Evaluation and correlation of risk recurrence in early breast cancer assessed by Oncotype DX®, clinicopathological markers and tumor cell dissemination in the blood and bone marrow

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Abstract. The Oncotype DX® assay is a validated genomic test that predicts the likelihood of breast cancer recurrence, patient survival within ten years of diagnosis and the benefit of chemotherapy in early-stage, node-negative, estrogen receptor-positive breast cancer. Further markers of recurrence include disseminated tumor cells (DTCs) in the bone marrow (BM) and circulating tumor cells (CTCs) in the blood, particularly stemness-like tumor cells (slCTCs). In this study, Oncotype DX, DTCs, CTCs and slCTCs were used to evaluate the risk of recurrence in 68 patients with human epidermal growth factor receptor 2-negative, early-stage breast cancer. Formalin-fixed, paraffin-embedded tissue sections were analyzed for the expression of 16 cancer genes and 5 reference genes by Oncotype DX, yielding a recurrence score (RS). G2 tumors were evaluated for urokinase-type plasminogen activator (uPA)/plasminogen activator inhibitor type 1 (PAI1) and Ki-67. Two BM aspirates were analyzed by immunocytochemistry for DTCs using the pan-cytokeratin antibody A45-B/B3. CTCs and slCTCs in the blood were detected using the AdnaTest BreastCancer, AdnaTest EMT and the AdnaTest TumorStemCell. Oncotype DX was performed in 68 cases, yielding a low RS in 30/68 patients (44%), an intermediate RS in 29/68 patients (43%) and a high RS in 9/68 patients (13%). DTCs were detected in 19/68 patients (28%), CTCs in 13/68 patients (19%) and slCTCs in 26/68 (38%) patients. Moreover, 8/68 patients (12%) with G2 tumors were positive for uPA, 6/68 (9%) for PAI1 and 21/68 (31%) for Ki-67. Ki-67, progesterone receptor (PR) and G3 tumors were significantly correlated with RS (P<0.001; P=0.006; and P=0,002, respectively), whereas no correlation was identified between DTCs, CTCs, slCTCs and RS. Ki-67 may support therapeutic decisions in cases where Oncotype DX is not feasible. Larger patient cohorts are required to estimate the additional detection of DTCs and CTCs for the determination of risk recurrence.

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Introduction

Risk assessment is crucial for the avoidance of overtreatment in primary breast cancer patients. In this regard, gene expression profiling has emerged as a useful tool for assessing the risk of distant recurrence in patients with early-stage breast cancer and has provided additional information to those obtained from traditional clinicopathological factors and biomarkers (1-6). The 21-gene recurrence score (RS) assay Oncotype DX® quantifies the risk of distant recurrence in patients with node-negative, estrogen receptor (ER)-positive, tamoxifen-treated breast cancer and has been validated in two independent data sets (7,8).

Other biomarkers involved in the estimation of risk recurrence include the urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI1), which have been used to determine the need for chemotherapy. However, these assays require fresh-frozen tissue samples, which is often not feasible. Furthermore, the expression of the cell cycle-regulated protein Ki-67 has frequently been used as a prognostic marker on formalin-fixed, paraffin-embedded tissue sections. However, no standardized immunochemical staining protocol and optimal cut-off points for the definition of prognostic subgroups for Ki-67 has been established. In the absence of a harmonized methodology, the International Ki-67 in Breast Cancer Working Group was unable to achieve a consensus regarding the ideal cut-off points to be used in clinical practice (9).

Apart from biomarker evaluation in tumor tissue, disseminated tumor cells (DTCs) in the bone marrow (BM) and circulating tumor cells (CTCs) in the blood are suggested to be potential surrogate markers for minimal residual disease, the precursor of metastatic disease. Their presence and

persistence in the blood and BM of primary breast cancer patients represents a strong independent prognostic factor for shortened disease-free and overall survival (10-14). More recently, several studies indicated that stemness-like tumor cells (slCTCs) and cells able to undergo epithelial to mesenchymal transition (EMT) are suggested as being the active source of metastatic spread in primary tumors and their presence has been detected in the blood of early and metastatic breast cancer patients (15-20).

The aim of this study was to correlate the RS with i) the Ki-67 proliferation assay and uPA/PAI1 and ii) the presence of DTCs in the BM and of different CTC populations in the blood, as well as clinicopathological patient data.

Patients and methods

Patient population and patient characteristics. This study was conducted at the Department of Obstetrics and Gynecology in Essen. In total, 68 primary breast cancer patients (pT1-3, pN0-1, M0) were investigated. The patient characteristics at the time of diagnosis are presented in Table I. All specimens were obtained after obtaining written informed consent and collected using protocols approved by the institutional review board of the University of Duisburg-Essen (114/2006A/05/2856).

Immunohistochemical analysis of the primary tumor. For each of the patients, the tumor type, TNM stage and grade were assessed according to the WHO Classification of breast tumors (21) and the TNM Classification, 6th edition (22). The ER and progesterone receptor (PR) status was determined by immunohistochemical analysis, as previously described by our group (23).

Determination of Ki-67 proliferation index. The recombinant mouse anti-Ki-67 monoclonal antibody [clone MSK018 (Zytomed Systems GmBH Berlin, Germany); dilution, 1:2,000; antigen retrieval with citrate buffer (pH 6.0) for 30 min in a hot water bath (95˚C)] was used on whole sections of excisional breast biopsies. Secondary and tertiary immunoreactions were performed using the Dako Autostainer Plus system (DakoCytomation, Carpinteria, CA, USA) with the anti-mouse IgG EnVision Plus detection kit (DakoCytomation). The reaction products were developed with diaminobenzidine, according to general protocols. Positive and negative control sections were included in each run, which demonstrated appropriate results. Three high-power fields (magnification, x40) were scored at the invasive edge of the tumors in hot spot areas. At least 100 tumor cells were counted in each section. Only nuclear staining in tumor cells and mitotic figures were assessed. The Ki-67 score was defined as the percentage of positively stained cells among the total number of malignant cells scored. Two specialized surgical pathologists independently performed a Ki-67 evaluation. In the case of inconsistencies, the mean value of the two assessments was used for the final score. A staining level of ≤15% was defined as Ki-67 low (negative) and a level of >15% as Ki-67 high (positive).

Analysis of uPA/PAI1. The tissue sampling for biological risk assessment was performed either by core needle or excisional biopsy. Following the confirmation of diagnosis of breast cancer by a pathologist, a representative piece of the tumor (100-300 mg) was immediately snap-frozen in liquid nitrogen. The uPA and PAI1 concentrations were measured in non-ionic, detergent-released, tumor tissue extracts (Triton X-100) using the FEMTELLE® ELISA kit (American Diagnostica Inc., Stamford, CT, USA). The determination of total protein was performed using the BCA Protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The method of measurement and cut-off evaluation were performed as previously described (24,25). The results were expressed as either ng uPA or ng PAI1/mg total protein in the tumor tissue extract being measured. Patients with a uPA concentration of <3 ng/mg total protein and a PAI1 concentration of <14 ng/mg total protein in the Triton X-100 tissue extract were classified as very low risk. uPA levels >3 and/or PAI1 levels >14 ng/mg total protein were considered to indicate a high risk of disease relapse.

Oncotype DX. The 21-gene recurrence score assay Oncotype DX (Genomic Health Inc, Redwood City, CA, USA) was performed on thin sections of formalin-fixed, paraffin-embedded tumor tissue samples, using a reverse transcriptase (RT) polymerase chain reaction (PCR) process to quantify the expression of specific mRNA for 16 cancer genes and 5 reference genes, combining the expression results into a single score, referred to as the RS, which is scaled between 0-100, corresponding to a specific likelihood of breast cancer recurrence within 10 years of the initial diagnosis. The RS may be used to assign a patient to one of three groups, according to the estimated risk of distant recurrence, as follows: low (<18), intermediate (18-31) and high (≥31) risk groups.

Collection and analysis of BM. Between 10-20 ml of BM were aspirated from the anterior iliac crest at the time of surgery and processed within 24 h. All the specimens were obtained following the provision of written informed consent, using protocols approved by the Institutional Review Board (05/2856). Tumor cell isolation and detection was performed based on the recommendations for standardized tumor cell detection published by the German Consensus Group of Senology, as previously described (23,26). The microscopic evaluation of the slides was performed using the Ariol Image Analysis system (Applied Imaging International Ltd., Newcastle, UK), consisting of a slide loader, camera, computer and software for the detection and classification of cells of interest according to particular color, intensity, size, pattern and shape.

Enrichment and molecular characterization of CTCs. Blood samples were collected for the isolation of CTCs prior to the application of therapeutic substances with an S-Monovette® (Sarstedt AG & Co., Nümbrecht, Germany) and were processed immediately or no later than 4 h after blood withdrawal. All the samples were subjected to immunomagnetic enrichment using the AdnaTest BreastCancerSelect (AdnaGen AG, Hanover, Germany), followed by RNA isolation and subsequent gene expression analysis by RT and multiplex PCR in separated tumor cells, using the AdnaTest BreastCancerDetect (AdnaGen AG) as previously described (20).

Table I. Association of clinical, histopathological and laboratory parameters with RS, according to the Oncotype DX® assay.

Variables	All	RS			P-value
		Low (<18)	Intermediate (18-31)	High (≥31)	
Patient no. (%)	68 (100)	30 (44)	29 (43)	9 (13)	
Median age at diagnosis, years (range)	59 (30-75)	58 (44-74)	55 (30-69)	61 (46-74)	0.196
Tumor stage					
T1	38	16	17	5	0.925
T2	26	12	11	3	
T3	4	2	1	1	
Lymph node status					
Negative	42	16	19	7	0.358
Positive	26	14	10	2	
Tumor grading					
G1	3	1	2	0	0.002
G2	54	27	23	4	
G3	11	2	4	5	
Progesterone receptor					
Negative	12	4	3	5	0.006
Positive	56	26	26	4	
Estrogen receptor					
Negative	8	1	2	1	0.654
Positive	60	29	27	8	
Histology					
Invasive ductal	43	17	19	7	0.188
Invasive lobular	8	6	2	0	
Other	5	2	1	2	
Ki-67					
Low	32	21	11	0	<0.001
High	21	3	11	7	
uPA					
Low	19	11	7	1	0.188
High	8	3	2	3	
PAI1					
Low	21	11	8	2	0.296
High	6	3	1	2	
CTCs					
Negative	25	17	10	8	0.100
Positive	13	4	8	1	
slCTCs					
Negative	18	10	5	3	0.232
Positive	26	8	13	5	
DTCs					
Negative	22	14	13	5	0.883
Positive	19	8	7	4	

CTCs, circulating tumor cells; slCTCs, stemness-like tumor cells; DTCs, disseminated tumor cells; RS, recurrence score; uPA, urokinase-type plasminogen activator; PAI1, plasminogen activator inhibitor type 1.

The AdnaTest BreastCancer for the evaluation of CTCs was considered positive if a PCR fragment of at least one tumor-associated transcript was clearly detected. The visualization of the PCR fragments was performed with a 2100 Bioanalyzer using the DNA 1000 LabChip kit (Agilent Technologies, Böblingen, Germany) and the Expert Software Package, version B.02.03.SI307 (Agilent Technologies). Peaks with a concentration of >0.15 ng/μl were positive for the transcripts GA733-2, MUC1 and human epidermal growth factor receptor 2. Peaks that were not detected in the above setting were considered as negative (concentration of <0.15 ng/μl).

Enrichment and characterization of slCTCs. The two tests were previously described (16) and require the enrichment of CTCs from 5 ml of blood using the AdnaTest BreastCancerSelect (AdnaGen) prior to the SinglePlex PCR assay to analyze aldehyde dehydrogenase (ALDH)1 (AdnaTestStemCell; AdnaGen) and the multiplex PCR assay to analyze EMT markers (AdnaTestEMT; AdnaGen), using actin as an internal control. Contaminating leukocytes (~1,500 per sample) were reduced 10-fold by using a special washing procedure (AdnaWash buffer; AdnaGen). This enabled the proper differentiation of EMT and tumor stem cell expression profiles with a specificity of >90%, which was confirmed in healthy donor samples. The cut-off values were 0.2 ng/μl for Akt2, 0.15 ng/μl for Twist-related protein 1, 0.25 ng/μl for phosphoinositide 3-kinase α and 0.15 ng/μl for ALDH1.

Statistical analysis. The analysis of clinical and histopathological data was performed using SPSS software, version 17.0 for Macintosh (SPSS Inc., Chicago, IL, USA). The potential associations of clinical and pathological parameters and all the analyzed biomarkers were assessed using the Chi-square test according to Pearson. Continuous variables were analyzed using the Kruskal-Wallis one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients. A total of 68 patients were included in the study. The clinical data and their association to RS are presented in detail in Table I. The majority of the patients had tumors <2 cm and were node-negative. Moreover, 64/68 patients (94%) were ER-positive and 56/68 (82%) were PR-positive. Node negativity was present in 42/68 (62%) patients. The majority of the patients had invasive ductal breast cancer (58/90, 64%) and G2 tumor (47/68, 69%). Moreover, 8/68 patients (12%) with G2 tumors were positive for uPA, 6/68 (9%) were positive for PAI1 and 21/68 (31%) were positive for Ki-67. Significant associations were observed between RS, tumor grade, PR and Ki-67 status (Table I).

Tissue analysis. Oncotype DX was performed in the 68 cases, yielding a low RS in 30/68 patients (44%), an intermediate RS in 29/68 patients (43%) and a high RS in 9/68 patients (13%). uPA/PAI1 and the Ki-67 proliferation index were assessed in 14/68 and 21/68 patients with G2 tumors, respectively, with 8/68 patients (12%) with G2 tumors being positive for uPA, 6/68 (9%) for PAI and 21/68 (31%) for Ki-67.

Detection of DTCs in the BM and CTCs in the blood. BM aspiration was performed in 52/68 patients, with a positivity rate of 28% (19/68 patients) for DTCs. The analysis of CTCs was performed in 49/68 evaluable patients, with a detection rate of 19% (13/68 patients), whereas slCTCs were detected in 26/68 evaluable patients (38%).

Correlation of RS with clinicopathological factors. When all the assessed biomarkers were correlated with clinicopathological factors, PR-positivity and G3 tumors were found to be significantly correlated with RS ($P=0.006$ and $P=0.002$, respectively). Furthermore, a low Ki-67 index was significantly correlated with a low RS ($P<0.001$). No correlation was observed between RS, uPA and PAI1 and the presence of DTCs in the BM and CTCs and slCTCs in the blood, or any of the other clinicopathological factors (Table I).

Discussion

Over the last 20 years, there has been a significant reduction in breast cancer mortality; a major contributor to this reduction is the administration of adjuvant medical therapy (27,28). Currently, clinicians use tumor size, nodal status, tumor grade and patient age to determine the risk of recurrence in patients with early breast cancer and decide on whether to recommend chemotherapy. However, these parameters alone are not sufficient for risk assessment, as they appear to result in the overtreatment of several patients. Therefore, novel markers for risk assessment are required in the clinical practice to minimize overtreatment, undertreatment or the administration of incorrect treatment (29). In this regard, the Oncotype DX Breast Cancer Test has been shown to predict the likelihood of adjuvant chemotherapy benefit and the 10-year risk of distant recurrence in patients with ER-positive, node-negative and node-positive early-stage breast cancer (5,7,30-32). Furthermore, Oncotype DX enables the identification of patients who are likely to benefit from chemotherapy and would not have been identified through standard prognostic methods and provides information additional to the classical clinicopathological factors, such as tumor size and grade, patient age and nodal status (5,33,34). Other factors that have been used in the estimation of the recurrence risk include biomarkers, such as Ki-67 and uPA/PAI1, gene expression profiling and the detection of CTCs and DTCs (9-20).

In this study, we evaluated the risk of recurrence in early breast cancer patients by using Oncotype DX, Ki-67, uPA/PAI1, DTCs, CTCs and slCTCs. Our main findings demonstrated a significant correlation between RS and Ki-67, PR and tumor grade. By contrast, no significant association was demonstrated when RS was correlated with uPA/PAI1 or with tumor cell spread to the blood and BM.

Similar to the PR status and tumor grade, which are well-known predictive markers in breast cancer, our findings appear to confirm the validity of the RS according to Oncotype DX. Among the 16 genes included in the Oncotype DX Breast Cancer test, Ki-67 is one of the 5 genes associated with cell proliferation (35). With regard to our results, the significant association between RS and the expression of Ki-67 emphasizes that these two parameters may be

valuable markers in the estimation of the recurrence risk. This finding may be particularly relevant in cases where Oncotype DX application is not available. The fact that high levels of Ki-67 expression are associated with worse prognosis in breast cancer has already been confirmed by previous studies (36-43). In this regard, Ki-67 was an independent prognostic factor for disease-free survival and the greatest benefits from Ki-67 assessment may be observed in patients with ER-positive breast cancers (44,45). However, to the best of our knowledge, the significant correlation between Ki-67 and RS, according to Oncotype DX, is described for the first time.

In our study, we also demonstrated a significant association between PR, tumor grade and RS, with PR-positive and G3 tumors predominantly exhibiting high RS. By contrast, no significant association was observed with Oncotype DX and uPA/PAI1.

Previous clinical studies have already addressed the association between these markers. In this context, the 'uPA/PAI1-algorithm' has achieved promising results in further assessing the risk of recurrence in patients with grade 2 breast cancer, in order to avoid unnecessary chemotherapy (46,48). Furthermore, uPA and PAI1 are the only biomarkers which have been proven to be useful in the clinical setting by a prospective clinical trial (Chemo N0) and a pooled analysis in <8,000 primary breast cancer patients with regard to risk group assessment (47,49). The 10-year results of the Chemo N0 trial revealed that half of the node-negative patients with low uPA/PAI1 values and no administration of systemic chemotherapy or endocrine therapy, exhibited a 10-year survival rate of ~90% (50). In addition, the final correlation analysis from the phase III West German Study Group plan B trial demonstrated that a high RS suggested a high risk associated with central grade 3, luminal B subtype (HR-positive and Ki-67 high) and high uPA/PAI1. The risk assessment within the low and intermediate RS risk groups exhibited substantial heterogeneity according to central grade, luminal subtype and uPA/PAI1. In our patient cohort, a high Ki-67 expression correlated significantly with a high RS. By contrast, no significant association was observed with regard to uPA/PAI1. This may be attributed to the small number of patients in our study for whom the analysis of all these markers was feasible.

All the markers mentioned above may be valuable tools for the assessment of tumor recurrence risk, provided a sufficient amount of tissue is available for these analyses (51). Furthermore, the analysis of tumor tissue is only feasible at the time of first diagnosis and not during follow-up. In this regard, the detection of CTCs and DTCs has been the focus of the research regarding risk assessment, since their presence in the blood and BM at primary diagnosis has been shown to be an independent prognostic factor for shortened disease-free and overall survival by several studies (11-13,17). In the present study, we did not observe a significant correlation between minimal residual disease and the other evaluated factors. As mentioned above, this may be due to the small number of patients in whom all markers could be assessed. However, we recently demonstrated that the presence of slCTCs was associated with resistance to conventional anticancer therapies and treatment failure in metastatic breast cancer patients and that these cells may even be present in the blood of early breast cancer patients (16,20). Those data were further confirmed by other studies (10-15,17-19).

The limited number of patients constitutes a limitation to our analysis. However, our findings appear to be plausible, although they require confirmation by studies including larger patient cohorts, to evaluate which of the assessed markers is the most reliable to estimate the risk of recurrence, assisting clinicians in therapeutic decision making, avoiding under- or overtreatment of early breast cancer patients. Furthermore, the observed association between Ki-67 and the RS according to Oncotype DX may prove to be useful for risk assessment when Oncotype DX is not feasible.

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