

Immunoglobulin Allotypes Influence Antibody Responses to Mucin 1 in Patients with Gastric Cancer

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Abstract

There are significant interindividual differences in naturally occurring antibody responses to the tumor-associated antigen mucin 1 (MUC1), but the host genetic factors that might contribute to these differences have not been identified. The aim of the present investigation was to determine whether the variation in naturally occurring antibody levels to MUC1 in patients with gastric cancer is associated with GM and KM allotypes, genetic markers of IgG heavy chains and κ -type light chains, respectively. A total of 169 Caucasian subjects with gastric cancer were allotyped for several GM and KM markers. These subjects were also characterized for IgG and IgM antibodies to MUC1. GM 3 23 5,13 phenotype was highly significantly associated with MUC1 IgG levels; subjects with this phenotype had lower antibody levels compared with those lacking this phenotype (median IgG level 65.5 relative units versus 91.0 relative units, $P = 0.0058$). In addition, this phenotype had an interactive effect with KM phenotypes on the levels of IgG antibodies to this antigen ($P = 0.0081$). Levels of MUC1 IgM antibodies were not associated with these genetic markers. These results show, for the first time, that GM and KM allotypes contribute to the interindividual differences in humoral immunity to MUC1. [Cancer Res 2008;68(11):4442–6]

Introduction

MUC1 is a membrane-bound glycoprotein that is expressed at low levels in healthy tissues but overexpressed in the majority of adenocarcinomas (1). MUC1 is immunogenic in its hypoglycosylated form expressed on tumors, and the tumor-bearing patients generate both cellular and humoral immune responses to this antigen (1, 2). High levels of anti-MUC1 antibodies are associated with a better prognosis in some adenocarcinomas (3, 4), an observation that has made MUC1 an attractive candidate for vaccines against these malignancies. Prophylactic vaccination, an approach that has been highly successful in preventing many infections, is also the most desirable strategy to prevent malignant diseases. Several vaccine trials involving MUC1 have been conducted but none to our knowledge have resulted in therapeutically beneficial immune responses (3).

Identification and understanding of the host factors that influence naturally occurring immune responses is an important

prerequisite to successfully designing a vaccine that would induce therapeutic responses. In contrast to our understanding of the naturally occurring immune responses to many infectious agents, our knowledge of the host factors that influence immune responsiveness to tumor-associated antigens is very incomplete. For MUC1, we know that there are significant interindividual differences in naturally occurring antibody responses, but apart from the identification of certain potential epidemiologic factors, no host genetic factors that might contribute to the differences in immune responsiveness have been identified (5).

Immunoglobulin allotypes are important candidates for controlling immune responsiveness, as documented by early animal studies (6). More recent studies in humans have shown that immune responsiveness to a variety of antigens—infected agents, vaccines, autoantigens, including some tumor-associated antigens—are associated with particular GM and KM allotypes, hereditary antigenic determinants of γ and κ chains, respectively (7–12). The aim of the present investigation was to determine whether the variation in naturally occurring antibody levels to MUC1 in patients with gastric cancer is associated with these genetic markers. We found highly significant associations between particular GM and KM phenotypes and the levels of IgG antibodies to MUC1. This is the first report to our knowledge implicating these genetic determinants in humoral immunity to MUC1.

Materials and Methods

Human subjects. Serum samples were obtained before treatment from 169 patients with histologically verified gastric carcinoma diagnosed at the Cancer Center of the North Estonian Regional Hospital. The study protocol was approved by Tallinn Medical Research Ethics Committee. All subjects provided informed consent. Age, gender, and stage of disease of the patients included in the study are listed in Table 1. Serum samples were stored at -20°C until required.

Detection of IgG and IgM antibodies to MUC1. IgG and IgM antibody levels to MUC1 were determined by a previously described ELISA (13). Briefly, a bovine serum albumin (BSA)-conjugated MUC1 60-mer tandem-repeat peptide (250 ng per well in PBS) and 1% BSA (control) were used to coat 96-well ELISA plates (Maxisorp, Nunc). After overnight incubation at 4°C , washing and blocking with 1% BSA in PBS, the serum diluted 1:100 and 1:500 for IgG and IgM antibody determination, respectively, was applied and the plates were incubated overnight at 4°C . The bound antibodies were detected with alkaline phosphatase-conjugated rabbit anti-human IgG or IgM (Dako) and developed with *p*-nitro-phenyl-phosphate (Sigma). Absorbance values were registered at 405 nm. A standard serum sample with a level of MUC1 IgG of ~ 1.0 absorbance unit was included in every plate to standardize the assay. We titrated some sera, including the control serum, and found that absorbance values for the serum dilutions used in this study were located in the middle part of the dose-dependent titration curves. The tested serum value was calculated as a percentage of the value of the standard serum (100%) and expressed in relative units (RU).

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GM and KM allotyping. Serum samples were typed for G1M (1/a, 2/x, 3/f, 17/z), G2M (23/n), G3M (5/b1, 6/c3, 13/b3, 21/g), and KM 1 and 3 allotypes by a standard hemagglutination-inhibition method (14, 15). In brief, a mixture containing human blood group ORh+ erythrocytes coated with anti-Rh antibodies of known GM/KM allotypes, the test sera, and monospecific anti-allotype antibodies were incubated in a microtiter plate. The test sera containing IgG of particular allotype inhibited hemagglutination by the anti-allotype antibody, whereas the negative sera did not. Linkage disequilibrium in the GM system is almost absolute and the determinants are transmitted as a group called haplotypes (16–18). The notation follows the international system for human gene nomenclature (19), in which haplotypes and phenotypes are written by grouping together the markers that belong to each IgG subclass, by the numerical order of the marker and of the subclass; markers belonging to different subclasses are separated by a space, whereas allotypes within a subclass are separated by commas.

Statistical analysis. GM and KM allotype determinations were made blinded with respect to the anti-MUC1 antibody status of the subjects, and the results were provided to an independent biostatistician (P.J.N.) who conducted the analyses. Because of almost absolute linkage disequilibrium between particular GM alleles in a given race, the allotype data were analyzed as a group (phenotypes), rather than the presence or absence of individual markers. Subjects with very unusual GM phenotypes and those whose frequency was <3% were combined as “other” for statistical analyses, so as not to have a test with too many degrees of freedom or categories with cell counts too small for appropriate analyses. For analysis purposes, antibody levels were log₁₀ transformed to obtain residual homoscedasticity. Analysis of covariance (ANCOVA) models were used to determine the statistical significance associated with the differences in antibody levels corresponding to different phenotypic groups.

Because previous studies in this cohort showed a possible association between the gastric cancer stage and MUC1 IgG levels (4), the ANCOVA models included cancer stage (I, II, III, or IV) as a covariate. A series of ANCOVA models was performed to determine whether GM and KM phenotypes individually, or in particular combinations, were associated with MUC1 IgG or IgM antibody levels. In all models, statistical significance was defined as *P* < 0.05.

Results

The distribution of GM and KM phenotypes among the 169 study subjects in relation to the median levels (relative units) of IgG and IgM antibodies to MUC1 is given in Table 2. The majority of the subjects possessed typical Caucasian GM phenotypes, which can be explained by postulating the segregation of four common haplotypes in this racial group—GM 1,17 21; GM 1,2,17 21; GM 3 5,13; and GM 3 23 5,13. Most subjects were KM 3 homozygotes (88.2%); a few were KM 1,3 heterozygotes (11.8%); and none were KM 1 homozygotes, which is not surprising because the gene frequency of KM 1 is only ~0.1 in Caucasians.

Table 1. Characteristics of patients with gastric carcinoma

Stage*	n	Males/females	Age (range), y
I	41	24/17	65.0 (24–81)
II	18	8/10	65.5 (29–80)
III	63	35/28	66.0 (45–84)
IV	47	32/15	65.0 (59–80)
All	169	99/70	66.0 (24–84)

*Pathologic tumor-node-metastasis classification of malignant tumors.

GM 3 23 5,13 phenotype was highly significantly associated with MUC1 IgG levels: Subjects with this phenotype had lower antibody levels compared with those lacking this phenotype (median IgG level 65.5 RU versus 91.0 RU, *P* = 0.0058). Figure 1 presents boxplots showing the distribution of MUC1 IgG antibody levels corresponding to various GM phenotypes. KM phenotypes were not independently associated with MUC1 IgG levels. However, a significant (*P* = 0.0081) interactive effect of the two KM phenotypes with the GM 3 23 5,13 phenotype on MUC1 IgG levels was observed (Fig. 2). Within KM1,3 subjects, there was, on average, a 124% increase in the median antibody levels for the GM 3 23 5,13 carriers compared with noncarriers (145.5 RU versus 65.0 RU), whereas in KM 3 subjects, however, there was a 34% decrease in the median antibody levels for GM 3 23 5,13 carriers compared with noncarriers (62.0 RU versus 94.0 RU). MUC1 IgM levels were not associated with GM or KM phenotypes.

As control antibody responses, we measured antibodies to *Helicobacter pylori* and to another tumor-associated antigen, Thomsen-Friedenreich disaccharide, in these patients. No GM and KM phenotypes, either individually or in particular combinations, were associated with antibody responsiveness to these antigens (data not shown).

Discussion

The results presented here show that gastric cancer patients with non-GM 3 23 5,13 phenotypes have a 39% higher median anti-MUC1 IgG antibody concentration than those with this GM phenotype. At least two mechanisms, which are not mutually exclusive, could explain the involvement of GM and KM allotypes in humoral immunity to MUC1. Memory B cells, which predominantly express IgG as the membrane-bound form of immunoglobulin (mIg), show enhanced response to antigen stimulation than cells expressing IgM on their surface (20, 21). Perhaps mIgG molecules with non-GM 3 23 5,13 phenotypes act as more compatible receptors for MUC1 and thus provoke a strong humoral immunity, whereas the mIgG molecules of GM 3 23 5,13 individuals form a less compatible receptor for the critical epitopes of this tumor-associated antigen. GM determinants could directly influence the conformation of the immunoglobulin variable (V) regions involved in antigen binding and thus cause changes in antibody specificity. Contrary to the current dogma in immunology that the V region of immunoglobulin is the sole determinant of antibody specificity, several studies have shown that structural variation in the constant (C) region affects the expression of certain idiotypes and causes variation in the specificity of V region-identical immunoglobulin molecules (22–25). A recent study has clearly established that amino acid sequence polymorphisms in the C region of the immunoglobulin molecule affect the secondary structure of the antigen-binding site in the V region (26). Amino acid substitutions associated with GM allotypes cause structural changes in the C region, which could impose structural constraints (conformation) on the V region, resulting in variation in antibody specificity to MUC1. It is also possible that the associations we have observed are due to linkage disequilibrium between particular GM/KM alleles and alleles of another locus, as yet unidentified, for humoral immune responsiveness to MUC1.

As mentioned before, high levels of anti-MUC1 antibodies are associated with good prognosis in some adenocarcinomas. The destruction of MUC1-bearing tumor cells by antibody-dependent cellular cytotoxicity (ADCC), which links the specific humoral

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Table 2. Distribution of GM and KM phenotypes in relation to IgG and IgM antibody levels to MUC1 in patients with gastric cancer

Phenotypes	No. subjects	Antibody levels (median, interquartile range)	
		MUC1 IgG (RU)	MUC1 IgM (RU)
GM 3 23 5,13	68	65.5* (41.5–121.5)	32.5 (20.2–49.0)
GM 1,3,17 23 5,13,21	32	84.5 (60.0–134.5)	35.5 (23.0–43.0)
GM 3 5,13	16	143.0 (51.0–283.0)	31.0 (19.5–41.5)
GM 1,3,17 5,13,21	14	71.5 (45.0–171.0)	45.5 (16.0–65.0)
GM 1,2,3,17 23 5,13,21	10	114.5 (101.0–149.0)	24.0 (20.0–41.0)
GM 1,2,3,17 5,13,21	10	158.0 (49.0–366.0)	37.5 (28.0–47.0)
Other GM	19	69.0 (49.0–172.0)	27.0 (15.0–42.0)
KM 1	0	NA	NA
KM 1,3	20	88.0 (40.0–166.5)	35.0 (18.5–52.5)
KM 3	149	82.0 (48.0–145.0)	33.0 (20.0–46.0)

Abbreviation: NA, not applicable.

*Significantly lower ($P = 0.0058$, obtained by analysis of covariance \log_{10} transformed data) compared with the rest of the GM phenotypes.

responses to the vigorous innate cytotoxic effector responses, could partly account for this observation (27). IgG-mediated ADCC is triggered upon ligation of Fc γ receptor (Fc γ R) to the Fc of IgG molecules (28). It follows that genetic variation in Fc γ R and Fc could contribute to the interindividual differences in ADCC. The majority of the GM markers are present on the Fc region of IgG molecules (17, 18). Fc γ Rs are genetically polymorphic and certain alleles are more efficient in causing ADCC than others. In one study, the Fc γ RIIIa genotype of the effector cells significantly influenced the ADCC against cells derived from a breast cancer cell line (29). GM allotypes have the potential to be effect modifiers of this phenomenon: Anti-MUC1 IgG antibodies with Fc of a parti-

cular GM genotype could preferentially associate with the Fc γ RIIIa or Fc γ RIIIa (expressed on natural killer cells) of a particular genotype (30) and influence the destruction of MUC1-expressing cells through ADCC (31).

We also observed interactive effects of GM and KM phenotypes on antibody responsiveness. The presence of GM 3 23 5,13 phenotype was associated with high anti-MUC1 IgG levels in KM 1,3 heterozygotes but with low antibody levels in KM 3 homozygotes. This suggests that the association of heavy and light chains in IgG antibodies directed against MUC1 may not be random. Only γ and κ chains carrying specific GM and KM allotypes might form a paratope with the necessary quaternary structure for an effective

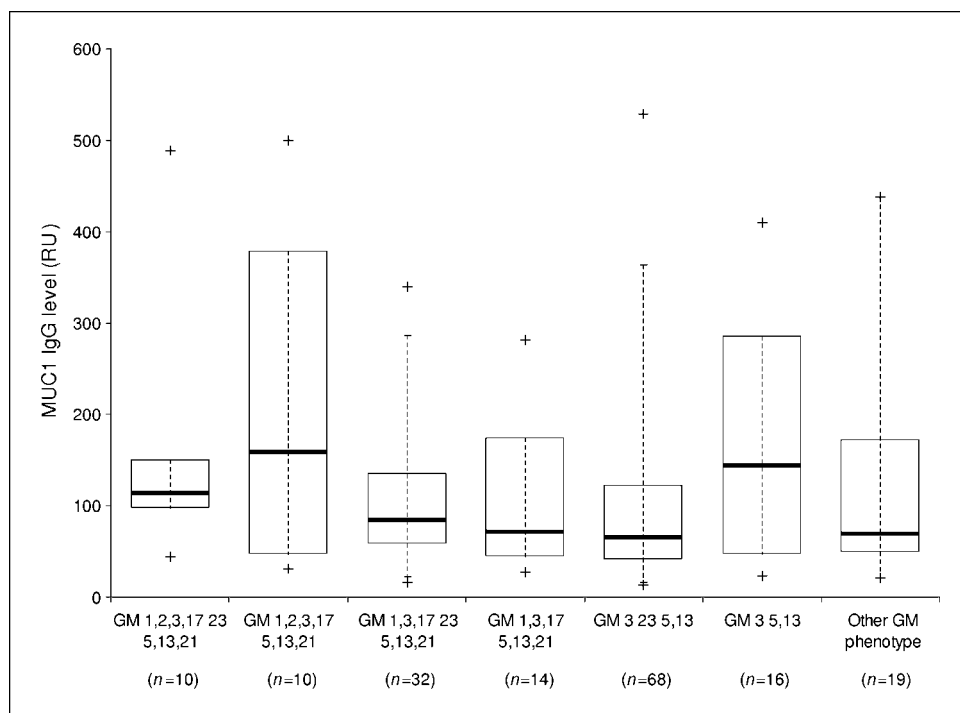
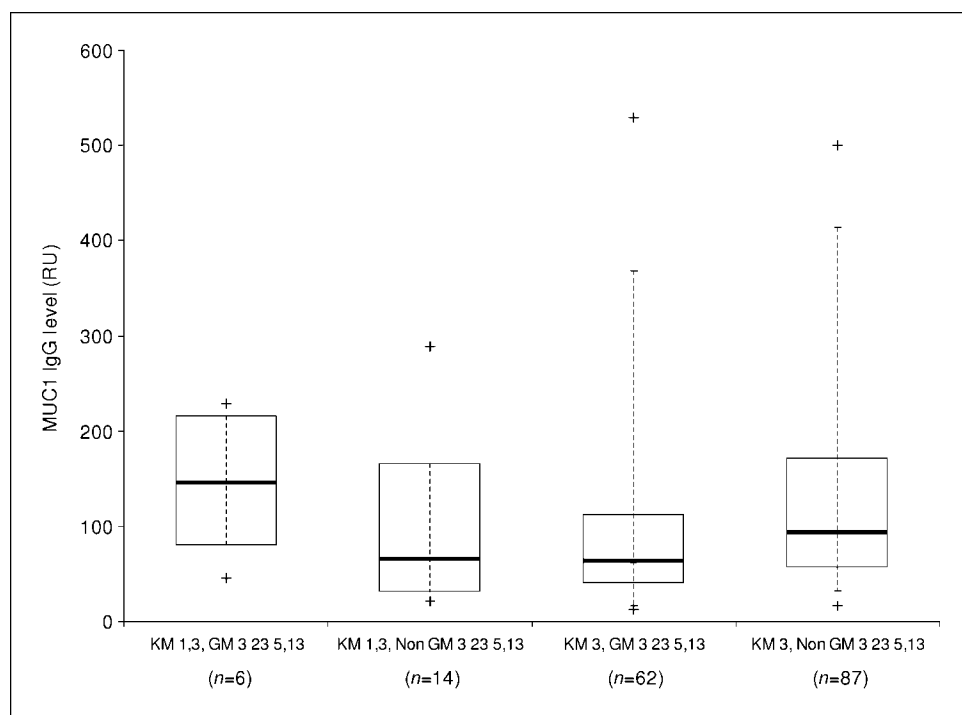


Figure 1. Boxplots of MUC1 IgG levels and GM phenotypes. **Bold horizontal lines**, median Ig levels corresponding to GM phenotypes; **boxes**, the interquartile ranges. **Dotted lines**, 5th to 95th percentiles; **+**, minima and maxima.

Figure 2. Boxplots of MUC1 IgG levels by KM and GM phenotype combinations. **Bold horizontal lines**, median IgG levels for the various KM-GM phenotype combinations; **boxes**, the interquartile ranges. **Dotted lines**, 5th to 95th percentiles; **+**, minima and maxima.



recognition of the MUC1 epitopes. Nonrandom pairing of heavy and light chains has been reported in experimental animals (32, 33). It may be of interest to note that particular GM and KM phenotypes have interactive effects on antibody responsiveness to EBV antigens in patients with Burkitt's lymphoma (34). Association of GM 3 23 5,13 with low levels of anti-MUC1 antibodies observed here is reminiscent of our findings in osteosarcoma, where this phenotype was associated with low antibody responses to osteosarcoma-associated antigens (12). (Subjects in the previous study were not typed for GM 23, so the GM 3 5,13,14 subjects in that study included those with and without GM 23.)

Association of non-GM 3 23 5,13 phenotypes with high responsiveness to MUC1, if confirmed in an independent study, could aid in identifying subjects who are more likely to benefit from MUC1-based vaccines. For individuals with the low responder GM 3 23 5,13 phenotype, MUC1 could be fused with appropriate adjuvants, such as heat shock proteins, to overcome the allotypic restriction in immune responsiveness (35). It is interesting to note that like MUC1, IgG antibody levels to certain heat shock proteins, too, are influenced by GM genotypes (36), making it conceivable to formulate a fusion MUC1-heat shock protein vaccine that could potentially generate high antibody responses in the majority of the population.

Although the associations reported here are very strong, it is nevertheless important to determine whether they can be repli-

cated in an independent study population. Furthermore, it is essential to study other racial groups, as each major race is characterized by a unique array of GM haplotypes (16), and putative phenotypes associated with responsiveness to MUC1 in other populations are likely to be different from those reported for the Caucasians in the present investigation. In future studies, inclusion of other candidate genes (e.g., *HLA*, *KIR*, *FcγR*, *IL-6*), and determining their possible epistatic effects with one another and with GM and KM alleles on immune responsiveness to MUC1 in a large study population, would significantly enhance our understanding of the mechanisms underlying natural immunity to this tumor-associated antigen. This knowledge would be valuable in formulating prophylactic vaccines for adenocarcinomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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