

## GENE EXPRESSION OF CIRCULATING TUMOUR CELLS AND ITS CORRELATION WITH TUMOUR STAGE IN BREAST CANCER PATIENTS

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### Abstract

**Background:** Breast cancer (BC) represents one of the leading causes of cancer related deaths worldwide. New tools for diagnostic staging and therapeutic monitoring are needed to improve individualized therapies and improve clinical outcome. The analyses of circulating tumour cells may provide important prognostic information in the clinical setting.

**Materials and Methods:** Circulating tumour cells (CTC) of 63 BC patients were isolated from peripheral blood (PB) through immunomagnetic separation. Subsequently, RT-PCR or mPCR for the genes *ga733.2*, *muc-1*, *c-erbB2*, *mgb-1*, *spdef* and *c-erbB2* were performed. Subsequently, expression data were correlated with the tumour stages. Fourteen healthy individuals served as controls.

**Results:** Significant correlations with tumour stages were found in single gene analyses of *ga733.2*, *muc-1* and in multi-gene analyses of *ga733.2/muc-1/mgb1/spdef*. Furthermore, a significant correlation of *Ca 15-3* and all studied genes was also observed.

**Conclusion:** Herein, we demonstrated a positive correlation of a gene signature consisting of *ga733.2*, *muc-1*, *mgb1* and *spdef* and advanced stages of BC. Moreover, all studied genes and gene patterns revealed a significant correlation with *Ca 15-3* positive cases.

**Key words:** Mamma carcinoma, gene signature, real-time-PCR

**Abbreviations:** BC = breast cancer; CTC = circulating tumour cells; PB = peripheral blood; *c-erbB2* = cellular avian erythroblastosis homologue B2; *ga733.2* = major gastrointestinal tumour-associated protein; *muc-1* = mucin-1; *mgb-1* = mammaglobin-typ1; mRNA = messenger RNA; PCR = polymerase chain reaction; RT-PCR = reverse transcriptase polymerase chain reaction; RT = reverse transcriptase; *spdef* = SAM pointed domain-containing ETS transcription factor; FD = First Diagnosis; MD = Metastatic disease

### INTRODUCTION

Even though new developments in clinical research on breast cancer (BC) have provided clinicians with a set of important diagnostic tools, there is still an urgent need for further, more sensitive staging techniques. Recent studies have shown that quantitative and qualitative analyses of circulating tumour cells (CTC) may provide prognostic information in BC [1, 2]. Attempts to isolate CTC in peripheral blood (PB) date back to more than 100 years [3]. Sandberg et al. isolated CTC from BC patients using centrifugation and fibrinogen to aggregate erythrocytes [4]. Notably, Hsieh et al. reported that the absolute count of CTC may be related to an aggressive course of disease in BC patients [5]. First attempts to isolate CTC through immunomagnetic separation were made by Berois et al using a CK-19 on BC cell lines [6]. This technique was recently successfully used by Hardingham et al. in patients with gastrointestinal neoplasias [7]. Combining immunomagnetic separation and real-time-PCR (RT-PCR), this group could positively correlate the presence of CTC in PB with shorter disease-free intervals.

Gene profile analyses of certain putative genetic markers may lead to sensitive and specific CTC detection and may enable an improvement in the outcome prediction [8-13]. In fact, PCR and RT-PCR can be applied to perform expression analyses of immunomagnetically isolated CTC. In a previous study we analysed the CTC of 63 BC patients in various disease stages and identified the genes *ga733.2*, *muc-1*, *mgb1* and *spdef* as putative markers for CTC detection. Based upon our previous data, in this study we performed correlation analyses between the detected gene expression levels and the known clinical staging of breast cancer. Furthermore, we compared the gene expression data with the detected *Ca15-3* serum levels. Increased serum levels of the tumour marker *Ca 15-3* have been associated with advanced disease and poor outcome in several studies [14, 15].

## MATERIALS AND METHODS

Our study population consisted of 63 BC patients and 14 healthy controls. 50 patients with first diagnosed disease (FD) and 13 patients with metastatic disease (MD) in various disease stages were enrolled. All patients provided written informed consent. After peripheral venous blood sampling, CTC were separated using an immunomagnetic method (Adnagen Breast Cancer Select Kit) according to the instructions of the manufacturer (Adnagen). Cell lysates underwent mRNA isolation and reverse transcription following the manufacturer's instructions (Promega). Subsequently, mPCR for the genes *ga733.2* and *muc-1* and RT-PCR for the genes *mgb1* and *spdef* were performed. The resulting gene profiles were then compared with tumour stage according to the TNM grading system and tumour stage of the American Joint Committee on Cancer (AJCC) [16]. Additionally, CTC profiling was compared to ELISA-determined *Ca 15-3* positivity. Correlations were performed either by Pearson's two-sided  $\chi^2$ -test for dichotomized values or by Pearson's bivariate correlation for values with two thresholds. Significance was defined by  $p < 0.05$ .

## RESULTS

## CORRELATION TO DISEASE STATE

Single gene expression and gene patterns were correlated to first diagnosed disease (FD) and metastatic disease (MD). Only *mgb1* was significantly correlated to MD ( $p = 0.019$ ) but not to FD, whereas *spdef*, *muc-1*, *ga733.2* and *c-erbB2* did not correlate with disease stage. Consecutive multiple gene evaluations

demonstrated significance for the pattern *ga733.2/muc-1* ( $p = 0.045$ ). The gene pattern *ga733.2/muc-1/spdef/mgb1* showed a significant correlation with MD ( $p = 0.023$ ).

## CORRELATION TO TUMOUR STAGE

Tumour stage was specified according to AJCC guidelines [16]. Since the tumour stage could not be identified in two BC cases, these patients were excluded from the study. Single positivity within the gene pattern *ga733.2/muc-1* was significantly related to advanced disease, with 10 out of 12 positive cases found in level IIIa or higher. Similarly, 11 out of 15 single positive results within the gene pattern *ga733.2/muc-1/mgb1/spdef* were observed in level IIIa or higher. Finally, 90% (9 out of 10) of twofold-positive results within the 4 gene pattern were documented in higher levels ( $p = 0.001$ ) (Table 1).

## CORRELATION TO NODAL STATUS

The gene expression profiles were further correlated with nodal status. Lymph node (N) status was not available in 4 patients. They were excluded from further analyses. N status was stratified in three levels, N0, N1 and  $\geq$  N2. Significant correlation was found between *ga733.2* ( $p = 0.042$ ) and *muc-1* ( $p = 0.039$ ) overexpression and higher N-levels. The analyses of *spdef*, *mgb1*, *ga733.2* and *muc-1* did not reveal a significant correlation. Interestingly, two-fold positive results within the gene pattern *ga733.2/muc-1/mgb1/spdef* were significant ( $p = 0.016$ ), whereas simple positive results within this gene pattern did not correlate with nodal status (Table 2).

Table 1. Correlation to disease staging. Correlation between gene expression and disease staging. Patients were stratified in groups I, IIa-IIb and IIIa or higher. Simple and twofold positivity of the gene profile *ga733.2/muc-1/mgb1/spdef* are shown (Pearson's bivariate correlation).

Analyzed gene	Disease Staging			<i>p-value</i>
	I	IIa-IIb	> IIIa	
<i>ga733.2</i>	0/18 %	1/16 6.3%	7/27 25.9%	0.009
<i>muc-1</i>	0/18 %	1/16 6.3%	9/27 33.3%	0.002
<i>spdef</i>	0/17 %	2/15 13.3%	5/25 20%	0.057
<i>mgb1</i>	0/16 %	1/15 6.7%	4/23 17.4%	0.063
<i>ga733.2/muc-1</i>	0/18 %	2/16 12.5%	10/27 37%	0.001
simple positive	0/16 %	4/15 26.7%	11/23 47.8%	0.001
twofold positive	0/16 %	1/15 6.7%	9/23 39.1%	0.001

Table 2. Correlation to nodal status. Correlation between gene expression and nodal status. Patients were stratified in groups N0, N1 and N2 or higher. Simple and twofold positivity of the gene profile *ga733.2/muc-1/mgb1/spdef* are shown (Pearson's bivariate correlation).

Analyzed gene	Nodal status			p-value
	N0	N1	> N2	
<i>ga733.2</i>	1/28 3.6%	4/19 21.1%	3/12 25%	0.042
<i>muc-1</i>	2/28 7.1%	3/19 15.8%	4/12 33.3%	0.039
<i>spdef</i>	1/27 3.7%	3/18 16.7%	2/10 20%	0.107
<i>mgb1</i>	2/26 7.7%	2/17 11.8%	0/9 %	0.640
<i>ga733.2/muc-1</i>	2/28 7.1%	5/19 26.3%	4/12 33.3%	0.034
simple positive	5/26 19.2%	5/12 41.7%	4/9 44.4%	0.143
twofold positive	1/26 3.8%	5/17 29.4%	3/9 33.3%	0.016

Table 3. Correlation to distant metastases. Correlation between gene expression and distant metastases. Patients were stratified in groups M0 or M1. Simple and twofold positivity of the gene profile *ga733.2/muc-1/mgb1/spdef* are shown (Pearson's bivariate correlation).

Analyzed gene	Presence of distant metastases		p-value
	M0	M1	
<i>ga733.2</i>	4/44 9.1%	4/19 21.1%	0.197
<i>muc-1</i>	4/44 9.1%	6/19 31.6%	0.025
<i>spdef</i>	3/39 7.7%	4/19 21.1%	0.148
<i>mgb1</i>	1/37 2.7%	4/18 22.2%	0.018
<i>ga733.2/muc-1</i>	5/44 11.4%	7/19 36.8%	0.018
simple positive	7/37 18.9%	8/18 44.4%	0.046
twofold positive	4/37 10.8%	6/18 33.3%	0.063

#### CORRELATION TO DISTANT METASTASES

Gene expression profiles were correlated with the presence of distant metastases. *muc-1* overexpression was significantly related to metastatic disease ( $p = 0.025$ ). Furthermore, 4 out of 5 patients overexpressing *mgb1* presented with distant metastases ( $p = 0.018$ ). Finally, the gene profiles *ga733.2/muc-1* ( $p = 0.018$ ) and *ga733.2/muc-1/spdef/mgb1* ( $p = 0.046$ ) significantly correlated with advanced disease (Table 3).

#### CORRELATION TO *Ca 15-3* STATUS

The gene expression data were also compared with ELISA-determined *Ca 15-3* serum levels. *Ca 15-3* could not be determined in 4 cases. Positive single gene and gene pattern strongly correlated with *Ca 15-3* positive cases. Statistical analyses revealed highly-significant results for all genes and gene patterns (Table 4).

Table 4. Correlation to *Ca 15-3*. Correlation between gene expression and presence of elevated *Ca 15-3* levels in PB. Simple and twofold positivity of the gene profile *ga733.2/muc-1/mgb1/spdef* are shown (Pearson's bivariate correlation).

Analyzed gene	<i>Ca 15-3</i> status		p-value
	negative	positive	
<i>ga733.2</i>	3/50 6%	4/9 44.4%	0.001
<i>muc-1</i>	4/50 8%	5/9 55.6%	< 0.001
<i>spdef</i>	2/46 4.3%	4/8 50%	< 0.001
<i>mgb1</i>	1/43 2.3%	3/8 37.5%	0.001
<i>ga733.2/muc-1</i>	5/50 10%	6/9 66.7%	< 0.001
simple positive	7/43 16.3%	7/8 87.5%	< 0.001
twofold positive	3/43 6.9%	6/8 75%	< 0.001

## DISCUSSION

Haematological dissemination of malignant cells is a prerequisite to distant organ metastases in many cancer entities [21-24]. Yet, several studies suggest that only a minority of tumour cells have the ability to invade and metastasize [17-19]. Therefore, it is crucial to investigate which genes ultimately facilitate tissue invasion and disease progression.

Here, we propose an association between a certain set of genes (*ga733.2*, *muc-1*, *mgb1* and *spdef*) and advanced tumour stages in BC. Notably, the studied gene signature exposed a significant correlation with stage levels IIIa or higher. Herein, *muc-1* expression significantly correlated with nodal and distant metastases, and hence higher staging levels. In line with these results, it has been recently shown that a *muc-1*-induced tumorigenesis gene signature could predict disease-free and overall survival in patients with breast and lung cancer [20].

All investigated genes and gene profiles were significantly correlated with *Ca 15-3* positivity. Since elevated levels of this tumour marker have been shown to predict the outcome in BC, the gene signature we analyzed is likely to hold prognostic relevance.

The present investigation poses several questions: First, since the analyses was not performed in a prospective manner, it is impossible to determine whether the detected gene profiles in patients with late disease might also be present in early stages of the disease. Second, it remains to be studied whether CTC exhibit a daily or hourly fluctuation in the gene expression levels. This could lead to false negative results in the proposed method.

In summary, our data indicate a notable correlation between the studied genes (*ga733.2*, *muc-1*, *mgb1* and

*spdef*) and advanced disease in BC. Accordingly, the studied genes might play a role in the process of metastases formation. However, prospective studies in a larger patient population are needed to further validate the proposed hypothesis.

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