Abstract To investigate the precise modality of association between SAA1 gene polymorphisms and the development of AA amyloidosis in patients with rheumatoid arthritis (RA), Japanese patients with RA (n = 153), among whom 29 were histologically diagnosed as having amyloidosis, were genotyped for three single nucleotide polymorphisms (SNPs), C-13T, C2995T, and C3010T, in the SAA gene. Pairwise linkage disequilibrium coefficients between each pair of SNPs were calculated and estimated haplotype frequencies were compared between patients with and without amyloidosis. Possible associations between these SNPs and amyloidosis were analyzed by a case–control study and by the Kaplan–Meier method, in which the endpoint was defined as the time of diagnosis of AA amyloidosis. The -13T and 2995C alleles, which were in a tight linkage disequilibrium, were more frequent in the patients with amyloidosis, and the groups with the -13TT and 2995CC genotype had worse survival curves than patients without these genotypes, whereas C3010T was not associated with amyloidosis. Moreover, the haplotype containing -13C and 2995T was found to be protective. Both C-13T and C2995T were associated with the development of amyloidosis. Examining both polymorphisms may be more useful than examining only one of them for estimating the risk of the development of amyloidosis.

Key words Amyloidosis · Genetic polymorphism · Rheumatoid arthritis (RA) · SAA1 · Single nucleotide polymorphism (SNP)

Introduction

AA amyloidosis is a major complication of chronic inflammatory diseases, such as rheumatoid arthritis (RA), which is responsible for about 60% of the cases of this severe complication. Although this complication is generally associated with severe, longstanding, and sustained inflammation, the incidence of AA amyloidosis varies among different ethnic groups. In Japan, AA amyloid deposition has been reported to be found in the gastric mucosa in 13.3% of RA patients by endoscopic biopsy and in more than 20% of RA patients in autopsy series, whereas among Caucasians in the United States, RA patients with AA amyloidosis are estimated to comprise less than 3% of RA patients. The difference in the frequency of AA amyloidosis among different races, and the fact that AA amyloidosis is not consistently related to the length and severity of chronic inflammation suggests that AA amyloidosis may be, at least in part, influenced by genetic factors.

AA amyloid deposits are largely made of polypeptides, which constitute about the amino terminal three-fourths of the serum amyloid A (SAA) protein. Among the human SAA family, SAA1 and SAA2 are well known as acute phase proteins, which are synthesized by the hepatocytes in response to inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF), and transported into the serum by the high-density lipoprotein (HDL) particles. The vast majority of the human AA proteins isolated from amyloid deposits are derived from SAA1. Although the relationship between the production of fibril precursor protein, the turnover of amyloid, and amyloidotic organ function is complex, it has been proved that outcome is favorable in AA amyloidosis when the SAA concentration is maintained below 10 mg/l.

There is an increasing body of evidence showing that polymorphisms in SAA1 gene participate in the development of AA amyloidosis. The presence of two single-nucleotide polymorphisms (SNPs), C2995T and C3010T, within exon 3 of the SAA1 gene define three haplotypes that correspond to the SAA1.1 (2995T-3010C), SAA1.2 (2995T-3010T), and SAA1.3 (2995C-3010T) haplotypes. The association between these polymorphisms and the risk of AA amyloidosis in Japanese patients with RA was investigated in this study.
(2995C-3010T), and SAA1.3 (2995C-3010C) alleles.\textsuperscript{9–11} As indicated in Fig. 1, both SNPs are accompanied by amino acid substitutions. In Japanese RA patients, SAA1.3 has been reported to be associated with increased risk of AA amyloidosis and SAA1.1 with decreased risk.\textsuperscript{12,13} While SAA1.1 was shown to be a risk factor for developing AA amyloidosis in a Caucasian population,\textsuperscript{14} An adequate explanation of this discrepancy has not been provided.

A more recent report evaluated another single nucleotide polymorphism (SNP) in SAA1, T-13C, and in that study the allele of SAA–13T was shown to be a risk factor for AA amyloidosis in both Japanese and Caucasian populations.\textsuperscript{15} However, little information has been available regarding the linkage disequilibrium between these polymorphisms of SAA1, and their impacts on the onset of AA amyloidosis have not been fully investigated. In addition, previous reports of association analysis between SAA1 polymorphism and the prevalence of AA amyloidosis in RA patients have been largely reports of case control studies, which did not take chronological factors into account in the analysis. In this study, we examined the three SNPs described above, a SNP upstream of exon 1 and two SNPs of exon 3, regarding their impact on the onset of AA amyloidosis using a case–control and time–to–event analysis. Furthermore, we evaluated the linkage disequilibrium between each pair of polymorphisms in Japanese patients with RA.

**Patients and methods**

**Study subjects**

The ethics committee of the Niigata University Graduate School of Medical and Dental Sciences approved the protocol for the genetic study. Written informed consent was obtained from all DNA donors. The study included 153 Japanese patients with RA. All patients fulfilled the 1987 revised criteria for the classification of RA by the American College of Rheumatology.\textsuperscript{16} In each patient, vigorous efforts were made to suppress the inflammatory activity as completely as possible: corticosteroids and/or other disease-modifying drugs were given at the discretion of each patient’s physician based on the standard protocol. Among 153 patients, 29 were diagnosed as having AA amyloidosis (Table 1). All patients with AA amyloidosis were identified by examining biopsy specimens, including gastro-duodenal ($n = 24$), rectal ($n = 1$) endoscopic biopsy, renal biopsy ($n = 3$), and abdominal fat biopsy ($n = 1$) specimens, and the sections were subjected to Congo red and immunohistochemical staining. In most cases, a work-up for amyloidosis was conducted because of renal injury manifested by either proteinuria, elevation of serum creatinine, or hematuria. Gastrointestinal symptoms such as diarrhea lasting more than 7 days that failed to respond to standard therapy were another reason to investigate for amyloidosis in some other cases. The mean age of the patients with AA amyloidosis was 65.5 years and their duration of RA ranged from 9.0 to 43.2 years with a mean of 20.4 years. The patients without amyloidosis ($n = 124$, 24 male), had a mean age of 61.8 years and their duration of RA ranged from 1.8 to 59.6 years with a mean of 12.3 years, and did not have any clinical symptoms associated with amyloidosis, such as refractory diarrhea, proteinuria, renal dysfunction, or nephrotic syndrome.

**Determination of SAA genotypes**

Genomic DNA from the peripheral blood cells was isolated with an automatic DNA isolation system (NA-1000; Kurabo, Osaka, Japan). The genotype of the SAA1 C-13T in the 5'-region of exon 1 was determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. The primers used for the PCR reaction were 5'-ACA TCT TGT TCC AGG TTG-3' (sense) and 5'-GCT GTA GCT GAG CTG CGG-3' (antisense).