PD1 Gene Promoter Polymorphism in Thymoma and Myasthenia Gravis

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INTRODUCTION

Myasthenia gravis (MG) is considered a phenotype for antibody-mediated neuromuscular disorder and autoimmune diseases, directed against the nicotinic acetylcholine receptor. Among MG patients, those with thymoma differ from the other groups by a lack of significant human leukocyte antigen (HLA) association, absence of sex preponderance, and a poor response to thymectomy [1]. There is also good evidence that paraneoplastic MG has a different pathogenesis from the common thymic lymphofollicular hyperplasia-associated MG [2]. MG [3] or thymoma is associated with the +49A/G single nucleotide polymorphism (SNP) of the cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) gene.

An imbalance of immune regulation affects tumor-specific T-cell immunity in the cancer microenvironment and reshapes tumor progression and metastasis [4]. The lack of immunostimulatory activation can be harmful if it impairs immune responses against cancer [5]. Many receptor-ligand interactions are known to trigger anti-apoptotic pathways that prevent activation-induced T-cell death [6,7]. Programmed death 1 (PD-1) protein, a T-cell coinhibitory receptor plays a central role in the ability of tumor cells to escape the host’s immune system. Blockade of interactions between PD-1 and PD-1 ligands enhances immune function in vitro and mediates antitumor activity in preclinical models [8,9]. Recent reports suggested that antibody-mediated blockade of PD-1 [10,11] induced durable tumor regression and prolonged stabilization of disease in some patients with advanced
cancers. The PD-1 molecule is a negative regulator of T cells. One of the SNP of PD-1 (PD1.3 G/A), a regulatory SNP located in intron 4, showed to be involved in susceptibility to SLE in Caucasian [12], however, Asian populations are not polymorphic at this SNP [13]. In addition, the PD-1 SNP statuses were not correlated with MG in Caucasians [14]. The polymorphism at PD-1 promoter was reported by Chinese, but vary rare in Caucasians [15]. The PD-1 promoter SNP statuses in tumors are not well investigated.

In this paper, we have investigated the PD-1 gene polymorphisms in Japanese thymoma with or without MG using real-time polymerase chain reaction (PCR) using TaqMan PCR in surgically treated cases. The findings were compared to the clinicopathologic features of thymoma/MG and PD-1 gene status.

### PATIENTS AND METHODS

**Patients**

The study group included thymoma patients (54.8±14.7 years old) and MG patients (38.4±18.9 years old) who had undergone surgery at the Department of Surgery, Nagoya City University Hospital between 1995 to 2013. All thymus tissue samples were immediately frozen and stored at -80 °C until assayed. Patient consent was obtained from the patients. The study was approved by the ethics committee of the University. The clinical and pathological characteristics of the 148 thymoma patients for PD-1 gene genotyping analyses were as follows; 65 (43.9%) were male 83 were female. 30(20.2%) were less than 40 years old. 40(27.0%) were with MG (male; 15, female; 25) and 84 were anti-acetylcholine receptor negative (male 43, female; 41). 49 (34.5%) were pathological stage I, 51 were stage II, 22 were stage III, and female (28.9%, p=0.5201). The GG ratio was not significantly different between male (30.8%) and female (26.5%, p=0.5680). The GG ratio was not different whether higher than 40 years old (27.1%) or lower than 40 years old (33.3%). The GG ratio was not correlated with pathological stages, if we compared stage I-III (20.4%) vs. IV (9.5%, p=0.1214), and GG was even smaller population in stage IV (Table 1). The PD-1 SNPs at promoter region were 10 AA, 17 GA and 5 GG (15.6%) in MG without thymoma patients. GG genotype was tendency towards lower when compared to the MG with thymoma patients (p=0.1003) (Table 2).

**PD-1** gene SNP status at intron 2 (rs 34819629) was very similar to the status for rs 36084323, and 93.9% identical. The PD-1 rs 34819629 SNPs were 35 AA, 67 GA and 46 GG in thymomas. GG genotype was not different between MG patients (35.0%), anti-acetylcholine antibody positive but not with MG patients (33.3%), and anti-acetylcholine antibody negative patients (28.5%). The GG ratio was not significantly different between male (33.8%) and female (28.9%, p=0.5201). The GG ratio was not different whether higher than 40 years old (29.7%) or lower than 40 years old (36.7%, p=0.4591). The GG ratio was tendency towards lower in pathological stages IV (8.7%) when compared to stage I-III (21%, p=0.0667) (Table 3). The PD-1 SNPs at intron 2 region were 10 AA, 16 GA and 6 GG (18.8%) in MG without thymoma patients. GG genotype was not significantly different with the MG with thymoma patients (p=0.1261).

**CTLA4** polymorphism status in Japanese thymoma and MG

**CTLA4** gene SNP status at exon1 (+49A/G, rs 231775) was 17 AA, 65 GA and 66 GG in thymomas. AA genotype was not different between MG patients (5%), anti-antibody positive but not with MG patients (20.8%), and anti-acetylcholine antibody negative patients (11.9%), and the ratio was lower in MG patients. The AA phenotype was significantly higher in male (20%) than in female (7.2%, p=0.0254). **CTLA-4** +49 SNP statuses was not different whether higher than 40 or lower than 40 (p=0.3190). **CTLA4** +49 SNPs was not correlated with pathological stages (I-III vs IV, p=0.8548) (Table 4). The **CTLA4** SNPs at +49 were 1 AA, 13 GA and 18 GG in MG without thymoma patients. AA genotype (31%) was tendency towards lower when compared to anti-
acetylcholine antibody negative thymoma patients (p=0.0928). Within 10 AA -606 PD-1 patients with non-thymomatous MG, 5 were GG at +49 CTLA4. Within 5 GG -606 PD-1 patients with non-thymomatous MG, no AA at +49 CTLA4. Thus, PD-1 and CTLA4 SNP was independent.

**DISCUSSION**

In this study, we focused on one of the programmed death 1, PD-1 gene SNP to know whether it might be a new molecular mechanism for thymoma. We have found that PD-1 gene SNP was tendency towards lower in MG patients (without thymoma) when correlated to MG with thymoma patients.

Human cancers harbor numerous genetic and epigenetic changes, generating neoantigens that are potentially recognizable by immune system [16]. Tumors develop multistep resistance systems, including local immuno-suppression, induction of tolerance, and systemic dysfunction in T-cell signaling [17-20]. In addition, tumors utilize several pathways to escape immune destruction. PD1 is a key immune-checkpoint receptor expressed by activated T-cells and mediates immuno-suppressions. Thus PD-1 might also act as a molecule target for tumor progression in cancers. In *in vitro*, inhibition of the interaction between PD-1 and PD-L1 could enhance T-cell responses and mediate preclinical antitumor activity [8, 9]. These observations made us our intensive efforts to develop immunotherapeutic approaches for cancer, including immune-checkpoint-pathway inhibitors such as anti-CTLA-4 antibody [21,22] and anti-PD-L1 therapy [11,12]. Anti-PD-1 antibody studies has been started in advances solid tumors [23]. The recent studies by Brahmer et al. [11] and Topalian et al. [12] have been reporting the safety and activity of anti-PD1 or PD-L1 immunotherapy in cancers. However, in our analysis, PD-1 or CTLA-4 polymorphism did not correlate with thymoma progression. PD-1 polymorphSNPs at thymoma patients were very similar to Asian healthy controls. These molecules might not have a role in thymoma itself.

The PD-1 belongs to the immunoglobulin receptor superfamily, encodes a 55-kd type 1 transmembrane inhibitory immunoreceptor, and is responsible for the negative regulation in T-cell activation and peripheral tolerance [24]. Expression of PD-1 was observed only in activated T and B cells and early lymphoid precursors [25]. Previous reports indicate that PD-1 is markedly upregulated on surface of exhausted virus-specific CD8+ T cells in mice with lymphocytic choriomeningitis virus infection [26], and in humans with human immunodeficiency virus (HIV) infection [27,28]. PD-1 -606G allele showed a significant association with Japanese salacutue sclerosing panencephalitis (SSPE) [29]. A haplotype having -606G allele with high promoter activity was associated with the development of SSPE [29]. Relative PD1 expression was higher in SSPE patients than in control [29]. PD-1 pathway might play a central role for the T cell dysfunction. Previous report demonstrated that -606G/A (previously called PD-1.1 at -531G/A) was associated with rheumatoid arthritis (RA) in Chinese [30]. However, this PD-1 SNP is rare in Europeans (1%) and Africans (4%) [15]. Other PD-1 SNP statuses were not significantly associated with MG in Sweden [14]. There is large variation in the frequencies of PD-1 SNP among different ethnic groups.

Studies on PD-1 deficient mice in different genetic backgrounds showed the development of lupus-like autoimmune diseases [30] and autoimmune cardio myopathy [31]. Various studies indicated that PD-1 gene SNP polymorphisms were associated with autoimmune diseases such as SLE [32], multiple sclerosis [33], rheumatoid arthritis (RA) [34] and type 1 diabetes [35], although most of the SNPs were Caucasian specific. Some reports on transcriptional levels have shown decreased

**Table 1:** Clinico-pathological data of 148 thymoma patients.

<table>
<thead>
<tr>
<th>Factors</th>
<th>No. of AA+GA</th>
<th>No. of GG</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>54.8±14.7</td>
<td>55.1±14.5</td>
<td>0.9078</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>35(33.7%)</td>
<td>14(33.3%)</td>
<td>I-III vs. IV 0.1212</td>
</tr>
<tr>
<td>II</td>
<td>33(31.7%)</td>
<td>18(42.9%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>15(14.4%)</td>
<td>7(16.7%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>21(20.2%)</td>
<td>4(9.5%)</td>
<td></td>
</tr>
<tr>
<td>MG+</td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>AchR Ab+</td>
<td>17(16.0%)</td>
<td>7(16.7%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>62(58.5%)</td>
<td>22(52.4%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>45(42.5%)</td>
<td>20(47.6%)</td>
<td>0.5680</td>
</tr>
<tr>
<td>Female</td>
<td>61(57.5%)</td>
<td>22(52.4%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40</td>
<td>20(18.9%)</td>
<td>10(23.8%)</td>
<td>0.5002</td>
</tr>
<tr>
<td>&gt;40</td>
<td>86(81.1%)</td>
<td>32(76.2%)</td>
<td></td>
</tr>
</tbody>
</table>

* MG: Myasthenia Gravis; AchR: Acetylcholine Receptor; Ab: antibody. N.S.: not significant
expression in Japanese DM 1 patients [36]. We have found the tendency towards lower GG phenotype at PD-1 promoter in MG without thymoma when compared to MG with thymoma patients. Thus PD1 low activity might favor the development of non-thymomatous MG. We could not demonstrate any significant association of the PD-1 gene SNP to MG, the possible reason could be sample size, fewer number of SNPs analyzed.

CTLA4 is a receptor mainly displayed on activated T-cells. CTLA4 plays a critical role in down regulating immune responses. Mice who lack the CTLA4 gene develop a lethal phenotype with massive T-cell activation and T-cell infiltrates in virtually all organs [37]. SNP of the CTLA4 gene. +49A/G in exon 1 has been shown to affect gene expression [38]. The frequency of allele G and genotype G/G at position +49 was increased in MG thymoma patients than healthy controls in Sweden [3]. In contrast, +49 A/A genotype were reported to be higher in MG thymomas than non-MG thymomas from German [1]. Our results were similar to the results from Wang et al. [3]. There might be also a large variation in the frequencies of CTLA4 SNP among different ethnic groups. AA might be lower in Asian [40]. Our AA ratio in thymomas is very similar to the previous Asian report [40].

In summary, PD-1 might have no role in thymomas. However, lower GG phenotype at promoter region of PD-1, as well as lower AA phenotype at CTLA4 +49 provided a candidate of its function as the autoimmune process. PD1 or CTLA4 low activity might favor the development of non-thymomatous MG. Larger cohort may be needed to determine the exact role of PD-1 and CTLA4 in MG.

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