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IGHG, *IGKC*, and *FCGR* genes and endogenous antibody responses to GARP in patients with breast cancer and matched controls

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ABSTRACT

Glycoprotein-A repetitions predominant (GARP) is a transmembrane protein that is highly expressed in breast cancer. Its overexpression correlates with worse survival, and antibodies to GARP appear to play a protective role in a mouse model. No large-scale studies of immunity to GARP in humans have yet been undertaken. In this investigation, using a large multiethnic cohort (1738 subjects), we aimed to determine whether the magnitude of anti-GARP antibody responsiveness was significantly different in patients with breast cancer from that in matched healthy controls. We also investigated whether the allelic variation at the immunoglobulin GM (γ marker), KM (κ marker), and Fcγ receptor (FcγR) loci contributed to the interindividual variability in anti-GARP IgG antibody levels. A combined analysis of all subjects showed that levels of anti-GARP antibodies were significantly higher in patients with breast cancer than in healthy controls (mean ± SD: 7.4 ± 3.5 vs. 6.9 ± 3.5 absorbance units per mL (AU/μL), $p < 0.0001$). In the two populations with the largest sample size, the probability of breast cancer generally increases as anti-GARP antibody levels increase. Several significant individual and epistatic effects of GM, KM, and FcγR genotypes on anti-GARP antibody responsiveness were noted in both patients and controls. These results, if confirmed by independent investigations, will aid in devising personalized GARP-based immunotherapeutic strategies against breast cancer and other GARP-overexpressing malignancies.

1. Introduction

Glycoprotein-A repetitions predominant (GARP) is a transmembrane protein that is highly expressed in many types of cancer, including breast cancer. According to The Cancer Genome Atlas (TCGA), the GARP-encoding gene, *Lrrc32*, is amplified in about 30% of patients [1]. Its overexpression correlates with worse survival, and antibodies to GARP appear to play a protective role in a mouse model of breast cancer [1]. These observations—coupled with the fact that GARP is the docking receptor for the cancer-promoting cytokine TGFβ—make GARP an attractive target for immunotherapy [2,3]. Since no animal model can fully replicate a human disease, a thorough understanding of the mechanisms responsible for natural immunity to GARP in humans is an important prerequisite to designing effective GARP-based immunotherapies. Such studies may also provide insights into the host immunosurveillance mechanisms that keep two-thirds of the human population cancer free [4].

In our previous immunogenetic studies of breast cancer, we have shown that particular alleles of GM (γ marker) and KM (κ marker) allotypes, encoded by immunoglobulin heavy chain G (*IGHG*) and immunoglobulin κ constant (*IGKC*) genes, respectively—contribute to the risk of breast cancer and to the magnitude of humoral immunity to breast tumor-associated antigens in a racially restricted manner [5–8]. Interactive effects of these genes with Fcγ receptor (FcγR) genes have also been noted. These results provide a strong rationale for investigating the role of GM, KM, and FcγR genes in endogenous antibody responses to GARP.

Additional rationale for investigating the KM alleles is provided by a large analysis of human gene expression, which identified the *IGKC* gene as a strong prognostic marker in human solid tumors, including

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breast cancer [9]. Identification of tumor-infiltrating plasma cells as the source of *IGKC* expression strongly suggests a role for humoral immunity in solid tumors, providing a compelling rationale for investigating the role of KM alleles, genetic variants of *IGKC*, in humoral immunity to tumor-associated antigens in breast cancer.

In this investigation, using a matched case-control design and archived specimens from a large multiethnic study population [10], we aimed to determine whether the magnitude of endogenous anti-GARP antibody responses in patients with breast cancer was significantly different from that in healthy controls, and whether these responses were associated with particular immunoglobulin GM, KM, and FcγR genotypes.

2. Materials and methods

2.1. Blood samples

Archived DNA and serum samples from an epidemiologic study of breast cancer were used in this investigation [10]. The study population consisted of breast cancer patients from several hospitals in Nagano, Japan, and São Paulo, Brazil. Controls were matched to patients by ethnicity, residential area, and age (within 3–5 years). A total of 1738 subjects participated in this investigation: 527 Caucasians (Brazil), 84 subjects of African descent (Brazil), 158 subjects of Japanese descent (Brazil), 167 subjects from the Brazilian mulatto population, 802 subjects from Nagano, Japan. The racial categories are self-identified. Data on possibly confounding variables were collected either by self-administered questionnaires or in-person interviews. These included: family history of cancer, menstrual and reproductive history, anthropometric factors, physical activity, smoking habits, and estrogen and progesterone hormone receptor status. The study protocol was approved by CONEP (Comissão Nacional de Ética em Pesquisa), Brasılia, Brazil, the Institutional Review Board of the National Cancer Center, Tokyo, Japan, and by the Institutional Review Board for Human Research, Medical University of South Carolina, USA.

2.2. Anti-GARP antibodies

Serum samples were frozen at −80 °C until used. Anti-GARP IgG antibody levels were determined by an ELISA. Recombinant human GARP was obtained from R&D Systems, Inc. (Minneapolis, MN). The recombinant GARP expressed in Chinese Hamster Ovary cell line, is a 70 kDa protein (Met1-Asn627) with a C-terminal 6-His tag. Ninety-six-well, round bottom microtiter plates were coated with 50 μL (100 ng/mL) of human GARP in phosphate buffered saline, pH 7.4, and incubated at room temperature for 3 h. Wells were washed five times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), and unbound sites were blocked with 1% BSA in PBS-T (BSA-PBST). Wells were again washed and incubated with diluted serum (1:50) from study subjects in BSA-PBST and incubated for overnight at 4 °C. All samples (patients and controls) were assayed at 1:50 dilution. Serial dilutions of the positive control were assayed to determine this optimal dilution which gives absorbance values in the linear range of a dose-dependent titration curve. Wells incubated with BSA-PBST alone (without patients' serum) were used as blank. Wells were washed further and incubated with anti-human IgG horseradish peroxidase (HRP) conjugate for 30 min at 37 °C. After final wash, 50 μL HRP substrate, hydrogen peroxide, along with tetramethyl benzidine (TMB) as chromogenic substrate were added to each well and incubated in the dark at room temperature for 20 min. The reaction was stopped by the addition of 50 μL of 2 N HCl, and the absorbance values were measured at 450 nm in an enzyme-linked immunosorbent assay (ELISA) reader. Plate to plate variations in the absorbance values were managed by running the anti-GARP positive serum sample as reference in each plate and adjusting the values accordingly. Anti-GARP antibody levels were expressed as absorbance units (AU) after multiplying the absorbance values with the dilution factor.

2.3. GM and FcγR genotyping

DNA samples from this study cohort were previously typed for GM alleles (3/f, 17/z, 23+/n+, 23−/n−, 5/b1, 21/g) by TaqMan® and PCR-RFLP genotyping methods [6]. The GM gene notation follows the international system for human gene nomenclature [11]. This system has recommended both alphameric and the numeric nomenclature. Some authors use the former, while others use the latter. We use the numeric system. For readers' convenience, we have provided both. The samples were also characterized for FcγRIIa alleles, histidine (H)/arginine (R) and FcγRIIIa alleles phenylalanine (F)/valine (V) by TaqMan® genotyping assays [10].

2.4. KM genotyping

The KM alleles 1 and 3 were previously determined [8], by a PCR-RFLP method [12].

2.5. Statistical analysis

Statistical analysis was performed in R v3.4.0 [13]. To estimate the antibody levels in patients with breast cancer and matched controls by ethnicity, we used a linear mixed regression model with a case status by ethnicity interaction term. A random term was included to account for the correlation of the case-control pairs. Linear combinations of the resulting model coefficients were used to test differences in antibody levels between cases and controls for each population group (Wald test). Several covariates were added to the model one at a time to determine their association with antibody levels, they included: age, hormone receptor status, family history of breast cancer, history of benign breast disease, menopausal status, number of births, age at first birth, history of breast feeding, smoking status, drinking status, physical activity levels in past 5 years, vitamin use, age at menarche, and BMI. We confirmed that the significance of associations of antibody levels with ethnicity and case status in the linear models remained as reported in the results even after adjustment for the genotypic effects at the 6 loci and smoking status. The regression analyses were done using the *lme* function in the nlme package v3.1–131 [14]. Linear combinations were constructed using the estimable function from the gmodels package v2.16.2 [15]. For each population group and case status, separate ANOVA models using the base *aov* function were fit to test within genotype associations (a single genotype), and between genotype two-way interactions (F-tests); all associations remained as reported after adjusting for smoking status. Lastly, to assess the functional form of the dose-response trend between case status and antibody levels, we fit a Loess curve with the base *loess* function (span = 0.7) of case status (0 = Control, 1 = Case) on antibody concentration for each ethnic category and plotted the predicted probability curve. All significance tests were two-sided $\alpha = 0.05$. Nominal p values are presented. Because this is an exploratory analysis, the p values were not adjusted for multiple testing. The findings need to be confirmed by independent investigations.

3. Results

3.1. Differences in anti-GARP IgG antibody levels between patients with breast cancer and matched controls

A combined analysis of all 1738 subjects showed that levels of antibodies were significantly higher in patients with breast cancer than in healthy controls (mean ± SD: 7.4 ± 3.5 vs. 6.9 ± 3.5 absorbance units per mL (AU/μL), $p < 0.0001$, Table 1). Stratified analyses by population demonstrated highly significant differences in antibody levels, with patients having higher levels than controls in whites and in