Detection of Circulating Anti-Mucin 1 (MUC1) Antibodies in Breast Tumor Patients by Indirect Enzyme-Linked Immunosorbent Assay Using a Recombinant MUC1 Protein Containing Six Tandem Repeats and Expressed in *Escherichia coli*

Yan Tang,1,3 Li Wang,2 Peiyin Zhang,1 Hongfei Wei,1 Rui Gao,4 Xinming Liu,3 Yongli Yu,2* and Liying Wang1*

Department of Molecular Biology1 and Department of Immunology,2 Norman Bethune Medical College, Jilin University, 126 Xin Min Street, Changchun 130021, China; Department of Oncology, the Second Hospital, Jilin University, 18 Zi Qiang Street, Changchun 130041, China1; and Department of Breast Cancer, Tumor Hospital of Jilin Province, 1018 Hu Guang Road, Changchun 130061, China4

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Mucin 1 (MUC1), a tumor-associated antigen, is a transmembrane glycoprotein expressed by normal epithelial cells and overexpressed by carcinomas of epithelial origin. Autoantibodies against MUC1 are often found in circulation, either free or bound to immune complexes, which might contribute to limit tumor outgrowth and dissemination by antibody-dependent cell-mediated cytotoxicity, and were found favorably predictive of survival in early breast cancer patients. There is no commercial enzyme-linked immunosorbent assay (ELISA) kit for detecting the anti-MUC1 antibodies in human serum thus far. To detect circulating anti-MUC1 antibodies, we established an indirect ELISA (I-ELISA) using a recombinant MUC1 protein containing six tandem repeat sequences of MUC1 after the antigenicity and specificity of the protein were confirmed. The I-ELISA had a sensitivity of 91.3% and a specificity of 94.1% when a competitive I-ELISA was used as a reference test. The results showed that more patients with benign breast tumors (*P* = 0.001) and breast cancer patients before primary treatment (*P* = 0.010) were found to have anti-MUC1 IgG than healthy women; anti-MUC1 IgG before primary treatment was found more than after primary treatment (*P* = 0.016) in breast cancer patients. Interestingly, the anti-MUC1 IgG serum level was reversely correlated to that of CA15-3 antigen in advanced-stage patients (*r* = −0.4294, *P* = 0.046). Our study has demonstrated the suitability of the established I-ELISA for detecting circulating anti-MUC1 antibodies in human serum. Furthermore, we found that circulating anti-MUC1 antibodies may still bind MUC1 shed into blood in stage IV breast cancer, which can support the use of MUC1-target immune therapy strategies.
MUC1 antibodies in human sera (13, 27). Alternatively, recombinant MUC1 VNTR containing peptide was also used as antigen for detecting circulating anti-MUC1 antibodies by Western blotting (9). Although the recombinant MUC1 VNTR containing peptide expressed in Escherichia coli cannot be glycosylated as in eukaryotic cells, it has been demonstrated to be efficient in detecting anti-MUC1 antibody because MUC1 is less or not glycosylated when expressed in tumor cells.

In the present study, we constructed a recombinant MUC1 protein, 8R-MUCPT, which contained six MUC1 VNTRs. After the antigenicity and specificity of the 8R-MUCPT were verified, we established an indirect ELISA (I-ELISA) using 8R-MUCPT as a coating antigen to detect anti-MUC1 antibodies in the sera of patients with benign breast tumors and breast cancer. The results have demonstrated the potential of this recombinant MUC1 protein as detecting antigen and the suitability of the established I-ELISA for detecting circulating anti-MUC1 antibodies. In addition, the results suggested that anti-MUC1 antibodies in serum may play a role in neutralizing MUC1 VNTR core peptides and forming MUC1-CIC. By analyzing the relationship between circulating MUC1 and anti-MUC1 antibodies in advanced-stage patients, we were able to deduce the same neutralizing role for the antibodies in stage IV breast cancer.

MATERIALS AND METHODS

Specimens. A total of 200 serum samples were obtained from 56 healthy women (median age, 58.5 years; range, 25 to 80 years), 22 patients with benign breast tumors (median age, 55 years; range, 22 to 70 years), and 122 breast cancer patients (median age, 57 years; range, 29 to 77 years) before or after primary treatment including surgery, postoperative radiotherapy, and/or chemotherapy in the Second Hospital of Jilin University and Tumor Hospital of Jilin Province, Changchun, China. Serum samples were collected and stored at −70°C until analyzed. Eleven cases of CA15-3 antigen-positive sera and eleven cases of CA15-3 antigen-negative sera in advanced stage were screened by using a Protein Chip kit for Multi-Tumor Marker Detection (Shanghai Health Digit Co., Ltd., Shanghai, China).

The patients with benign breast tumors included seven with fibroadenoma, three with fibroadenosis, five with nomenclature adenosis, four with hyperplasia, and 10 with fibroadenosis. The breast cancer patients included 69 with infiltrating duct carcinoma, 20 with adenocarcinoma, 17 with infiltrating lobular carcinoma, 3 with intraductal carcinoma, 4 with mucinous adenocarcinoma, 1 with atypical medullary carcinoma, and 1 with medullary carcinoma. The group of healthy women included 2 pregnant and 54 nonpregnant women, 26 lactating and 54 nonlactating women, and 4 nulliparous women.

Preparation of recombinant 8R-MUCPT protein. Human MUC1 VNTR encoding sequence containing six tandem repeats was directly obtained by PCR using pET2sa-HSP65-MUC1 plasmid, which was previously constructed in our lab (14), as a template. The MUC1-encoded gene was then subcloned into prokaryotic expression vector pET26bH11001, which was previously constructed in our lab (14), as a template. The MUC1-encoded gene was then subcloned into prokaryotic expression vector pET26bH11001, which was previously constructed in our lab (14), as a template. The MUC1-encoded gene was then subcloned into prokaryotic expression vector pET26bH11001, which was previously constructed in our lab (14), as a template. The MUC1-encoded gene was then subcloned into prokaryotic expression vector pET26bH11001, which was previously constructed in our lab (14), as a template.
MUCPT on the dot, such as 5 MAb when its amount was high enough to neutralize 8R-MUCPT could be recognized by the sera of a natural anti-mimic antigenicity. The dot blot and inhibition test showed that MUCPT possesses high antigenic capacity and natural MUC1- as well as anti-MUC1 MAb (Fig. 2A), which revealed that 8R-PAb could recognize MUC1 expressed on breast cancer tissue, as no histochemical staining of breast cancer tissue. The anti-MUC1 polyclonal antibody (PAb; anti-MUC1 PAb) for immunoprecipitation in "I". The results showed that 8R-MUCPT was highly expressed in E. coli and able to be specifically recognized by anti-MUC1 VNTR MAb.

To confirm the antigenicity and specificity of 8R-MUCPT for measuring circulating anti-MUC1 antibodies, three tests were performed. Rabbit anti-8R-MUCPT serum was used as anti-MUC1 PAb for immunohistochemical staining of breast cancer tissue. The anti-MUC1 PAb could recognize MUC1 expressed on breast cancer tissue, as well as anti-MUC1 MAb (Fig. 2A), which revealed that 8R-MUCPT possesses high antigenic capacity and natural MUC1-mimic antigenicity. The dot blot and inhibition test showed that 8R-MUCPT could be recognized by the sera of a natural anti-MUC1 antibody that could be inhibited by anti-MUC1 VNTR MAb when its amount was high enough to neutralize 8R-MUCPT on the dot, such as 5 μg/ml (Fig. 2B), which demonstrates that natural anti-MUC1 antibody could recognize an epitope on 8R-MUCPT very similar to that of anti-MUC1 VNTR MAb. A blocking experiment was set up which showed that 8R-MUCPT could efficiently neutralize natural anti-MUC1 antibody in the sera, but poly-R or poly-H could not (Fig. 2C), and fused partners of poly-R or poly-H in 8R-MUCPT did not dramatically affect 8R-MUCPT as a detecting antigen. The result suggests that 8R-MUCPT possesses high specificity.

For verifying whether the established I-ELISA could be used to detect anti-MUC1 antibodies in the sera of patients with breast tumors, 40 serum samples were examined. Detection was performed by I-ELISA using 8R-MUCPT as a coating antigen, and a competitive I-ELISA with commercial anti-MUC1 VNTR MAb as a reference test for the CI-ELISA (Fig. 2D) could be used as a qualitative method for detecting MUC1. In the CI-ELISA, anti-MUC1 VNTR MAb at different dilutions was used to bind 8R-MUCPT first for competing with natural anti-MUC1 antibody in human sera. The anti-MUC1 antibody level detected by I-ELISA and CI-ELISA was consistent, except for one sample that was determined to be positive in the I-ELISA but negative in the CI-ELISA and two samples that were determined to be negative in the I-ELISA but positive in the CI-ELISA. Accordingly, I-ELISA had a calculated sensitivity of 91.3% and a specificity of 94.1%.

Detection of circulating antibodies against MUC1 VNTR in the sera of the patients with breast tumors and health women by the I-ELISA. The established I-ELISA method was used to detect anti-MUC1 IgG and IgM in the sera of patients with breast tumors or healthy women. The results are presented in Table 1. Anti-MUC1 IgG was detected in 32.8% of the breast cancer patients, while IgM was detected in 15.8% of the breast cancer patients; a statistically significant correlation was found between the two antibodies (r = 0.5943). IgG antibodies to MUC1 were found in 23.2% of the healthy women, 63.6% of the patients with benign breast tumors (P = 0.001 versus healthy women), and 50.0% of the patients with breast cancer before primary treatment (P = 0.010 versus healthy women). Comparatively, the positive rate of anti-MUC1 IgG antibodies in breast cancer patients before primary treatment was significantly higher than that (26.7%) in breast cancer patients after primary treatment (P = 0.016). It was also noted that there were no significant differences in anti-MUC1 IgM levels among healthy women, benign breast tumor patients, and breast cancer patients. The levels of circulating anti-MUC1 antibodies were also similar in breast cancer patients in different stages, or with or without lymph node metastasis.

Relationship between circulating MUC1 antigen and anti-MUC1 IgG in patients with advanced carcinomas. To analyze the relationship between serum MUC1 antigen and anti-MUC1 antibodies in patients with advanced-stage cancer, we selected 11 cases of CA15-3 antigen-positive sera and 11 cases of CA15-3 antigen-negative sera that were screened out by using a Protein Chip kit for Multi-Tumor Marker Detection. By the established I-ELISA, we evaluated the circulating anti-MUC1 antibody levels of the selected 22 serum samples. Circulating anti-MUC1 antibodies showed a negative correlation with serum CA15-3 expression, and it was statistically significant only between the serum level of CA15-3 antigen and the serum level of anti-MUC1 IgG (r = -0.4294, P = 0.046). As shown in Fig. 3, the serum samples were divided into three groups. In one group, positive anti-MUC1 IgG was detected together with negative CA15-3 antigen. In the second group, negative anti-MUC1 IgG was shown with negative CA15-3 antigen. In the third group, both negative anti-MUC1 IgG and positive CA15-3 antigen were found. These results suggest that circulating levels of anti-MUC1 antibodies are negatively correlated with the level of MUC1 antigen in the advanced-stage patients.
DISCUSSION

In this study, we analyzed circulating anti-MUC1 antibodies in patients with benign or malignant breast tumors by using an I-ELISA with *E. coli*-expressed recombinant MUC1 VNTR-
containing peptide. To establish a credible ELISA method for detecting serum antibodies against MUC1 VNTR, we expressed recombinant MUC1 VNTR-containing peptide consisting of six tandem repeats of MUC1 VNTR (8R-MUCPT) in E. coli. The 8R-MUCPT, with no glycosylation, is be able to mimic the naturally expressed MUC1 in tumor cells. Compared to synthetic peptides, 8R-MUCPT contains more epitopes and is more efficient for coating the plates. In addition, 8R-MUCPT possesses antigenicity of naturally expressed MUC1 and high specificity, having the crucial property as a coating antigen of detecting the anti-MUC1 antibodies in clinical samples.

With high specificity, the CI-ELISA has been used to quantify anti-MUC1 antibody and to determine the sensitivity and/or specificity of the established I-ELISA. A capture-antibody anti-MUC1 VNTR MAAb was used for firm adhesion in the experiment, and its validity has been verified by the inhibition results. The comparison results indicated that the detection capacities of the two ELISAs were nearly equivalent. The differences in epitopes that we observed were due to antigen captured by an antibody versus antigen bound to a plate when comparing the CI-ELISA to the indirect ELISA, but the difference is less because the anti-MUC1 MAAb only binds one tandem repeat in the recombinant MUC1 protein with six tandem repeats. In addition, the serum samples were diluted 1:40 for the I-ELISA and diluted 1:2 for the CI-ELISA, which suggests that the I-ELISA developed here is more sensitive.

In agreement with the findings of Croce et al. (4), the anti-MUC1 IgG and IgM serum levels of breast cancer patients in our study were 32.8 and 15.8% positive, respectively, whereas in the study of Croce et al. the levels were elevated in 32 and 14%, respectively, although different ELISA method cutoff values might have led to differences in the positive rates. In addition, we also found a positive correlation between the two antibodies.

Consistent with the results of von Mensdorff-Pouilly et al. (25), we found that positive rates of circulating anti-MUC1 IgG in patients with benign breast tumors and breast cancer before primary treatment were higher than those in healthy women. After statistical analysis, we found that our data demonstrated statistical significance, whereas the earlier findings (25) did not, possibly due to the relatively few samples and sample sources. However, similar results were reported in colorectal cancer patients; Nakamura et al. (16) reported that the positive rate of anti-MUC1 IgG showed a significant increase in colorectal cancer and a higher frequency in benign disease than in healthy subjects. According to Croce et al. (4), frequent detection of anti-MUC1 IgG may be related to an early stimulation, such as pregnancy and lactation, in which a second challenge may induce immunoglobulin switching. That is, the second challenge or the presence of tumor may lead to an increase in anti-MUC1 IgG. Furthermore, we infer that the circulating level of the natural anti-MUC1 antibody may correlate with the frequency and the amount of exposed MUC1 core peptide.

Our study showed that primary treatment was correlated with decreasing positive rates of circulating anti-MUC1 antibodies in breast cancer patients, although similar findings were also reported in other cancer patients (10). Compared to the level before primary treatment, the circulating anti-MUC1 IgG level in breast cancer patients after primary treatment was low ($P = 0.016$). Moreover, for anti-MUC1 IgM, the circulating level was lower after primary treatment than before primary treatment, although it was not statistically significant. We postulate a possible reason for this: in the course of primary treatment, including surgery, chemotherapy, or radiotherapy, membrane-bound MUC1 antigens following cell necrosis are shed into the blood to neutralize circulating anti-MUC1 antibodies (24), leading to the reduced levels of circulating anti-MUC1 antibodies. In addition, a longer course of disease and latent micrometastasis after primary treatment may also cause the increase of MUC1 antigen, and thus the numbers of circulating anti-MUC1 antibodies are reduced for greater neutralization (5, 12, 23).

According to the results of other groups before primary treatment (4, 25), we also found slightly elevated serum levels of anti-MUC1 IgG in patients with stage II compared to those detected in stage I patients, and slightly decreased serum levels in stage III patients, whereas there were again elevated levels in patients with stage IV breast cancer after primary treatment. As for circulating anti-MUC1 IgM, stage II, III, and IV patients had slightly increased levels compared to stage I patients, an observation also noted in an earlier study (4). Therefore, there are many similarities in the changes of circulating anti-MUC1 antibodies at different stages of breast cancer before and after primary treatment, which implies these changes are related to the stages of the disease but not to the primary treatment. We presume that the slightly elevated serum levels of anti-MUC1 antibodies in patients with stage II may be caused by more exposed circulating MUC1 as direct immunogen, and the slightly decreased levels of anti-MUC1 IgG in stage III may be due to more neutralization from MUC1 antigen.

A negative correlation between circulating anti-MUC1 antibodies and MUC1 antigen has been found in patients with some malignant tumors (4, 20, 23). In the present study, we found a significantly negative correlation between the CA15-3 (MUC1 antigen) serum level and that of the anti-MUC1 IgG antibody in patients with an advanced stage of breast cancer.

Stage IV breast cancer patients presented more circulating MUC1 and anti-MUC1 antibodies, but the MUC1-CIC was low, which seemed to indicate that anti-MUC1 antibodies were of low affinity in stage IV patients (4, 26). However, in the present study, we found that circulating MUC1 and anti-MUC1 antibodies in stage IV breast cancer patients showed a significantly negative correlation. That meant high levels of circulating MUC1 should correspond to a low level of anti-MUC1 antibody or that a low level of circulating MUC1 should correspond to a high level of anti-MUC1 antibody in an individual patient. The simultaneous increase in serum MUC1 and anti-MUC1 antibodies should thus be a characteristic in later-stage breast cancers. Accordingly, we deduced that anti-MUC1 may still have the ability to bind MUC1 in stage IV breast cancer patients. This deduction may be in agreement with a recent report in which the overexpression of MUC1 antigen was associated with the absence of both regional recurrence and distant metastasis (18).

In summary, we examined serum samples from patients with benign and malignant breast tumors using the established I-ELISA. Our findings indicate that anti-MUC1 antibodies in
serum play a role in binding MUC1 VNTR in breast cancer, including in stage IV.

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