

Epithelial-mesenchymal transition was identified as a potential marker for breast cancer aggressiveness using reverse transcription-quantitative polymerase chain reaction

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Abstract. The primary cause of breast cancer-associated mortality is the formation of distant metastasis. During the metastatic process, single tumor cells dissolve from the primary tumor site and undergo various changes in cell adhesion and motility properties. The tumor cells invade the blood stream and travel to different sites of the body, where they may initiate outgrowth. These cells are referred to as circulating tumor cells (CTCs). The process of changing cellular properties is known as epithelial to mesenchymal transition (EMT). As a different set of genes is upregulated during EMT, such genes may serve as marker genes for the detection of CTCs based on reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Therefore, EMT- and breast cancer-related genes were selected as RT-qPCR markers. These genes were tested for performance in a model system of blood samples from healthy donors, to which a number of various breast cancer cell lines were added. The genes with optimal performance were subsequently used in RT-qPCR with 35 breast cancer patient samples. The genes which showed the highest and most consistent increase in gene expression with the increase in the number of cancer cell line cells added were CK19, Snail, FoxC2 and Twist. Following RT-qPCR for all patient samples, two subgroups were arranged: One group in which all genes were downregulated and the second group with at least one gene indicated an upregulation of gene expression. Comparisons were made between the tumour characteristics from these two groups. Results suggested that carcinomas of the first group exhibited a less aggressive tumor biology compared with those in the second group. The present study indicated a novel RT-qPCR based test for tumor malignancy.

Introduction

Breast cancer is, although diagnosis and treatment improved a lot in the past years still the most frequent malignancy and causes the most cancer-related death in women worldwide. Thereby it is not the primary tumor itself, which is deadly, but the outgrowth of remote metastases, devastating vital organs. The mechanisms leading to metastasis formation had been investigated and described in detail (1): Single tumor cells detach from the primary tumor mass, enter blood vessels and travel throughout the body via blood stream. When they leave circulation, they can settle in remote spaces of the body and are considered to be the seed for metastatic outgrowth (2). As long as these detached cells are in the blood they are called circulating tumor cells (CTCs) (3-5), but if these CTCs manage to invade bone marrow, they turn to be 'DTCs', disseminated tumor cells, which can even form tumor reservoirs within the bone marrow (6-10). It had been shown, that the occurrence of CTCs/DTCs strongly influences patients' prognosis towards a worse outcome (5,10-12).

The most important step in the formation of CTCs/DTCs is a process called epithelial to mesenchymal transition (EMT). During EMT the epithelial tumor cells change their phenotypic characteristics like cell adhesion and motility, acquire invasiveness and loose epithelial markers (13-15). At the end of the EMT process, it gets hard to recognize these cells as tumor cells, because they adopt a mesenchymal-like appearance (16,17). But the EMT process is reversible, and as the cells leave the circulation to establish metastatic outgrowth in a different organ, mesenchymal to epithelial transition (MET) takes place (18-20).

Here we report a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) based approach for the detection of CTCs from patient blood samples, using EMT-associated genes as PCR targets. The genes, which are described in the following get upregulated, when EMT process is started, but are not upregulated in normal mesenchymal cells like blood cells, so they can be detected by RT-qPCR even in a background of white blood cells (for this reason Vimentin, which is a frequently described EMT-marker was not included in our analysis, as it is also upregulated in normal blood cells). We decided to concentrate on the following genes: Cytokeratin 19, which is also used in the immunohistochemical detection of cancer cells (APAAP-staining) (21,22) and is

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a marker for shorter disease free survival and reduced overall survival in breast cancer patients (23-26). Furthermore it is especially expressed in metastatic breast cancer cells (27,28) and was already used as a biomarker for CTCs in the blood of breast cancer patients (29). Furthermore CK19 was shown to be expressed in CTCs from early and metastatic breast cancer patients, together with the EMT-markers Twist and Vimentin (30), so it could be concluded, that this epithelial marker is not downregulated by EMT. Snail is a zinc-finger domain transcription factor, which is known to trigger EMT by repression of E-cadherin expression (31-33). It is upregulated in recurrent tumors (34), provokes loss of epithelial markers (33) and is regarded as a marker of metastatic potential (31). Slug also represses E-cadherin expression (35), but snail and slug have a non-equivalent role in EMT and need different cofactors for DNA binding (36). Additionally, Snail and Slug seem to have high expression levels in early and metastatic breast cancer patients (30). FoxC2 is also a transcription factor and is involved in tumor relapse and metastasis formation (37,38). Slug and FoxC2 are in turn upregulated by snail and twist and are another driving force for EMT (39). FoxC2 is induced during the EMT process, promotes mesenchymal differentiation (40) and is also involved in angiogenesis (41). Together with Twist, FoxC2 is associated with grading and a shorter time to recurrence (38). Twist in turn regulates cell migration, a knockdown results in reduced cell migration and invasion (42), and EMT is induced more moderately (43). Twist also seems to play a role in metastasis formation (44) and is regulated by the Wnt/ β -catenin pathway (45). Snail and Twist are regarded as inducers for EMT process, (39) are misregulated in breast cancer and correlate with poor clinical outcomes (46,47). Furthermore Twist upregulates the expression of the proto-oncogene Akt2 (48). Akt2 in turn upregulates integrin-expression (49), leading to an enhanced EMT-like morphological conversion (50), increased migration and invasion and resistance to Paclitaxel (48). ALDH1, last but not least, was described as marker for EMT, which has elevated expression levels in breast cancer patients (46,51), is a marker of stem cells and contributes to a poorer prognosis (52). It is associated with a larger tumor size, higher grading and the occurrence of lymph node metastasis, and hence is related to a more aggressive phenotype and a poorer prognosis (53-55). ALDH1, Twist and Akt2 were especially found to be upregulated in a group of CTC-positive breast cancer patients (56).

These genes were first analysed in an artificially created model system: Blood samples from healthy donors were withdrawn and breast cancer cell line cells (MCF-7 and MDA-MB231) were added in certain amounts (0-1,000 cells/ml blood sample). The samples were processed just like the patient samples and RT-qPCR was carried out with all selected marker genes. The genes which performed best in this model system were then used in the patients RT-qPCR, so that precious patient material could be saved. The genes for Cytokeratin 19 (CK19), Snail, FoxC2 and Twist were then used to analyse 35 patient samples, which were selected randomly. After analysis of the RT-qPCR experiments patient samples could be divided into two subgroups: in the first group all genes were downregulated, in the second group at least one gene had an RQ-value greater than 1, meaning that the gene is upregulated. In the comparison of the samples from the two groups it could be

seen, that the subgroup of only downregulated genes consisted of less aggressive tumors while in the group, in which at least one of the marker genes was upregulated, tumours had a more aggressive tumor biology, what means, that RT-qPCR analysis of those 4 genes could already give a hint towards tumor aggressiveness.

Materials and methods

Model system samples. As a model system blood samples from healthy donors (all female, average age of 35 years) were withdrawn and processed as described for the patient blood samples, but before addition of TRIzol LS reagent (Invitrogen; ThermoFisher Scientific, Darmstadt, Germany) a certain number (10/100/1,000 per ml blood sample) of breast cancer cell line cells were added (both cell lines used were added to the blood sample in equal parts). Breast cancer cell lines which were used in the experiments were MCF-7 (ATCC: HTB-22) and MDA-MB-231 (ATCC: HTB-26). The cells were subcultured as described in the ATCC product sheet and counted with a Neubauer improved counting chamber to determine exact amounts which had to be added to the blood samples.

Patients. Written consent was received from 35 patients included in the study and conformed to the declaration of Helsinki. Furthermore, ethical approval was received from the ethics committee of LMU Munich (Munich, Germany) (LMU 148-12). 20 ml blood were withdrawn from each patient and processed as described. RT-qPCR was performed on the patient samples and afterwards patient samples could be divided in two subgroups depending on gene expression values. Patient characteristics (as completely as possible) are shown in Table I.

Blood samples. A total of 20 ml blood were withdrawn from each patient and from some healthy volunteers and diluted with PBS to 30 ml. To enrich the leucocyte/CTC-fraction a density gradient centrifugation was carried out. Therefore the blood samples were layered onto 20 ml of Histopaque 1,077 (Invitrogen; ThermoFisher Scientific) and centrifuged at 400 x g for 25 min at room temperature. Afterwards the buffy coat was aspirated carefully, transferred into a fresh tube and washed with PBS by centrifuging at 250 x g for 10 min at 4°C. Supernatant was discarded and the cell pellet containing leucocytes and CTCs was stored at 80°C until further processing.

RNA isolation. For RNA isolation the cell pellets were dewatered and resuspended in 1 ml Trizol LS reagent. 0,2 ml Chloroform (Merck KGaA, Darmstadt, Germany) were added, samples were vortexed vigorously and subsequently centrifuged at 12,000 x g and 4°C for 15 min. After centrifugation the clear upper phase was carefully aspired and transferred into a fresh Eppendorf tube. A total of 0,5 ml isopropanol (Merck KGaA) were added and samples were placed at -20°C overnight. The next day the samples were centrifuged at 12,000 x g, 4°C for 10 min. to precipitate RNA. Supernatant was removed and RNA-pellet was washed with 75% ethanol (Merck KGaA) and centrifugation at 12,000 x g and 4°C for 10 min. Supernatant was removed again and RNA-pellet was air dried before it was resuspended in RNase-free water. RNA concentration

Table I. Patient subgroups and tumor characteristics.

No. of sample	Age	Tumor size	Nodal state	Metastasis state	Grading	ER state (%)	PR state (%)	Her2 state	Menopausal state
1	62	pT1b (8 mm)	pN0 (0/1)	pMx	G1	80	60	neg	post
2	50	pT1b (8 mm)	pN0 (0/5)	pMx	G1	80	95	neg	pre
3	73	pT1c (13 mm)	pN0 (0/2)	pM0	G1	99	99	neg	post
4	54	pT1c (13 mm)	pN0 (0/3)	pMx	G1	100	100	neg	climact
5	72	pT2 (35 mm)	pN1a (3/12)	pM0	G1	90	90	neg	post
6	72	cT1b (8,6)	pNx	pMx	G1	90	80	neg	post
7	82	pT1b (7 mm)	pN0 (0/6)	pMx	G3	75	0	pos	post
8	67	pT1b (8 mm)	pN0 (0/1)	pMx	G3	95	90	neg	post
9	48	pT1b (9 mm)	pN0 (0/1)	pMx	G3	0	0	neg	n.a.
10	65	pT1c	pN0 (0/3)	pMx	G3	85	85	pos	n.a.
11	73	pT1c (14 mm)	pN0 (0/1)	pM0	G3	80	80	neg	post
12	35	pT1c (14 mm)	pN0 (0/4)	pMx	G3	10	10	neg	pre
13	65	pT2 (34 mm)	pN1 (1/2)	pM0	G3	99	15	neg	post
14	62	pT1c (12 mm)	pN0 (0/3)	pMx	G2	100	100	neg	n.a.
15	48	pT1c (14 mm)	pN0 (0/2)	pM0	G2	95	95	neg	pre
16	60	pT2 (22 mm)	pN0 (0/1)	pMx	G2	90	15	n.a.	post
17	74	pT3	pN0 (0/8)	pM0	G2	90	90	neg	post
18	49	pT2 (28 mm)	pN1a (2/2)	pMx	G2	80	80	neg	pre
19	65	pT1c	pN1a (2/19)	pMx	G3	100	33	pos	n.a.
20	52	pT1c (19 mm)	pN0 (0/1)	pM0	G3	0	0	neg	post
21	73	pT2 (40 mm)	pN0 (0/4)	pMx	G2	75	85	neg	n.a.
22	84	pT1b (8 mm)	pN1a (1/24)	pM0	G2	90	90	neg	n.a.
23	64	pT1c (12 mm)	pN1 (1/3)	pMx	G2	100	70	neg	post
24	50	pT1c (14 mm)	pN1a (1/4)	pMx	G2	99	99	neg	n.a.
25	51	pT1c (19 mm)	pN1a (1/1)	pMx	G2	90	90	neg	pre
26	65	pT2	pN1a (1/3)	pMx	G2	100	100	neg	n.a.
27	75	pT3 (65 mm)	pN1a (3/3)	pM0	G2	81	81	neg	post
28	60	pT3	pN1a (1/11)	pMx	G2-3	100	100	neg	post
29	57	pT4b	pN2a (4/9)	pM1	G3	0	0	neg	n.a.
30	54	pT1c	pN0 (0/11)	pM1	G3	n.a.	n.a.	neg	post
31	55	pT1c	pN0 (0/11)	pM1	G3	n.a.	n.a.	neg	post
32	84	pT1b (8 mm)	pN1 (1/18)	pM1	G3	70	80	neg	post
33	76	pT3 (63 mm)	pN1a (2/7)	pM1	n.a.	90	40	neg	post
34	56	pT2 (22 mm)	pN3c	pM1	G3	90	20	neg	post
35	75	pyT3	pN1a (3/12)	pM1	G2	99	0	neg	post

n.a., data not available; neg, negative; pos, positive; post, postmenopausal; pre, premenopausal; climact, climacterium; ER, estrogen receptor; Her2, human epidermal growth factor receptor 2; PR, progesterone receptor; pM0, no metastasis detected; pM1, metastasis detected; pMx, no metastases confirmed.

and ratio were measured photometrically (Implen, Munich, Germany) and only RNAs with a ratio between 1,7 and 1,9 were used in further experiments. Additionally RNA integrity was controlled by denaturing gel electrophoresis.

Reverse transcription. For reverse transcription 4 µg of the isolated RNA in a max. Volume of 6 µl were used. Reverse transcription was carried out with the SuperScript III First Strand Synthesis SuperMix (Invitrogen; ThermoFisher Scientific), according to manufacturer's instructions. In brief: annealing buffer and oligo (dT)-Primers were added to the

RNA and volume was adjusted to 8 µl with water. After an incubation at 65°C for 5 min and a short cooling on ice 10 µl of 2X first strand reaction mix and 2 µl of the enzyme mix (SuperScriptIII/RNaseOUT) were added and the samples were incubated at 50°C for 50 min. Afterwards enzymes were denatured by a 5-min incubation at 85°C, then samples could be stored at -20°C until use.

qPCR. For qPCR 2 µl of the previously generated cDNA of each sample were pipetted into a 96-well qPCR plate and 18 µl of a mastermix, consisting of 7 µl water, 1 µl TaqMan®

Table II. Reverse transcription-quantitative polymerase chain reaction probes.

Probe/Gene	Order no. of probe	Fragment size	Manufacturer
18S	Hs99999901_s1	187 bp	Thermo Fisher Scientific, Inc. (Waltham, MA, USA)
GAPDH	Hs03003631_g1	69 bp	Thermo Fisher Scientific, Inc.
CK19	Hs00761767_s1	116 bp	Thermo Fisher Scientific, Inc.
Snail	Hs00195591_m1	66 bp	Thermo Fisher Scientific, Inc.
Slug	Hs00161904_m1	79 bp	Thermo Fisher Scientific, Inc.
FoxC2	Hs00270951_s1	102 bp	Thermo Fisher Scientific, Inc.
Akt2	Hs01086099_m1	70 bp	Thermo Fisher Scientific, Inc.
Twist	Hs01675818_s1	85 bp	Thermo Fisher Scientific, Inc.
ALDH1	Hs00946916_m1	61 bp	Thermo Fisher Scientific, Inc.

PCR-primer (Hydrolysis probes used are listed in Table II; they were not validated, as Applied Biosystems claims a PCR-efficiency of $100 \pm 10\%$ for their hydrolysis probes) and $10 \mu\text{l}$ 2X RT-PCR reaction mix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) per sample, were added. Every sample was at least analysed as duplicates, 18S was used as a reference gene. Negative (water) controls were included. The qPCR reaction was carried out in an Applied Biosystems 7500 fast machine and the following program was run: 95°C for 20 sec. As an initial denaturation followed by 40 cycles 95°C for 3 sec and 60°C for 30 sec. Increase in fluorescence was measured after every elongation step and thereof gene expression (RQ=relative quantification) was calculated by the SDS V 1.3.1 software using $2^{-\Delta\Delta\text{C}_q}$ method (57). RQ-values are displayed in Table IIIA and B.

Evaluation. The relative gene expression of every gene in respect to the expression of 18S was calculated by the SDS-software. The SDS-files can be displayed in MicrosoftTM Excel[®], and it was also used to generate graphical data for the model system samples, showing gene expression in dependence on the number of breast cancer cell line cells added to the respective blood sample.

Statistical analysis. All statistical analyses were performed with SPSS v.23 (IBM Corp., Armonk, NY, USA). Kolmogorow-Smirnov-Test was applied to test, if distribution of data was similar. The average RQ-values were compared with the two-tailed t-test and the medians with the Mann-Whitney-U-Test.

Results

Results from model system analysis. The analysis of a model system-blood samples from healthy donors with breast cancer cell line cells added in predefined amounts-was used to decide, which marker genes should be used in the patient samples without wasting precious patient material. The strongest increase in gene expression with increasing number of tumor cells added to the blood samples could be found for CK19. Snail, FoxC2 and Twist also had increasing gene expression values and were therefore selected as marker genes for the analysis of patient samples. The expression

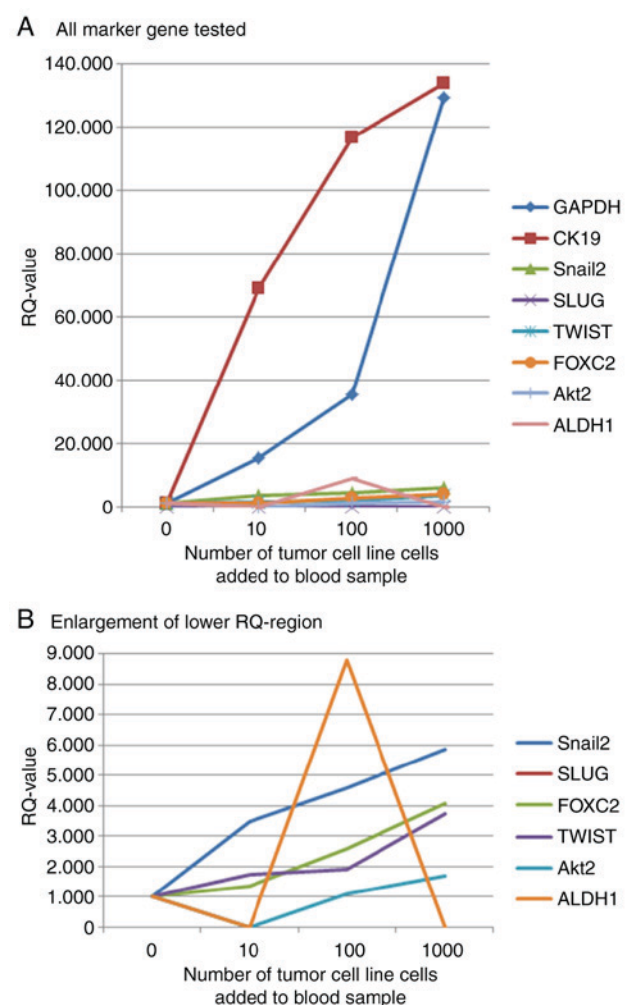


Figure 1. Marker gene expression in model system samples. (A) All marker genes tested; x-axis: Number of tumor cells added, y-axis: RQ-value. (B) Enlargement of lower RQ-region; x-axis: Number of tumor cells added, y-axis: RQ-value. RQ, relative gene expression value.

of Slug could not be detected in the qPCR reaction, maybe the Hydrolysis probe was not suitable enough for the probes used in our experiments. ALDH1 showed a decrease in gene expression in the sample with most tumor cells added, so it was also excluded from further analysis. The increase in gene

Table III. Gene expression values in patient samples.

A, Subgroup of less aggressive tumors with all marker genes downregulated

No. of sample (corresponding to Table I)	RQ CK19	RQ Snail	RQ FoxC2	RQ Twist
1	0.396	0.361	0.984	0.439
2	0.107	0.393	0.513	0.269
3	0.334	0.152	0.829	0.566
4	0.063	0.073	0.19	0.171
5	0.132	0.269	0.201	0.163
6	0.011	0.183	0.031	0.024
7	0.033	0.597	0.023	0.021
8	0.177	0.433	0.432	0.322
9	0.021	0.354	0.102	0.132
10	0.097	0.326	0.248	0.082
11	0.854	0.339	0.281	0.741
12	0.073	0.216	0.17	0.164
13	0.009	0.37	0.044	0.035
14	0.148	0.031	0.786	0.364
15	0.131	0.078	0.648	0.334
16	0.014	0.494	0.091	0.054
17	0.017	0.327	0.068	0.086
18	0.16	0.787	0.703	0.567

B, Subgroup of aggressive tumors with at least one marker gene upregulated

No. of sample (corresponding to Table I)	RQ CK19	RQ Snail	RQ FoxC2	RQ Twist
19	0.038	10.747	0.15	0.181
20	0.572	0.237	1.479	1.09
21	0.401	15.387	1.948	2.15
22	0.068	1.635	0.418	0.647
23	0.027	3.143	0.191	0.15
24	0.01	2.103	0.062	0.056
25	1.037	0.502	1.657	1.231
26	1.251	1.007	4.117	3.72
27	1.315	0.453	2.486	1.685
28	0.441	0.246	2.236	0.579
29	1.555	2.301	1.096	0.945
30	0.016	1.565	0.068	0.089
31	0.491	4.566	1.435	1.65
32	0.094	2.442	0.227	0.277
33	0.735	1.965	2.922	2.224
34	0.038	1.279	0.269	0.207
35	0.806	2.072	2.818	0.958

Bolded items are gene expression values ≥ 1 . RQ, relative gene expression value.

Table IV. Statistical comparison of gene expression values between the two patient groups.

Statistical test	CK19	Snail	FoxC2	Twist
Kolmogorow-Smirnof (data have similar distribution)	0.018	0.000	0.002	0.002
Two-tailed t-test (comparing average values)	0.008	0.007	0.002	0.002
Mann-Withney U-Test (comparing medians)	0.062	0.000	0.011	0.028

CD19, cluster of differentiation 19.

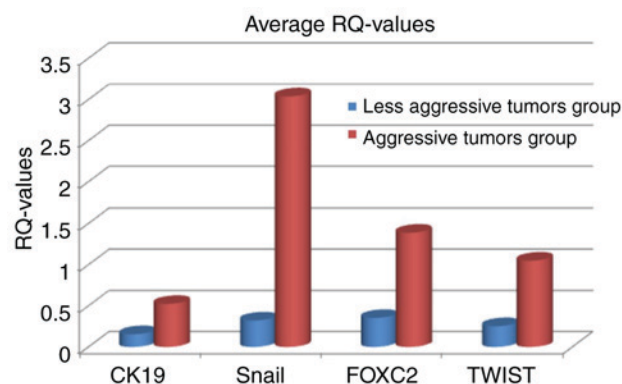


Figure 2. Graphical representation of PCR results from patient probes. RQ-values were averaged over all samples from each subgroup. RQ-values are higher for all four genes in the group of more aggressive tumors (red) in comparison to less aggressive tumor subgroup (blue). (RQ-values are relative gene expression values, calculated by $2^{-\Delta\Delta C_q}$ -formula). RQ, relative gene expression value.

expression shown by Akt2 was lower than that seen for Snail, FoxC2 and Twist. Therefore it was decided not to use Akt2 in further experiments. Curves for marker gene expression in artificial model system samples are shown in Fig. 1.

Results from analysis of patient samples. After Real-Time PCR analysis patient samples could be subdivided into two subgroups: In the first group of 18 samples all RQ-values of the used marker genes were below 1, what means, that the genes are downregulated in comparison to the reference sample (Table IIIA). In the second group in contrast, which consisted of 17 samples, at least one of the genes analysed was upregulated, having a RQ-value greater than 1 (Table IIIB). Comparing the subgroups a trend seems to become apparent: Tumors in the first group have a less aggressive biology than the ones in the second subgroup. In the first group some tumors still have a G1-grading (samples 1-6), while in the second group all malignancies have a grading of 2 or more. Furthermore in none of the samples from the first group metastasis formation could be detected, while 7 samples (samples 11-17) of the second group show up with remote metastasis. Also for the nodal status a difference can be seen

between the two subgroups: while in the first group only three samples have a nodal status of N1 (samples 5, 13 and 18) in the second group only four samples have an N0 status (samples 2, 3, 12 and 13), whereas sample No. 2 is a rather large tumor, sample three belongs to the group of aggressive TNBC-tumours and samples 12 and 13 are of the metastatic subcategory. Additionally in the 'less aggressive' category, only one, relatively small, triple-negative tumour can be found (sample 9), while in the second group two triple negative tumours can be found (samples 2 and 11), which also have large tumour sizes, and there are two tumours, for which hormone receptor status was not determined, so it is not sure if they might also be of the rather aggressive TNBC tumor group (samples 12 and 13). Averaging RQ-values of all four genes in both tumor subgroups shows, that RQ-values are higher in the group of aggressive tumors than in the group of less aggressive tumors (Fig. 2). The average and median RQ-values are also statistically significant different between the two groups (Table IV). Only for CK19 the P-values is at a borderline significance level (0.062).

Discussion

The marker genes, which were used in patient sample analysis are all known as markers for a poor clinical outcome. CK 19 is a marker for metastatic breast cancer (27,28) and late relapse (26), so it is not surprising, that it is upregulated in some of the tumors with aggressive biology, but it still has to be clarified, why it is only upregulated in one of the metastatic breast cancers analysed in our study (sample 11). The other three cases in which it's expression is upregulated have a positive lymph node status, although no coherence between CK19 and lymph node metastasis was shown in the literature. Snail in contrast is upregulated in most aggressive tumor samples, and therefore seems to be a rather important marker. That could be explained by the fact, that snail triggers early events during EMT in its role as a transcription factor (31-34). Fox C2 is upregulated in more than 50% of the tumors with aggressive biology (9 out of 17), but no coherence between its function in metastasis formation (40,41) its correlation with grading (38) and lymph node metastasis could be seen from the results of the presented study. Twist plays a more general role within the EMT process, and is upregulated in 7 out of 17 cases in the on hand study. Therefore it could be concluded, that Twist also plays a basic role in tumor cell transformation, but seems not to be such important as Snail. A special role in metastasis formation (44) could not be confirmed from the experiments presented here.

Taking together these results as a rough conclusion can be said, that tumors, for which all of the four used marker genes are downregulated, have a less aggressive tumor biology than those samples, in which at least one gene is upregulated. But a lot of work confirming this thesis is still necessary. First of all, the analysis should be amplified to a larger group of patient blood samples and the marker gene panel could also be enlarged. It still has to be clarified, in which tumor situation a certain gene is upregulated, and the significance of only one in contrast to two or more upregulated genes has to be elucidated. Differences in gene expression between TNBC-samples should also be enlightened. It can be said, that still lots of work has to be done in this field of research, but the results we present in the on hand study are already giving a certain hint

and direction for further research in order to improve diagnostics and refine therapeutical approaches. A further research in this filed could thus help to personalize treatment, thereby reducing side-effects while increasing treatment efficiency, so that research would be rather worth while.

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