Mucin 1-specific B cell immune responses and their impact on overall survival in breast cancer patients

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Considering the diverse functions of B cells, responses to tumor-associated antigens (TAA) have been thought to be the main source of B cell-mediated antitumor immunity. Polymorphic epithelial mucin (MUC1) is considered one of the most specific TAA in patients with breast cancer. The present study aims to dissect the level and subclasses of naturally occurring anti-MUC1 antibodies in regard to tumor biologic parameters, clinical characteristics and overall survival. In 288 primary, non-metastatic breast cancer patients, pretreatment serum levels of anti-MUC1 immunoglobulin G (IgG) and its subclasses G1–4 as well as immunoglobulin M (IgM) were analyzed via ELISA. With respect to overall survival (Kaplan–Meier analysis), tumor biologic parameters as hormone receptor status, human epidermal growth factor receptor 2 (Her2), Ki-67 expression and tumor grading have been correlated as well as clinical characteristics as nodal involvement, tumor stage and patients’ age at the time of diagnosis. Median follow-up time was 148 mo (IQR: 73.1–158.5 mo). A significant increase in IgG antibody titers was correlated highly significantly with an improved overall survival of patients. In multivariate analysis, total IgG proved to be an independent prognostic marker for overall survival (p = 0.002). IgG subclass analysis did not reveal any correlation of IgG1, IgG3 and IgG4 levels with overall survival, while increased immunoglobulin G2 (IgG2) values, although statistically not significant, tended to correlate with prolonged patient survival. MUC1-specific IgM antibodies were shown not to be predictive of overall survival. Altogether, humoral immune responses appear to play a crucial part in the tumor immunity of breast cancer patients. The present data confirms the positive impact of tumor-specific IgG on prolonged overall survival in breast cancer patients. MUC1-antibody testing might be a useful tool to identify high-risk patients who may need adjuvant therapy and potentially might benefit from MUC1-directed immunotherapy.

B cells are well known for their contribution to antitumor immune responses by secreting natural antibodies and inducing antibody-dependent cellular cytotoxicity (ADCC). In contrast, arising evidence also suggests a tumor-promoting role of B cells by several mechanisms.1

B cells, on the one hand, may infiltrate into premalignant lesions in low numbers and, on the other hand, exert a distant effect on cancer development by secreting antibodies that are deposited at the tumor site as immune complexes.2,3 In line with the pivotal role of Fcγ receptors (FcγRs) for ADCC,4 it was recently demonstrated that immunoglobulins in the tumor stroma elicit at least two pro-inflammatory pathways, namely, by activating the complement system and via the engagement of FcγRs on the surface of immune cells.5 The activation of FcγRs in B cells was found to be mandatory for the maintenance of a tumor-promoting stroma. Through Fcγ-activating receptors binding to the above immune complexes, myeloid cells are recruited to the tumor site and macrophages become primed as tumor promoters. Domschke et al.6,7 confirmed these findings and demonstrated the presence of tumor-specific T-cell responses in the bone marrow of 40% of breast cancer patients which correlated with better prognosis, while tumor-specific natural antibodies against MUC1 were detectable in approximately half of the patients and correlated with both the absence of bone marrow tumor-specific T cells as well as advanced tumor stage. In addition to that, recent results suggest a tumor-promoting role for immunoglobulin subclass G4 as mediated by FcγRs on immune cells.8

Several studies, meanwhile, reported on a prolonged survival for breast cancer patients with elevated levels of tumor-specific immunoglobulins.9,10 The chosen approach to estimate those humoral responses by plasma cells is based on the expression of
mucin 1 (MUC1), which is a glycoprotein with aberrant post-translational modifications in terms of an extensive O-linked glycosylation of its extracellular domain. It is considered one of the most specific and validated antigens in breast cancer patients.11

The epithelial mucin MUC1 is detectable in more than 90% of adenocarcinomas (e.g., breast, colorectal, pancreatic and ovarian cancers). The soluble MUC1 (CA 15.3) is routinely used in the clinical setting as a biomarker to monitor treatment progress in metastatic disease of breast cancer patients. The MUC1 gene is located on chromosome 1q21–24.14–16. The mucin encoded by that gene is a high-molecular-weight (400 kd) transmembrane glycoprotein, located at the apical cell surface of normal glandular epithelia and overexpressed in most epithelial cancers.12 The polymorphic epithelial mucin MUC1 protects glandular cell surfaces from pathogens by reducing intercellular adhesion and stimulating the production of a cell surface lubricant. It is also involved in the cell sheet differentiation.13 The extracellular domain of the molecule consists mainly of an extended highly glycosylated protein chord located 200 – 500 nm above the plasma membrane and the glycocalyx. The configuration is essentially dominated by numerous tandemly bound peptide repeats with a highly conserved sequence of 20 amino acids. Each repeat contains 5 sites with an O-linked glycosylation in the mature MUC1 molecule. In cancer, the regular structure of MUC1 is modified specifically. In contrast to other molecules on the cancer cell surface, the glycocalyx is less and aberrantly glycosylated, while the whole molecule is expressed in the cell surface.14 In cancer patients, the modified version of MUC1 is causing cellular and humoral immune responses after contact with cellular components in the periphery at the tumor site as well as in lymph nodes.

In view of the vast majority of reports in the literature indicating prolonged survival for breast cancer patients with elevated levels of tumor-specific immunoglobulins,9,10 in the present study we aim to dissect the level of naturally occurring MUC1-specific immunoglobulins and their distinct subclasses as well as the impact on survival and clinicopathologic parameters in breast cancer patients.

Results

Prevalence of MUC1-specific immunoglobulins

In the present study, a total of 288 non-metastasized breast cancer patients (median age 57 y, IQR 48.2–64.6 y) with a median follow-up time of 148 mo (IQR: 73.1–158.5 mo) were included at the time of primary diagnosis. First, levels of naturally occurring MUC1-specific IgG and IgM as well as specific subclasses IgG1, IgG2, IgG3 and IgG4 were analyzed in the peripheral blood of participating patients (Table 1). In 61 out of 288 patients (21.2%), MUC1-specific IgG antibodies and in 89 out of 288 patients (30.9%) anti-MUC1 IgM antibodies were detectable in the ELISA assays. In the subclass analysis we found a response rate of 24 (8.3%), 30 (10.4%), 37 (12.8%) and 27 (9.4%) patients for IgG1, IgG2, IgG3 and IgG4, respectively. The proportional representations within IgG subclasses were calculated as 25.3% (IgG1), 31.6% (IgG2), 38.9% (IgG3) and 28.4% (IgG4), respectively, including 23.2% with a combined detection of IgG 1–4.

MUC1-specific immunoglobulins relating to patient survival

We next correlated the prevalence of MUC1-specific immunoglobulins with patients’ overall survival. The Kaplan–Meier analysis for overall survival (Fig. 1) revealed a 10-y survival rate for MUC1-specific IgG non-responders of 71% (95% CI, 64–77 mo), while the corresponding survival value for responders came to 91% (95% CI, 80–96 mo) (p = 0.003). On the other hand, relating to the occurrence of anti-MUC1 IgM, 10-y survival rates of non-responders (76%; 95% CI, 69–81 mo) and those of responders (75%; 95% CI, 64–83 mo) were not different (p = 0.842). Of note, also in patients developing metastatic disease during follow-up (n = 37), survival rates of initial MUC1-specific IgG responders compared favorably with those of non-responders (p = 0.034; Fig. 2).

In contrast, analysis of all 288 breast cancer patient samples for the immunoglobulin subclasses IgG1, IgG2, IgG3 and IgG4, respectively, did not reveal any significant link of the subclasses to the overall survival of individual patients. Only immunoglobulin G2 demonstrated a non-significant trend for longer survival of patients with higher titers. Relating to IgG1, IgG3 and also IgG4, however, neither significant correlations nor relevant trends could be detected (Table 2).

Subgroup analysis of anti-MUC1 IgG responders

As the Kaplan–Meier analysis for overall survival had revealed a significantly improved survival rate for MUC1-specific IgG responders, we next dissected distinct clinical and tumor biologic characteristics in the patient cohort (Table 3). In this context, firstly age, tumor size and lymph node involvement have been taken into consideration. However, no significant differences between IgG responders and IgG non-responders were detected in this matter. The same held true with regard to tumor biologic characteristics as tumor grading, proliferation index and Her2 status. Interestingly, however, immune responses relating to MUC1-specific IgG production were observed more often in patients without estrogen receptor (ER) expression (p = 0.04) and by tendency (p = 0.054) also without progesterone receptor (PR) expression (Table 3).
Independent prognostic impact of MUC1-specific immunoglobulin G

The obviously significant role of total anti-MUC1 IgG was tested multivariately by the Cox proportional hazards regression model. In this analysis, we included all established prognostic parameters as tumor size, lymph node involvement, tumor grading, hormone receptor expression, Her2 status and the immunophenotype presentation. As expected, the MUC1-specific IgM response as well as the tested IgG subclasses did not turn out to be independent prognostic markers (Table 4). Total IgG, however, remained a highly significant prognostic parameter in regard to breast cancer patients’ overall survival ($p = 0.002$). This substantiates increased serum levels of MUC1-specific IgG as statistically significant prognostic marker independent of established clinical and tumor biologic characteristics (Table 4).
well as Her2 status. Findings were regarded as significant at \( p < 0.05 \). Patients with missing values in a parameter were excluded from the respective analysis.

Table 3. Clinico-pathologic and tumor biologic characteristics of the total study cohort (\( n = 288 \)) and the subgroups as defined by anti-MUC1 IgG response. Correlations of IgG response and different parameters were evaluated by Fisher’s exact test or the Mann–Whitney \( U \) test as appropriate. Findings were regarded as significant at \( p < 0.05 \) (*). Patients with missing values in a parameter were excluded from the respective analysis.

| Age (y, median) | All patients | IgG responder \( n = 61 \) | IgG non-responder \( n = 227 \) | \( P \) |
|----------------|--------------|--------------------------|-----------------------------|------|
| Tumor size 1/2 | 57.3 IQR (48.2–64.6) | 58.1 IQR (45.7–62.5) | 57.7 IQR (45.8–65.4) | 0.08 |
| Tumor size 3/4 | 261 | 3 (4.9%) | 24 (10.6%) | 0.22 |
| N + | 107 | 21 (34.4%) | 86 (37.9%) | 0.66 |
| N - | 181 | 40 (65.6%) | 141 (62.1%) | 0.50 |
| G 1 | 34 | 9 (14.8%) | 25 (11.0%) | 0.054 |
| G 2/3 | 254 | 52 (85.2%) | 202 (89.0%) | 0.054 |
| ER + | 199 | 36 (60.0%) | 163 (74.4%) | * 0.04 |
| ER - | 80 | 24 (40.0%) | 56 (25.6%) | 0.17 |
| PR + | 170 | 30 (50.0%) | 140 (63.9%) | 0.054 |
| PR - | 109 | 30 (50.0%) | 79 (36.1%) | 0.054 |
| Her2 + | 205 | 41 (69.5%) | 164 (78.5%) | 0.17 |
| Ki-67 < 20% | 137 | 26 (56.5%) | 111 (59.4%) | 0.74 |
| Ki-67 ≥ 20% | 96 | 20 (43.5%) | 76 (40.6%) | 0.74 |

Tumor size (cm): \( T1 \leq 2 \text{cm}; T2 \leq 5 \text{cm}; T3 > 5 \text{cm}; T4 \) chest wall/skin infiltration or inflammation.

Lymph node involvement: \( N +/– \) with/without local lymph node involvement.

Grading/differentiation of tumor tissue: \( G1 \) well differentiated (low grading); \( G2 \) moderately d. (intermediate g.); \( G3 \) poorly d. (high g.).

Estrogen receptor expression: \( ER +/– \) with/without estrogen receptor expression.

Progesterone receptor expression: \( PR +/– \) with/without progesterone receptor expression.

Her2/neu expression: \( Her2++/– \) with/without Her2/neu overexpression.

Proliferation index Ki-67: low proliferation (< 20%), high proliferation (≥ 20%).

than carriers without cancer.\(^{21}\) Thus, anti-MUC1 antibodies may serve some immunosurveillance function in carcinogenesis.

The observed humoral antibody response against MUC1 is based on natural properties of the mucin superfamily as well as due to aberrantly glycosylated MUC1 as expressed on the surface of most epithelial adenocarcinomas, including breast and ovarian cancer.\(^{22}\) Besides findings of elevated MUC1 levels in physiological conditions (e.g., pregnancy, lactation period),\(^{12,23}\) MUC1 is believed to play a pivotal role in the adhesion processes of cells (cell-cell and cell-extracellular matrix interactions), failure of immune recognition and in immunosuppressive effects.\(^{24}\) In this context, the positive prognostic impact of MUC1-specific immunoglobulin serum levels in breast cancer patients was associated with the function of MUC1 as a ligand for the intercellular adhesion molecule 1 (ICAM-1) as well as for E-selectin.\(^{9,25-27}\) Worth mentioning is also that cancer-associated MUC1 was shown to have immunosuppressive effects on T-cell proliferative responses.\(^{24}\) Interestingly, a significant correlation was found between the presence of circulating anti-MUC1 antibodies and the ability to isolate polymorphic epithelial mucin (MUC1)-specific B cells from tumor-draining lymph nodes of epithelial cancer patients.\(^{28}\) Especially on the latter point, however, studies are scarce.

Unexpectedly, in their initial study, von Mensdorff-Pouilly et al.\(^ {9} \) did not observe a significant disease-specific survival benefit in stage I breast cancer patients while they did so in stage II disease women. In contrast, our analysis revealed a positive prognostic impact of MUC1-specific IgG independent of clinico-pathologic parameters. This disagreement between the earlier and our findings may be due, at least in part, to the different definition of MUC1-specific IgG responses as employed in both studies. Von Mensdorff-Pouilly et al.\(^ {3} \) arbitrarily defined a positive MUC1 IgG antibody result as MUC1 IgG levels equal to or greater than the corresponding median value obtained in the entire breast cancer population. On the other hand, in the present study, a positive MUC1 IgG response was strictly defined as a significant increase (\( p < 0.05 \)) in the optical density of serum samples as compared to negative controls. Irrespective of these procedural differences, the reported significant benefit in disease-specific survival of breast cancer patients as associated with a positive test result for MUC1 IgG antibodies appears to suggest that naturally occurring anti-MUC1 IgG may control, at least to some extent, hematogenic tumor cell dissemination and micrometastatic seeding by effecting MUC1-specific tumor cell killing.
A further issue that should be considered is the prevalence of MUC1-specific antibodies in cancer patients compared to healthy donors. Although this question was not addressed in the present paper, von Mensdorff-Pouilly et al. have already shown MUC1 IgG antibody levels to be significantly higher in breast cancer patients than in the total control population, while no significant difference was found for IgM antibody levels. Furthermore, MUC1 IgG levels were significantly higher in patients with benign ovarian tumors and in patients with ovarian or endometrial cancer than in healthy controls or patients with endometriosis. In a former report, our group analyzed naturally occurring MUC1 IgG and IgM levels in 11 healthy donors. In accordance, we detected no MUC1-specific IgG antibodies in the control cohort and MUC1-specific IgM in only two cases. This, also, implies a significant increase of MUC1-specific antibodies in cancer patients. Taken together, those findings hint at a de-novo production of at least MUC1-specific IgG antibodies in cancer patients.

In the above ADCC process against MUC1-positive cancer cells, the various IgG subclasses obviously play a complex role which to date is not fully understood. In breast cancer patients and healthy controls with high levels of total anti-MUC1 IgG, von Mensdorff-Pouilly et al. mostly detected IgG subclass 2, lesser responses of IgG1 and IgG3, but no IgG4. Interestingly, also in this analysis, no difference in IgG subclasses was found between breast cancer patients and healthy controls. More recently, IgG4 subclass antibodies were shown to impair antitumor immunity in melanoma with serum IgG4 levels being inversely correlated with patient survival. IgG4 could be demonstrated to prevent the activation of antitumor effector functions in melanoma patients as well as to compromise the potency of tumoricidal IgG1 in a human melanoma xenograft mouse model. This IgG4 blockade worked via reduction of Fcγ receptor I activation.

With the above data serving as background, we analyzed the prognostic impact of MUC1-specific IgG subclasses in our patient cohort. Although statistically not significant, there was a trend of IgG2 levels to correlate with prolonged overall survival, while IgG subclass 1, 3 and 4 did not show any correlation with patient survival. Admittedly, it is difficult to reconcile the highly significant prognostic value of total anti-MUC1 IgG antibodies as to patients’ overall survival with the failing role of individual IgG subclasses in this regard. Naturally, one might hypothesize that in different patients there are different combinations of the individual IgG subclasses whose interactions may result in mutual augmentation (e.g., via IgG2) or neutralization (e.g., via IgG4) of antitumor immunity. Additionally, genetic variants of IgG subclasses may contribute individually to the overall immune responsiveness. It is in line with this notion that Pandey et al. reported on a highly significant association between the GM 23-carrying IgG2 phenotype and high anti-MUC1 IgG antibody levels in breast cancer patients. Clearly, factors along these lines need further identification. In accordance with an earlier publication, in our study even significantly elevated serum levels of MUC1-specific IgM had, by themselves, no impact on disease-specific survival. Correspondingly, available data rather suggests an immune accelerating function for IgM in that an early primary, relatively low-affinity IgM antibody response to antigens may lead to maturation of the B cell humoral immune response to yield higher-affinity antibodies, most of which are IgGs.

Finally, we detected IgG responses to MUC1 especially in ER-negative patients which corresponds with previous results. In breast cancer, distinct differences in the intratumoral cytokine microenvironment have been shown to occur in regard to the tumor biology and immunophenotype. Low levels of IFN-α and high levels of TGFβ1 were found in lowly differentiated breast cancer types, which are associated with ER negativity. In particular, high levels of INF-α were associated with ER expression. Further, tumor-specific B-cell responses correlated highly with increased TGFβ1 and reduced IFN-α within the tumor tissue. Therefore, MUC1-specific antibodies turned out to be predominantly detectable in patients with lowly differentiated and receptor-negative tumors. This data suggests immune responses to be linked to distinct intratumoral cytokine microenvironments and to correlate with prognosis-relevant differences in tumor pathobiology.

Taken together, the present data of breast cancer patients demonstrates MUC1-specific IgG as a valuable prognostic marker, independent of clinico-pathologic and tumor biologic parameters. Patients with a positive humoral response to MUC1 have a significantly better disease-specific survival, possibly due to MUC1 antibody-mediated control of hematogenic tumor cell dissemination and micrometastatic seeding. Accordingly, MUC1-specific IgG testing may become instrumental as a diagnostic means to identify high-risk patients who might need additional adjuvant therapy. Detection of autoantibodies against MUC1 may also provide a minimally invasive procedure for earlier diagnosis of breast cancer. Currently, relevant research focuses on the elaboration of suitable tumor-associated antigen panels, which regularly contain MUC1 as one component. Last but not least, MUC1 antigens may serve, too, as attractive targets for the development of antibody-based immunotherapy, vaccines and therapeutic drug inhibitors. Vaccine strategies against MUC1 being systematically under investigation include DNA vaccines, peptide vaccines, glycopeptide vaccines, fusion proteins as well as recombinant vaccines delivered by various viral vectors.

Material and Methods

Patients and human samples
Pretherapeutic blood samples of 288 patients diagnosed with primary operable breast cancer were obtained at the Department of Gynecology and Obstetrics at the Heidelberg University Hospital during the years 1999–2003. The study had been approved by the ethical review board of the University of Heidelberg and all investigations were conducted according to the declaration of Helsinki. In line with this notion, all patients provided their written informed consent before study inclusion.

Of these 288 patients, 251 were diagnosed with early breast cancer according to pathological stage (125 stage I, 126 stage II;
Axillary lymph node involvement was present in 107 patients. Patients with metastatic disease at the time of diagnosis had been excluded. Thirty-seven patients developed metastatic spread to distant organs during follow-up observation. All patients received local therapy by either breast conserving therapy (n = 204) or mastectomy (n = 84) and axillary dissection for lymph node involvement. Irradiation of the breast/thoracic wall was performed in 235 patients including all those with breast conserving therapy. The entire cohort of breast cancer patients received systemic treatment in terms of endocrine therapy (n = 199) or chemotherapy (n = 159) with or without additional trastuzumab depending on the patients’ Her2 status. Histopathologic and tumor biologic analyses were carried out in the Department of Pathology at Heidelberg University Hospital by determination of tumor size, nodal involvement, tumor grading, proliferation index (Ki-67) as well as expression levels of ER, PR, and Her2/neu. All immunohistochemical stainings for Her2/neu, Ki-67, ER and PR were performed according to protocols of the Department of Pathology, Heidelberg University Hospital. Her2 3+ tumors were clearly considered Her2 positive. Her2 2+ tumors with Her2 amplification upon fluorescence in situ hybridization (FISH) were finally classified Her2 positive too. ER and PR statuses were analyzed by the immunoreactive score (IRS) according to Remmele and Stegner. The cut-off level for the analysis of nuclear Ki-67 expression was 20% in order to differentiate tumors with lower proliferative activity from those with a higher one. According to these results, hormone receptor positive – or luminal – tumors with either low grading (G1) or Ki-67 <20% were defined as ‘luminal A’. Naturally occurring antibodies to MUC1 were analyzed with an enzyme-linked immunosassay (ELISA). Peptides corresponding to MUC1 tr(37–57)5-IgG and IgM as well as the negative control in terms of HIV pol peptide (ILKEPVHGV) were adsorbed in alternate rows in 96-well ELISA plates. After overnight incubation stored at 4°C and washing steps using phosphate buffered saline (PBS), the wells were incubated with gelatin 1% to block non-specific binding. Following the above washing steps, serum samples at dilutions of 1:10, 1:50 and 1:100 were added and incubated overnight at 37°C. Then, after another two washing steps, antibodies specifically bound to MUC1 were incubated with a secondary antibody. Finally, streptavidin was used as a substrate and the reaction was quantified photometrically at 450 nm in an ELISA reader. Each serum sample was tested separately for each immunoglobulin subclass. The assay was performed for each serum sample in triplicate.

### Statistical analysis

A positive MUC1 antibody test result was defined as a significant increase of optical density on photometric measurement in triplets compared to an unspecific control triplet (HIV pol peptide) as analyzed by two-sided Student’s t-test with the threshold of significance set at α = 0.05. Survival curves were calculated by the Kaplan–Meier method, and univariate and comparison between survival curves were made using the two-tailed log-rank test. Univariate and multivariate analysis of potential predictors of survival was done using the Cox proportional hazards model. The following biological or clinical parameters were included in the multivariate model: tumor size, nodal involvement, tumor grading, hormone receptor (ER and PR) and Her2 status. Patients with missing values in any of the covariates were excluded from the analysis. Correlation of MUC1 IgG response and tumor biologic as well as clinical parameters was evaluated by Fisher’s exact test or the Mann–Whitney U test as appropriate. Findings were regarded as significant at p < 0.05. All statistical analyses were performed using the Statistical Analysis System, Version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
