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p53 Codon 72 Proline/Arginine Polymorphism and Autoimmune Thyroid Diseases

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p53 protein participates in the processes of apoptosis, which is involved in a number of immunological reactions. In order to test whether the *p53* gene could be used as a genetic marker for the prediction of the development of autoimmune thyroid diseases (AITD), we screened, by using polymerase chain reaction (PCR) analysis, for the C (CCC)/G (CGC) polymorphism at the *p53* codon 72 (Pro 72/Arg 72) to determine the genotypes of 107 Hashimoto's thyroiditis (HT) and 90 Graves' disease (GD) patients, and 105 normal controls. The data demonstrated that, for the genotype analysis, HT patients featured an enhanced numerical ratio for the Arg/Arg homozygous genotype (33.7%) and a diminished ratio for the Arg/Pro heterozygous genotype (41.1%) at the *p53* codon 72 than was the case for normal controls (Arg/Arg: 17.1% and Arg/Pro: 61.9%; $P = 0.005$). The odds ratio for the risk of the Arg/Arg genotype's appearance, compared with that of the Arg/Pro and Pro/Pro genotypes combined, for the HT patient group was 2.450 (95% confidence interval: 1.274–4.716). With respect to allelic analysis, we did not observe significant difference in the frequency of appearance of the Arg allelic variant and the Pro allelic variant for the *p53* codon 72 when comparing the HT patient group with the control group ($P = 0.208$). On the other hand, GD patients presented no significant difference in distribution for both genotype and allelic frequencies ($P = 0.344$ and 0.245, respectively) when compared with normal controls. In conclusion, HT patients feature a greater ratio of arginine homozygosity at *p53* codon 72 than in the case for normal subjects. The *p53* codon 72 proline/arginine polymorphism may be a genetic marker to predict the increased susceptibility of development of HT. J. Clin. Lab. Anal. 22:321–326, 2008. © 2008 Wiley-Liss, Inc.

Key words: autoimmune thyroid diseases; Graves' disease; Hashimoto's thyroiditis; *p53*; polymorphism

INTRODUCTION

Hashimoto's thyroiditis (HT) and Graves' disease (GD) are both common organ-specific autoimmune diseases of the thyroid gland (AITD), which are most common in middle-aged women. The pathogenesis of HT involves the antibody (Ab), such as antimicrosomal Ab (AMA), -dependent cytotoxicity and cytotoxic T lymphocyte (CTL)-induced direct cell destruction or programmed cell death, i.e., apoptosis (1,2), whereas it would appear that GD is mainly caused by the stimulatory effect upon thyroid cells of thyrotropin receptor Ab (TRAb) (3). Histologically, the thyroid glands of patients suffering from either of these two

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disorders virtually always present with diffuse lymphocyte infiltration of the gland with germinal-center formation and, to a greater or smaller degree, the presence of fibrosis (4). Furthermore, there typically exists an increased obliteration of thyroid follicles by widespread apoptosis for HT sufferers, a feature that also appear, but less remarkably, in the thyroid glands of GD patients, however (5,6). Clinically, both disorders are characterized by the presence of diffuse thyroid enlargement (goiter), although HT patients always appear euthyroid or hypothyroid, whereas GD patients usually present with hyperthyroidism.

Intrathyroidal lymphocyte infiltration, the pathognomonic feature of AITD, is typically composed of T cells and B cells, implying a contribution to the pathogenesis of both disorders from cell-mediated as well as humoral immunity (7). Typically such B cells are able to produce Abs, such as AMA and antithyroglobulin Ab (ATA), and TRAb (1,3). With respect to T cells, they mediate cascades of cellular and humoral immune reactions via, respectively, T-helper (Th) 1 and Th2 pathways (8). Obviously thus, lymphocytes with their mediated immunological reactions play a critical role in the development of AITD.

The *p53* is usually recognized as a tumor-suppressor gene because its encoding protein, p53, participates in the processes of cell-cycle arrest and apoptosis (9). Apoptosis not only prevents tumor generation but also normally regulates the maturation and control of both T- and B-cell immune responses, the basic pathogenetic components of AITD, via removing autoreactive or nonreactive immune elements (10,11). In general, p53 is expressed to varying degrees in various cancer cells, however, it has also been detected in noncancerous thyroid tissue (12–14). Using immunohistochemical staining techniques, Chetty *et al.* and Okayasu *et al.* have independently, observed the expression of p53 in thyroid tissue derived from HT sufferers (12,13). Further, in 2000, Fenton and colleagues also detected the presence, in serum, of Ab against the p53 for patients suffering from AITD (14). Thus, it seems reasonable to propose that there is a linkage between the *p53* (and p53) and an individual's susceptibility to AITD.

The genetic mechanism underlying AITD would appear to be quite complex, and to the best of our knowledge, most studies that have attempted to deal with this mechanism have focused upon the human leukocyte antigen (HLA) region and the cytotoxic T lymphocyte-associated protein-4 (*CTLA-4*) (15–17). It would be appear, however, that other genes are also involved in the inheritance of AITD. Codon 72 proline/arginine allelic variants are a very common polymorphism of the *p53* and they have been reported to feature markedly different apoptotic potentials (18). The degree of proline homozygosity in this site has been found to constitute a risk factor for an individual's susceptibility to thyroid cancer (19,20). To the best of our knowledge, however, from a thorough review of the literature, at the time of writing there would not appear to have been any literature-published studies that have reported on the association between the *p53* polymorphisms and AITD. In order to test whether the *p53* could be used as a genetic marker for the prediction of AITD development, we screened the *p53* codon 72 proline (Pro 72, C<u>C</u>C)/arginine (Arg 72, C<u>G</u>C) polymorphism, using polymerase chain reaction (PCR) analysis, so as to compare the distributions for both genotype and allelic frequencies for both HT as well as GD patients with normal controls from a selected Taiwanese study population.

METHODS

Patient Selection

One hundred and seven unrelated Chinese HT patients (97 women) aged between 17 and 69 years (mean 36.0 ± 12.5) and 90 GD patients (73 women) aged between 17 and 71 years (mean 35.3 ± 12.5) were enrolled in this study that continued from January 2003 through to January 2005 inclusively. All study-participating patients were of the Han race and resided in central Taiwan. None of the HT patients participating in this study revealed any current or any previous history of hyperthyroidism or any thyroid-associated ophthalmopathy. None of the study-participating female patients were pregnant at the time of the study, and none had delivered a baby/babies within the entire year period prior to study enrolment. The presence of a palpable goiter, a positive serum AMA titer (1:100 or greater), either featuring an elevated serum ATA titer or not, and negative serum TRAb were used as the definitive criteria to specifically define HT. On the other hand, the presence of hyperthyroidism, a diffuse goiter, and a positive serum TRAb ($>10\%$), supported by infiltrative ophthalmopathy and a positive serum AMA and/or ATA, were used to define GD. Ultrasonographic examination revealed a diffuse hypoechogenic pattern with no evidence of nodular lesions in thyroid gland for individuals from both groups of patients. The control group consisted of 105 (90 women) ethnically and residentially matched healthy volunteers over the age of 40 years who featured neither goiter nor any evidence of thyroid dysfunction. None of them revealed any positive antithyroid Ab in their sera. Further, these individuals also exhibited no previous personal or family history of thyroid disease or any form of autoimmune disease. This study was approved by the institutional ethics

committee of our institution, and informed consent was requested of, and obtained from, each study subject before their enrolment in this study.

Polymerase Chain Reaction

Experimental genomic DNA was prepared from peripheral blood using the genomic DNA-isolation reagent kit (Genomaker Inc.; Taipei, Taiwan). A conventional PCR was used to identify the genotypes of *p53*. This reaction was carried out in a total volume of 25 μL, containing genomic DNA; 2–6 pmol of each primer; 1 × Taq polymerase buffer (1.5 mM $MgCl_2$); and 0.25 units of AmpliTaq DNA polymerase (Perkin-Elmer; Foster City, CA). The primer "Pro 72" was designed specially for the *p53* codon 72 in proline form and for "Arg 72" in arginine form, according to a procedure described by Storey *et al.* (21). PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (PerkinElmer). Cycling conditions for Pro 72 were set as follows: one cycle at 94°C for 5 min, 35 cycles at 94°C for 15 sec and 52°C for 20 sec, and 72°C for 30 sec, with one final cycle of extension at 92°C for 7 min. Conditions for Arg 72 were the same as for Pro 72, apart from a change from 52 to 50°C, for annealing. The PCR products of Pro 72 and Arg 72 from the same individual were mixed together and 10 μL of this mixture was loaded into a well of a 3% agarose gel containing ethidium bromide, following which the gel was subjected to electrophoresis under normal conditions.

Genotype analyses for both patients and controls were uniformly performed in the same laboratory by a single well-trained professional technician contemporaneously, and all resultant gels were inspected by investigators who were blinded to the clinical phenotypes of the individuals being studied. If a slightly doubtful or unclear reading resulted from PCR, the PCR procedure was repeated by another trained technician in order to attempt to determine the exact genotype for each study-participating individual.

Statistical Analysis

The genotype and allelic frequencies of the polymorphism for individuals from the HT and from the GD patient groups were separately compared statistically with the corresponding data for the control group. For this purpose, we used the χ^2 test performed with the Statistical Package for Social Sciences (SPSS) Version 8.01 software (SPSS for Windows, SPSS Inc.; Chicago, IL). Results were considered statistically significant when the probability of findings occurring by chance was less than 5% ($P < 0.05$), which was further examined with calculation of power. Odds ratios (OR) with associated 95% confidence intervals (CI) were calculated for disease susceptibility associated with specific genotypes and alleles.

RESULTS

The PCR products for Pro 72 and Arg 72 were, respectively, 177-base pairs and 141-base pairs.

As regards genotype analysis, HT patients featured an enhanced numerical ratio for the Arg/Arg homozygous genotype (33.7%) and a diminished ratio for the Arg/Pro heterozygous genotype (41.1%) at the *p53* codon 72 than was the case for normal controls (Arg/Arg: 17.1% and Arg/Pro: 61.9%; χ^2 test, $P = 0.005$, power = 0.85; Table 1). The OR for the risk of the Arg/Arg genotype's appearance, compared with the appearance of the Arg/Pro and Pro/Pro genotypes combined, was 2.450 (95% CI: 1.274–4.716) for the HT patient group. Further, however, we noted no difference in the distribution of genotype frequencies for GD patients as compared with normal controls ($P = 0.344$; Table 1).

With respect to the allelic analysis undertaken, unlike the results for genotype analysis, we observed no significant difference as regards frequency of appearance for the Arg allelic variant (54.2 and 48.1%, respectively) and the Pro allelic variant (45.8 and 51.9%, respectively) for the *p53* codon 72 when comparing the HT patient group with the corresponding data from the control group (χ^2 test, $P = 0.208$; Table 2). Further, we also failed to observe any statistically significant difference as regards the allelic analysis for the GD patient group compared with the normal control group (χ^2 test, $P = 0.245$; Table 2).

Further subgroup analysis for HT patients was performed, and no significant association between the serum TSH levels (< 5 or ≥ 5 mIU/L) and genotypes was detected ($P = 0.892$; Table 3).

DISCUSSION

To the best of our knowledge, *p53* is generally recognized as a tumor-suppressor gene. Wild-type *p53* has been reported to be able to reduce the opportunity

TABLE 1. Genotype Frequencies of *p53* Codon 72 Between Healthy Control Subjects, HT and GD Patients

Genotype	HT patients[a] $n = 107$ (%)	GD patients[b] $n = 90$ (%)	Controls $n = 105$ (%)
Arg/Arg[c]	36 (33.7)	13 (14.4)	18 (17.1)
Arg/Pro	44 (41.1)	50 (55.6)	65 (61.9)
Pro/Pro	27 (25.2)	27 (30.0)	22 (21.0)

[a] $P = 0.005$, compared with controls (power = 0.85).
[b] $P = 0.344$, compared with controls.
[c] OR for Arg/Arg = 2.450 (95% CI = 1.274–4.716), compared with Arg/Pro and Pro/Pro combined, for HT patients.

TABLE 2. Allelic Frequencies of *p53* Codon 72 Between Healthy Control Subjects, HT and GD Patients

Allelic variant	HT patients[a] $n = 214$ (%)	GD patients[b] $n = 180$ (%)	Controls $n = 210$ (%)
Arg	116 (54.2)	76 (42.2)	101 (48.1)
Pro	98 (45.8)	104 (57.8)	109 (51.9)

[a] $P = 0.208$, compared with controls.
[b] $P = 0.245$, compared with controls.

TABLE 3. Genotype Frequencies of *p53* Codon 72 Between HT Patients With Euthyroidism (TSH < 5 mIU/L) or Hypothyroidism (TSH ≥ 5 mIU/L)

Genotype[a]	Euthyrodism $n = 53$ (%)	Hypothyroidism $n = 54$ (%)
Arg/Arg	20 (37.7)	22 (40.8)
Arg/Pro	20 (37.7)	18 (33.3)
Pro/Pro	13 (24.6)	14 (25.9)

[a] $P = 0.892$.

for certain tumor formation because its encoding protein, p53, is capable of activating either cell-cycle arrest or apoptosis in an attempt to prevent the proliferation of DNA-damaged cells. It would appear, however, that mutant variants of *p53* lose this "protective" ability and thus their appearance has been suggested to be associated with an increase in the incidence of various cancers for individuals featuring such mutant variants (9). The appearance of the *p53* polymorphism has been linked with an individual's susceptibility to thyroid cancer, as revealed by investigations of Boltze *et al.* (19) and Granja *et al.* (20), both groups having (separately) observed that the presence of proline homozygosity at codon 72 of *p53* increases the risk of susceptibility to, respectively, undifferentiated and differentiated thyroid cancers for involved individuals. Interestingly, we report here on a novel finding that *p53* is also related to the development of AITD, our data demonstrating that there appeared to be an increased frequency of presence of the Arg/Arg homozygous genotype for the *p53* codon 72 for HT patients as compared with corresponding data for normal controls ($P = 0.005$, power = 0.85). This suggests that the *53* codon 72 Arg/Pro polymorphism could represent a viable candidate genetic marker for the purpose of predicting the increased susceptibility to the development of HT.

HT and GD are both AITD, and undoubtedly, humoral as well as cell-mediated immunity play an important role in the development of both disorders (7,8). It should be recognized, however, that apoptosis also contributes to an individual's susceptibility to AITD, at least partially by way of regulating the maturation and control of the immune response mediated by both T and B lymphocytes (10,11). Further, apoptosis may also be directly involved in the pathogenesis of AITD. For example, upon histological examination, the thyroid glands of HT patients typically present a markedly increased level of apoptosis of thyrocytes, a scenario, however, which appears to be less significant for cases of GD (5,6). Apoptosis is a coordinated cycle of programmed events that results in cell death, the process of apoptosis being complex and involving multiple factors, including the *p53*-encoded protein, p53 (22). p53 is actually a transcriptional factor that activates apoptosis by upregulating the proapoptotic genes, such as *Bax*, and by downregulating the antiapoptotic genes, such as *Bcl-2* (21,23). Furthermore, p53 has also been implicated in the overexpression of a specific cell-death receptor, Fas, and this is believed to be an underlying cause of apoptotic thyrocyte destruction for sufferers of HT (24,25). Thus, p53 may contribute in some way to the development of AITD, especially HT.

p53 features an abundance of mutation, with more than a thousand variants having been identified among human tumor samples to date, indicating that the proper functioning of p53 is very sensitive to even slight changes in amino-acid sequence (9). The Pro (C<u>C</u>C)/Arg (C<u>G</u>C) polymorphism at codon 72 is a very common genetic feature of *p53* (26). Proline and arginine are two different types of amino acid featuring disparate physical and chemical characteristics (27). Proline is a hydrophobic amino acid that does not readily fit into an ordered secondary structure of a polypeptide and thus, in theory, reduces the ability of p53 to activate apoptosis (27). This may explain why porline's homozygosity increases the risk of an individual's susceptibility to the suite of thyroid cancers (19,20). By contrast, arginine contains a fully positively discharged side chain, which is able to form ionic bonds with other charged species within the cell and can therefore enhance the ability of p53 to activate apoptotic process (27). Such observation is quite compatible with our finding that, as compared with normal healthy controls, there exists an increased frequency of the Arg/Arg homozygous genotype among patients suffering from HT, a disease that features marked apoptosis. Unlike our finding for the HT patient group, we failed to observe any relationship between the *p53* codon 72 polymorphism and an individual's susceptibility to GD. This apparent discrepancy of results derived from our study may be attributable to the somewhat different degree of contribution by apoptosis to the genesis of these two disorders (5,6).

Our data demonstrate significant difference in the distribution for *p53* codon 72 genotypes, but not for allelic variants, between HT patients and normal controls. This statistical disagreement also appeared in the study for thyroid cancer (20) and is partly because of the relatively high appearance rate of heterozygous genotype in control groups. Because p53 is actually a tetramer and this structure is necessary to exert its biological activities (28), different *p53* genotypes will result in the production of p53 with different conformations and, in theory, consequently influence the activities of p53. To the best of our knowledge, the effect of *p53* codon 72 genotypes on the biological activities of p53 has not been conclusively studied yet. However, in an in vitro study using primary mouse fibroblasts model, Lowe and coworkers observed that homozygously wild and mutant *p53* and hemizygous *p53* displayed different degrees of effect to modulate the cytotoxicity of anticancer agents (29). Further, the genotype of *p53* codon 72 was also suggested to be a genetic marker for early diagnosis of lung cancer (30). In our research, we also investigated the effect of *p53* on clinical status of HT. However, we failed to observe significant difference in genotype distribution for *p53* codon 72 between HT patients with euthyroidism ($TSH < 5\,mIU/L$) or hypothyroidism ($TSH \geq 5\,mIU/L$). Further investigation is clearly needed to elucidate the effect of the polymorphism at *p53* codon 72 to the biological activities of p53.

Patients suffering from AITD are typically diagnosed subsequent to the development of symptoms, primarily owing to a lack of available predictive markers for early diagnosis of the disease. Logically, therefore, the determination of a reliable genetic marker for AITD should lead to earlier diagnosis of the malady. Our data indicate that the Arg/Pro polymorphism at the codon 72 of the *p53* may potentially be a candidate genetic marker for screening for an individual's susceptibility to certain AITD. As direct evidence for a clear association between the presence of p53 and an individual's AITD susceptibility would still appear to be lacking, further study is clearly necessary in this realm in order to elucidate the role of the *p53* polymorphism with respect to the pathogenesis of certain AITD.

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