muc-1 double staining
% of stained cells	47.40	34.45	18.15

All stained cells of the analysed samples were subdivided for their staining properties. *: every double staining was carried out on single cover slips.

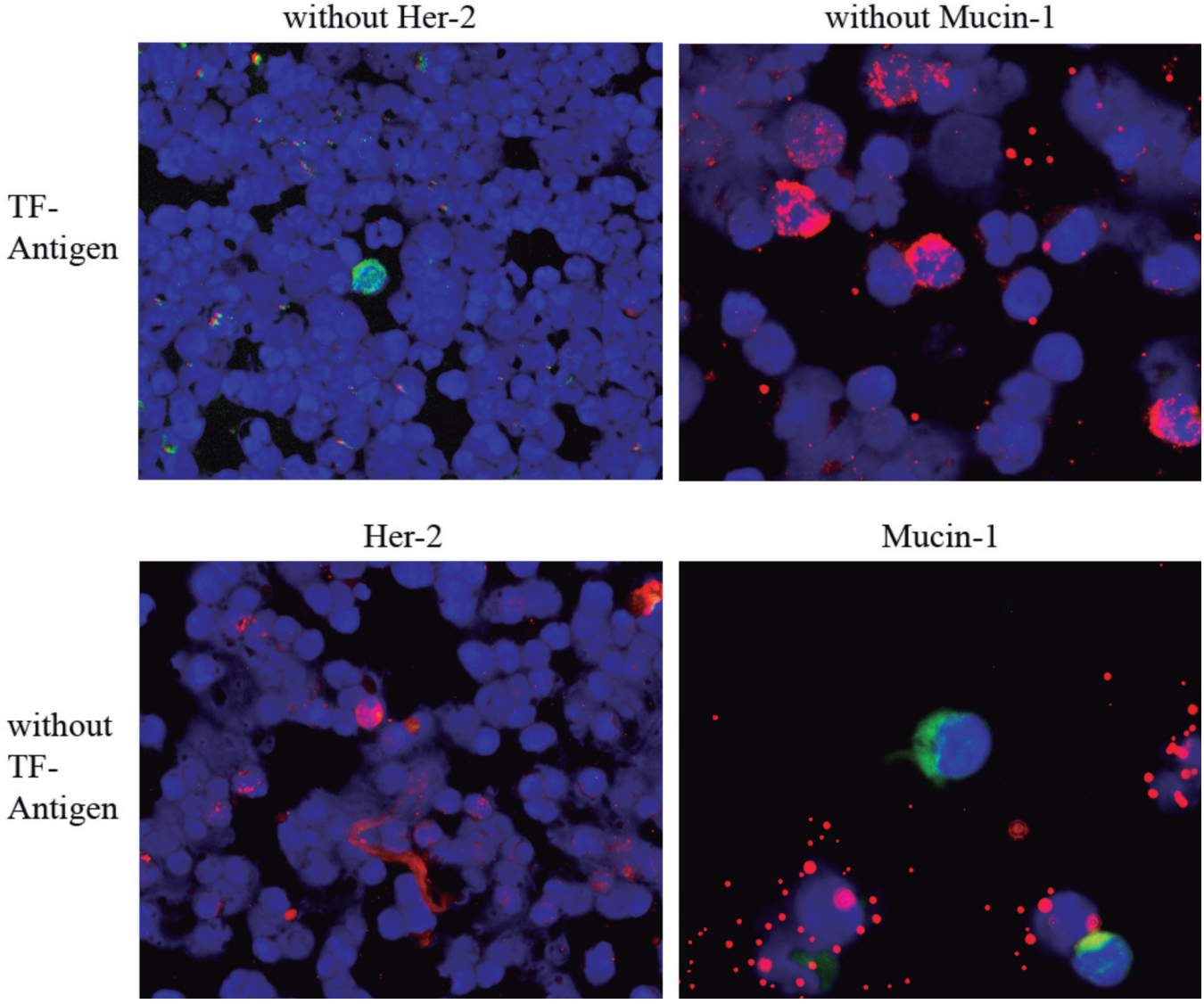

Fig. 2. Control stainings II – Exclusion of antibody crossreactions. Staining of bone marrow with only one primary (upper row: TF primary antibody used, Her-2 or Mucin-1 omitted; lower row: TF primary antibody omitted, Her-2 or Mucin-1 used) but both secondary antibodies.

staining of bone marrow with both primary but only one secondary antibody (Fig. 2) and with both primary but only one secondary antibody (Fig. 3).

Table 3. Correlations between Her-2 antigen switch and primary tumour size.

	Antigen switch (Her-2)	No Antigen switch (Her-2)
Overall	N=15; 55.5%	N=12; 44.4%
pT1	N = 9; 33.3%	N= 5; 18.5%
pT2	N=4; 14.8%	N=5; 18.5%
pT3	N= 2; 7.4%	---
pT4	---	N =2; 7.4%

27 Samples were compared for Her-2 status in primary tumour and in DTCs. Total numbers and percentages are given.

Co-staining of TF and Her-2 increases sensitivity of DTC detection

In 32 out of 41 patients (78.05%) at least one cell per 10^6 bone marrow cells was detected with positive stains for Her-2 or TF. The number of detected cells varied between 1 and 24 per 10^6 total cells (Fig. 8). Co-staining of TF and Her-2 helped not only to identify more patients with DTCs, but also facilitated the discovery of multiple DTCs in the same patient. TF staining was able to uncover 1 patient with more than 10 DTCs, double-staining detected 7 samples with the maximum number of 24 DTCs (Fig. 8, Table 2).

Her-2 switch in small tumours

According to previous reports (Solomayer et al., 2006; Krawczyk et al., 2009), a switch in Her-2 marker

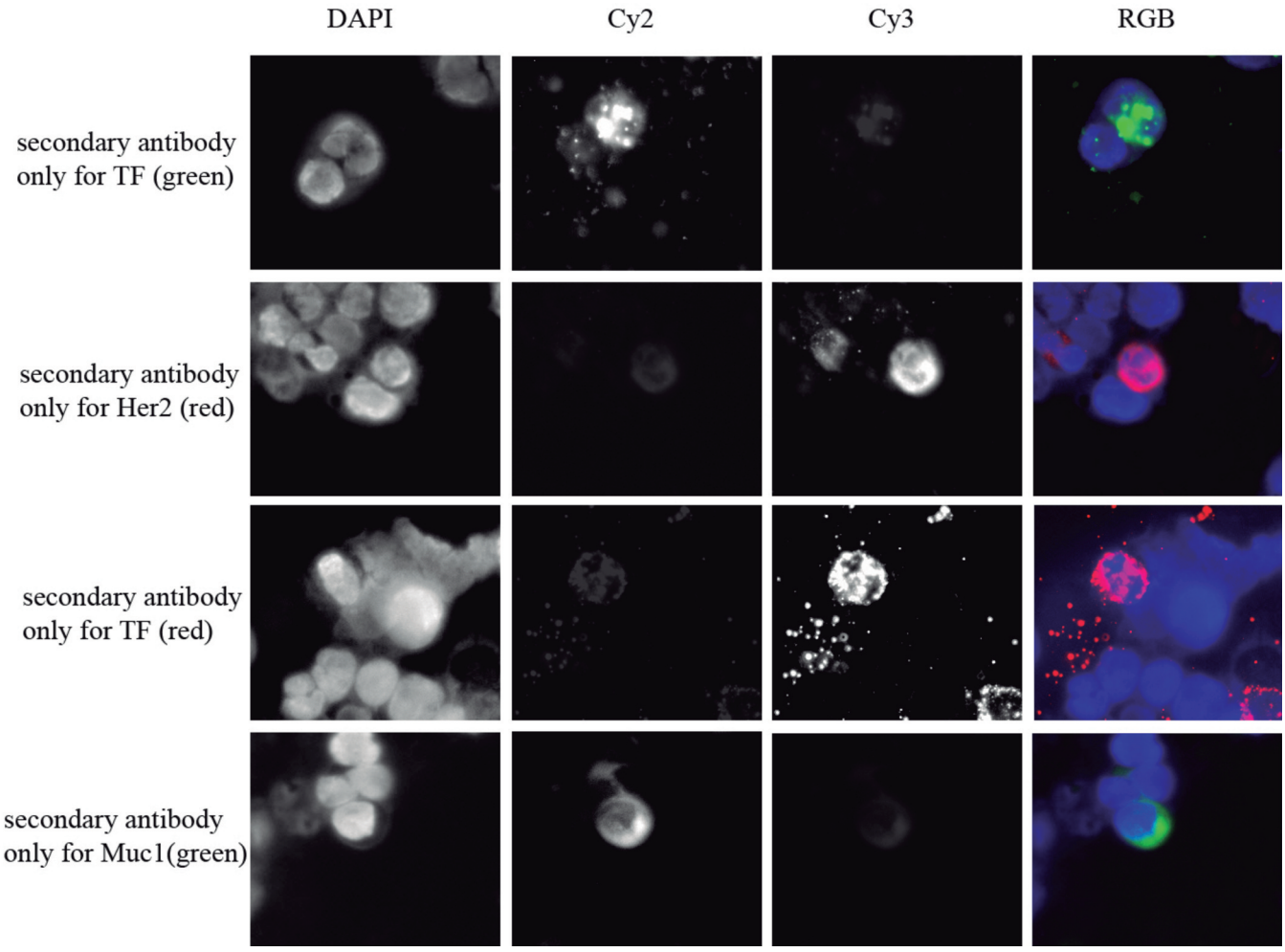

Fig. 3. Control stainings III - Exclusion of antibody crossreactions. Staining of bone marrow with both primary antibodies, omitting one of the secondary antibodies (a slight bleeding through between Cy2 and Cy3 is seen because of overlapping emission spectra of fluorochromes).

expression can occur. This phenomenon describes that DTCs can become positive for Her-2, although their primary tumour is Her-2 negative and vice versa. We report here that 22 out of 41 patients (55.5%) had a switch of Her-2 comparing primary tumour data with DTC stainings. When analysed in detail, smaller tumours (pT1) especially seem to favour this phenomenon (Table 3).

Discussion

The Thomsen-Friedenreich antigen (TF, CD176) is an eminent marker for the detection of DTCs since only malignant cells seem to express it, in contrast to healthy cells (Schindlbeck et al., 2005a,b). Hence, we hypothesized that immunohistochemical detection of TF in combination with the established marker Her-2, or

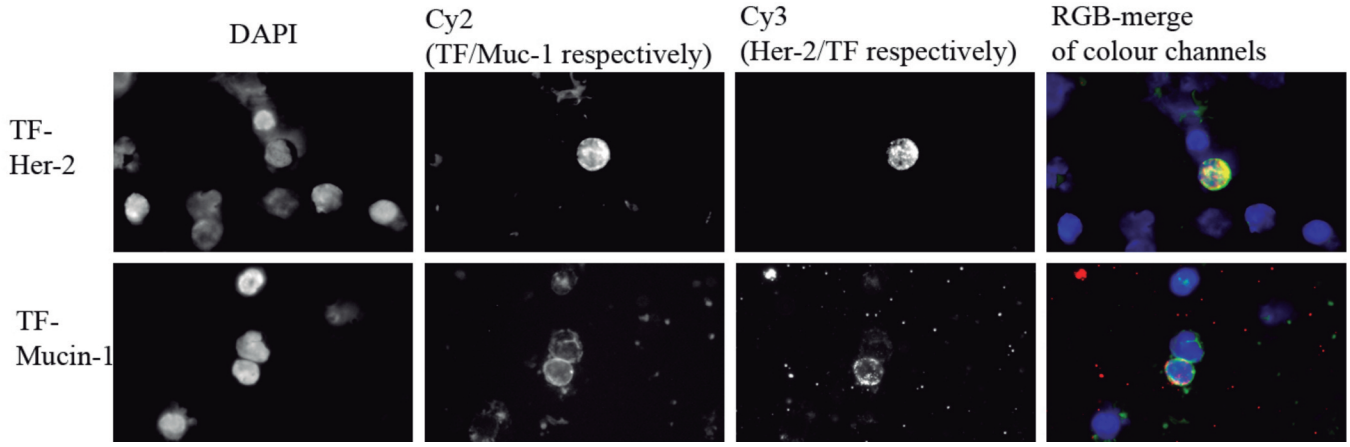

Fig. 4. Fluorescent staining of bone marrow sample with TF and Her-2 (upper row) and TF and Muc-1 (lower row); x 40. magnification. TF/Her-2 Staining: Cy2 corresponds to TF, Cy3 to Her-2. TF/Muc-1 Staining: Cy2 corresponds to Muc-1, Cy3 to TF. TF, Her-2 and Muc-1 stainings are concentrated at the cellular membrane.

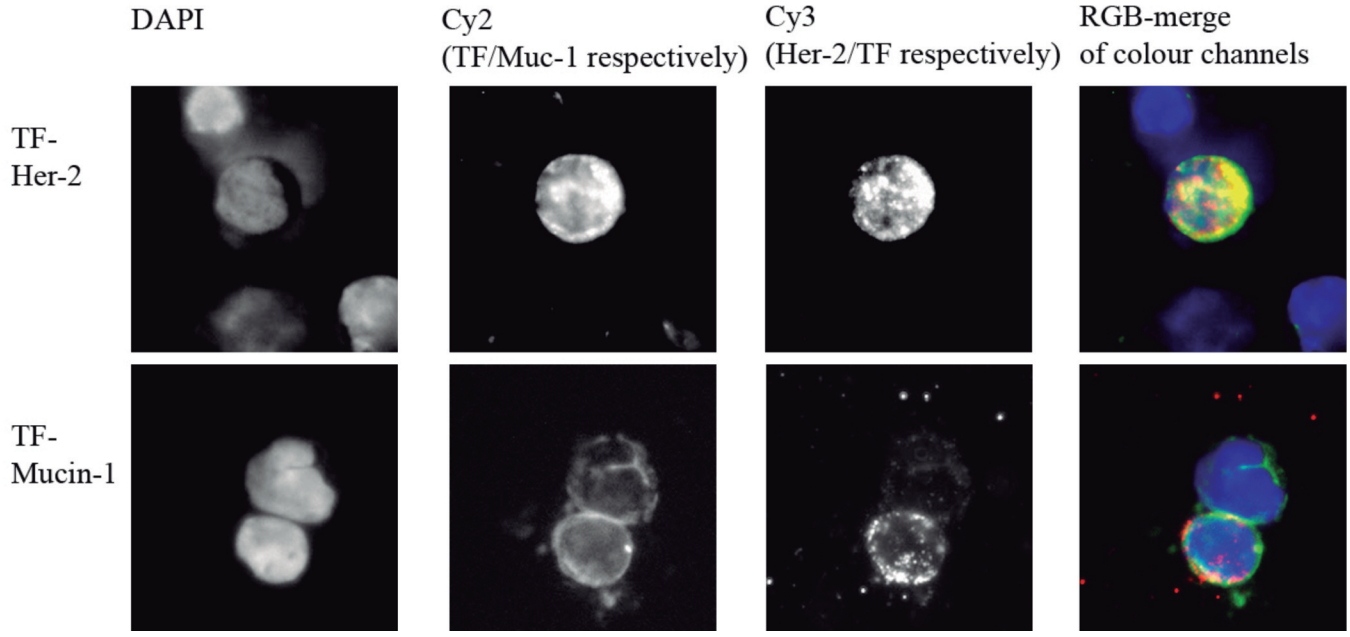

Fig. 5. Fluorescent staining of bone marrow sample (zoom of stained cells) with TF and Her-2 (upper row) and TF and Muc-1 (lower row), Magnification of the stained cells TF/Her-2 Staining: Cy2 corresponds to TF, Cy3 to Her-2 TF/Muc-1 Staining: Cy2 corresponds to Muc-1, Cy3 to TF TF, Her-2 and Muc-1 stainings are concentrated at the cellular membrane.

other markers like MUC-1, could enhance ways of finding and characterizing DTCs in breast cancer patients.

Further important markers for the detection of DTCs are cytokeratins (CKs), which were not stained in the on hand study, as there is already data showing a coexpression of CKs with TF in many cell types (Jeschke et al., 2002). It was furthermore shown that DTCs, which stain for CK also stain for TF in 96% of all cases, while normal bone marrow cells without CK-staining, do not stain for TF as well (Schindlbeck et al., 2005a). Therefore cells which are stained for TF can be regarded as DTCs.

The advantage of the described methodology is its independence of EpCAM-based enrichment methods. It is known that after detachment from the primary tumour cells can undergo epithelial mesenchymal transition and might therefore no longer express epithelial cell surface antigens like EpCAM. This also means that an essential part of these EMT-transformed cells would escape detection (Bednarz-Knoll et al., 2011). Here, we use a simple and cost-effective enrichment method and subsequently proceed to a DTC detection method with a combination of cell surface markers which have been largely described not only as markers for malignancy, but also for therapeutic meanings.

In detail, the combination of TF and Her-2 significantly increased the rate of detected DTCs (46.14% double stained cells). Several recent investigations on DTCs have shown a high variability in DTC detection rates (Janni et al., 2005; Slade et al., 2005; Wolfle et al., 2006; Effenberger et al., 2011, 2012; Sanger et al., 2011). The here detected high percentage of Her-2 positive stained DTCs might be due to the relatively small number of patients analysed. If a bigger subgroup was examined numbers of Her-2 positive DTCs would drop to the levels which are normally described in the literature.

Interestingly, the Her-2 status of the primary tumour compared to the DTCs does not correspond in more than 50%. That means that up to 50% of DTCs displayed positive Her-2 expressions, whereas the primary tumour tissue did not or vice versa. This phenomenon has been described as Her-2 switch (Solomayer et al., 2006; Krawczyk et al., 2009). This switch is only explicable for DTCs displaying certain plasticity in altering their Her-2 expression patterns before or after spreading from the primary tumour. Also, it could be a reaction to different micro environmental cues of the metastatic niche in the bone marrow. Differences in staining of Her-2 in primary tumours and DTCs due to different staining methods can almost be excluded, as both stainings show high sensitivity.

In this work, we classified tumour sizes in subgroups with and without Her-2 antigen shift and we found that especially in stages with small tumour sizes, such as pT1, it is much more likely to observe Her-2 antigen switches. Bigger primary tumour sizes could not be correlated to significant antigenic switches. This latter point could indicate that there might be additional benefits of targeted therapy against Her-2, especially in early tumour stages, when the primary tumour is still small (≤2cm). Furthermore, in order to avoid a relapse, our data presented here suggest that patients with negative Her-2 primary tumours might benefit from a Her-2 targeted therapy in order to eliminate minimal residual diseases which had undergone a switch in Her-2 expression. By the use of Trastuzumab or Lapatinib, disseminated and circulating tumour cells that have switched to a Her-2 positive status could be targeted.

Several isoforms of Mucin-1 are expressed on breast cancer tissues, and have been reported to be of clinical relevance (de Roos et al., 2007). Interestingly, TF was reported to be linked to Mucin-1 via Galectin-3

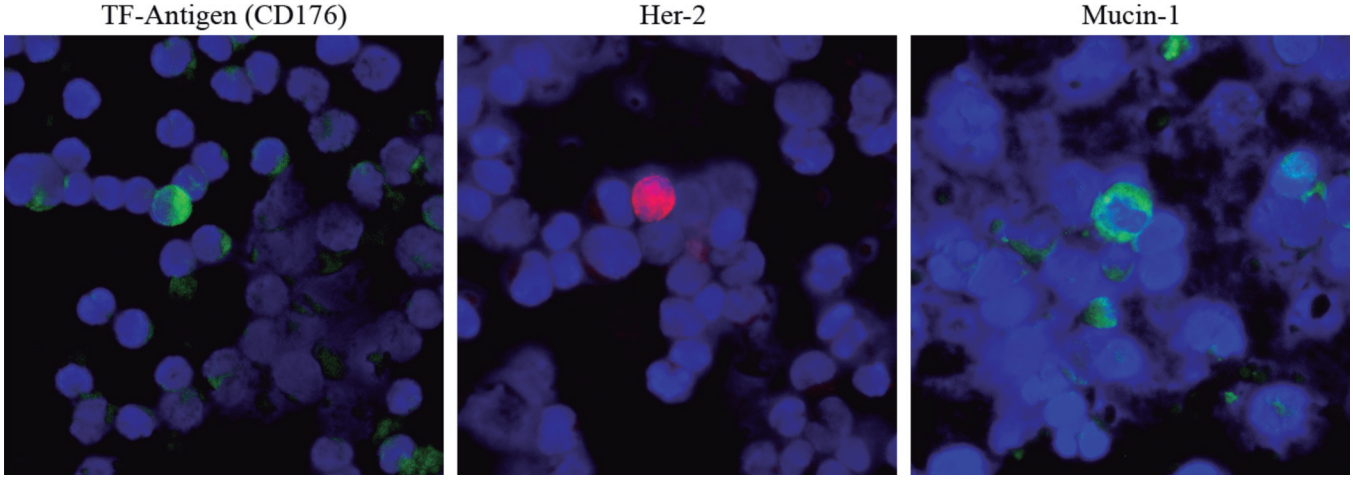

Fig. 6. Fluorescent staining of bone marrow samples (single stainings). Single Stainings of TF, Her-2 and Mucin-1. Nuclei were counterstained with DAPI. x 40 magnification.

interactions. Thus, the use of a combination of both antigens for DTC detection appears to be reasonable (Khaldoyanidi et al., 2003; Yu et al., 2007). Furthermore, Mucin-1 expression is a prognostic indicator for clinical outcome in breast cancer patients (van der Vegt et al., 2007).

In our experiments, more than 50% of the detected DTCs using TF and Muc-1 double staining showed positive signals for Mucin-1 only. However, only a smaller proportion of these cells were positive for both markers. This might be due to the existence of several Muc-1 isoforms or a Muc-1 positive and another Muc-1 negative subpopulation of TF positive DTCs. We speculate that these different subgroups could represent two states of the same cell type, e.g. cells that are in quiescence or a fast-cycling cell pool; alternatively, it might be a matter of different stages of stemness and differentiation according to the cancer stem cell hypothesis (Tan et al., 2006).

The detection of DTCs in bone marrow may affect the prognosis of breast cancer patients and is linked to an increased risk for future relapse and inferior outcome (Janni et al., 2005; Banys et al., 2009; Tjensvoll et al., 2012). Therefore, it appears to be useful not only to focus on primary tissue characteristics, but also taking CTC and DTC characteristics into consideration when decisions on medical treatment are being made.

The detection and characterization of DTC's is a useful tool to examine the actual bone marrow status of a breast cancer patient. However, BM-aspiration is an exhausting and painful procedure for the patient that cannot be repeated very often to control disease progression. Hence, combining different markers for the detection of DTCs by using established tumour markers like Her-2 or markers like Muc-1 and further markers, such as the TF-antigen, might facilitate the decision on subsequent therapies for the patients.

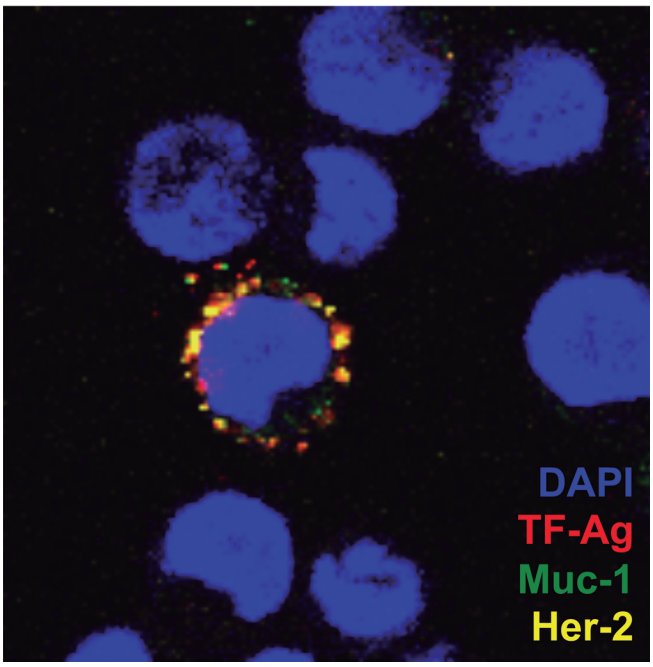

Fig. 7. Confocal image with 63-fold magnification. Four colour staining of bone marrow with antibodies against TF (red), Her-2 (yellow) and Muc-1 (green). Nuclei were counterstained with DAPI

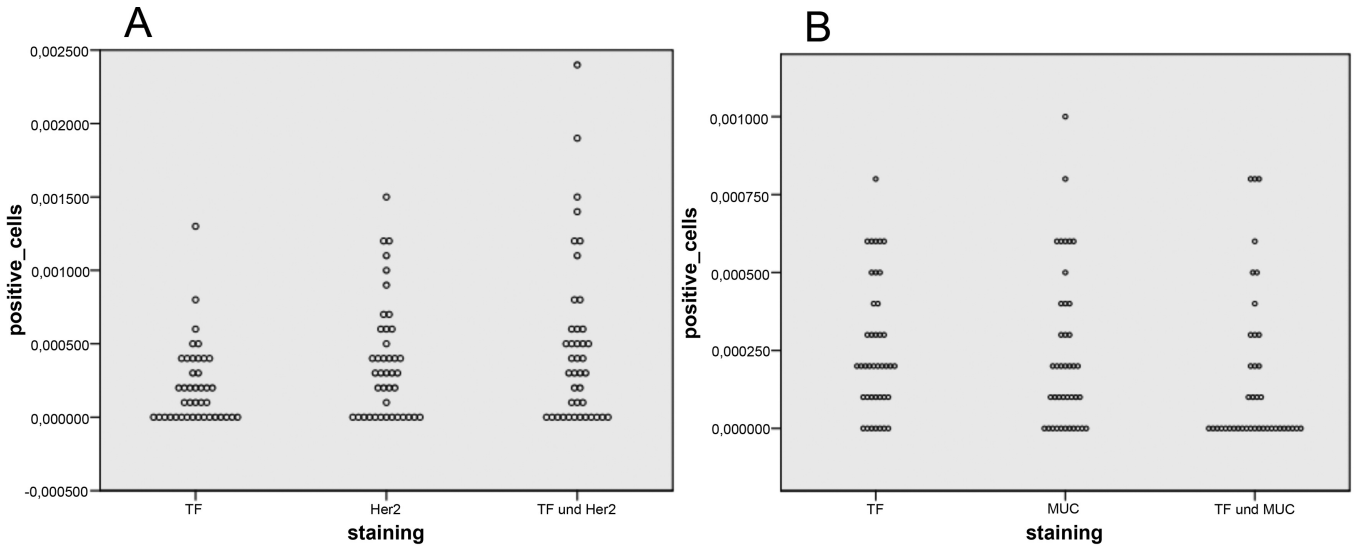

Fig. 8. Frequency of positive TF (CD176) and Her-2/Muc-1 stained cells in comparison to surrounding bone marrow cells-. **A.** TF and Her-2. **B.** TF and Muc-1.

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