**Article**

# Laboratory Characterizations on 2007 Cases of Monoclonal Gammopathies in East China

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**Monoclonal gammopathies are characterized by the presence of monoclonal immunoglobulin in patients with or without evidence of multiple myeloma (MM), macroglobulinemia, amyloidosis (AL), or a related plasma cell proliferative disorder. This study aims to evaluate laboratory diagnostic characters of monoclonal gammopathies and investigates the correlation between monoclonal gammopathies and transforming growth factor β1 (TGFβ1). Immunofixation electrophoresis (IFE), serum protein electrophoresis (SPE), nephelometry and urine light chain ELISA were used for laboratory identification of monoclonal immunoglobulins. Plasma TGFβ1 was detected with double-antibodies ELISA. Lightcycler was used for single nucleotide polymorphism (SNP) analysis. Totally 2,007 cases of monoclonal immunoglobulin (M protein) were identified in 10,682 samples. The isotypes of M protein were IgG type 47.1%, IgA 23.0%, IgM 8.7%, IgD 5.3%, free light chain κ 6.1%, λ 9.8%. In reference to IFE, the coherency of diagnosis was serum light chain ratio (κ/λ) 94.4%, quantitation of Igs 83%, light chain quantitation 80.9%, and urine light chain ratio (κ/λ) 58.0%. Plasma TGFβ1 was elevated significantly compared to normal control. The allelic frequency of codon 10 (C > T) was neither associated with the existence of the M protein nor with the M protein isotype. Monoclonal gammopathies can be identified with the combination of IFE, SPE, Igs quantitaion and urine light chain determination. Although TGFβ1, an important cytokine in immune regulation, was elevated in monoclonal gammopathies, the SNPs in coding region of TGFβ1 gene did not confer susceptibility to the development of monoclonal gammopathies in this study. *Cellular & Molecular Immunology*. 2008;5(4): 293-298.**

**Key Words:** monoclonal gammopathies, laboratory diagnosis, TGFβ1, SNP

## Introduction

Monoclonal gammopathies are characterized by the presence of monoclonal immunoglobulin (M protein, paraprotein, or called myeloma protein) in patients with or without evidence of multiple myeloma (MM), macroglobulinemia, amyloidosis (AL), or a related plasma cell proliferative disorder (1). This group of diseases is most commonly seen in elderly patients, with the average incidence age above 50 (1). With the improvement of laboratory diagnostic techniques, availability of medical facilities as well as the increasing number of elderly generation, the incidence of this group of diseases seems to be increased significantly these years in China. Now the group of diseases (monoclonal gammopathies) is generally considered as heterogenesis with different prognosis, clinical course and response to therapeutic interventions in different subjects (2). But the etiology of this group of diseases remains unknown. Several cytokines were reported to have pathological effect on the pathogenesis of the diseases. IL-6, TNF-α, IL-1 as well as transforming growth factor β1 (TGFβ1) were among these cytokines, which might be correlated with the disease severity, prognosis and predisposition (3-5). Biological studies supported a role for aberrant class switch recombination early in the natural history of the disease group suggesting that factors both in genetic and the environment may interact with this mechanism (6). Recent studies revealed that the genetic predisposing factors involved in microsatellite polymorphism (7), single nucleotide polymorphism (SNP) (8, 9), methylation (10), mutation as well as deletion (11). Various cytokines such as IL-6, TNF-α, IL-1β and immune regulatory molecules like CTLA-4 (cytotoxic T-lymphocyte antigen-4)

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as well as proto-oncogene such as p53, p16, nuclear factor IκB, etc., are reported to be the predisposing factors (11-15). In order to investigate the laboratory diagnostic characters of monoclonal gammopathies in large scale in China as well as to analyze the putative genetic predisposing factors, here in this study, we studied the immunological features of 2,007 cases of monoclonal gammopathies from 10,682 suspected samples for laboratory diagnostic confirmation or exclusion with serum protein electrophoresis (SPE), immunofixation electrophoresis (IFE), immunoglobulin (Ig) quantitation (rate nephelometry) as well as urine free light chain ELISA. TGFβ1, the important immune inhibitory cytokine (16), was probed for its association of expression, SNPs with monoclonal gammopathies.

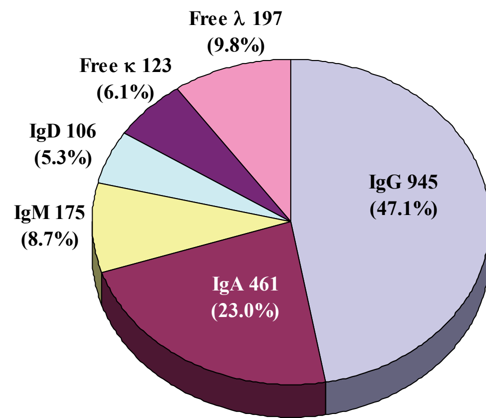


**Figure 1. Distribution of serum M protein in 2,007 positive cases identified from 10,682 samples.** Totally 2,007 cases of positive M protein were identified from 10,682 suspected samples for diagnostic confirmation or exclusion as required by clinicians with the combination of diagnostic techniques including IFE, SPE, rate nephelometry for Ig quantitation and ELISA for urine free light chain measurement. The percentage indicates the subtype distribution among all M protein positive cases.

## Materials and Methods

*Sample collection and laboratory study*

Totally 10,682 candidate samples for M protein analysis were collected from patients in Shanghai and neighboring East China region in the past 10 years. Identification of M protein was required by clinical doctors for all these samples in order to exclude or confirm the diagnosis.

Serum Igs and light chains (κ, λ) were measured with rate nephelometry on Beckman Array 360. SPE and IFE were run with REPII and SPIFE 2000 (Helena Laboratories, USA), respectively. M-spike was quantitated after band scanning and EZ1.51 (Helena Laboratories, USA) software analysis.

Urine light chain was determined with ELISA using rabbit anti-human κ or λ antibody (DAKO) for detection antibody. Qualitative result was obtained according to the ratio of κ/λ in reference to negative and positive control.

The diagnosis of M protein and consensus isotype was based on IFE, SPE, Igs as well as light chain measurement. Among these methods, SPE was used for screening test, while IFE was for confirmation and isotype identification. Measurement of Igs and light chain were used for M protein quantitation and confirmation that was complementary to IFE and SPE.

*Determination of TGFβ1 in sample plasma*

Totally 146 cases of EDTA-$Na_2$ anti-coagulated whole blood were collected and centrifuged at 3,000 g for 15 min, platelet-poor plasma was collected and acidified for 1 h in ice and then neutralized to pH 7.0. Double antibodies sandwich ELISA was applied to determine TGFβ1 in sample plasma according to manufacturer's instruction (Jingmei Biotech, Shanghai, China). One hundred and sixty-four ethnically matched unrelated healthy controls were randomly recruited from the healthy blood donors in Shanghai, China.

*TGFβ1 gene SNP analysis in lightcycler system*

Sequence variants at codons 10, 25 and 263 of human TGFβ1 gene (TGFβ1) were determined in an LC-system (Roche, Mannheim, Germany). Genomic DNA was extracted from peripheral blood cells using the QIAamp DNA Blood Mini extraction kit (Qiagen, Hilden, Germany). The primers, cycling conditions, and melting curve analysis were in referring to our previous publication (17). The fluorescence-labeled primers were obtained from Metabion (Metabion, Martinsried, Germany), and the common PCR-primers were synthesized at MWG-Biotech AG (Ebersberg, Germany).

*Sequence analysis*

Representative amplification products were purified by gel electrophoresis and sequenced on the ABI PRISM 310 DNA sequencer served by Genecore Biotech, Shanghai, China.

*Gene frequency calculation and statistical analysis*

Student's *t* test was used for the statistical analysis of laboratory parameters, especially the distribution and subtype of M protein. The allele frequencies of TGFβ1 gene were calculated assuming Hardy-Weinberg equilibrium. The consistency of genotype frequencies was visualized with help of the triangular de Finetti diagram (31, Elston and Forthofer 1977), employing software developed by Wienker and Strom (http://www.ihg.gsf.de/ihg/snps.html).

## Results

*General features of M protein*

Totally 2,007 cases of positive M protein were identified from 10,682 samples for diagnostic confirmation or exclusion as required by clinicians. The positive rate was 18.8%. The ratio of male to female in positive cases was about 3:2. The average age for these positive cases was 62.1 ± 12.0 with the youngest being 18 and the eldest being 93. For subtypes of these monoclonal gammopathies, type of intact monoclonal Ig molecules was account for 84.1%.

**Table 1.** Case distribution of M-spike position in SPE in different types of M protein

| M protein type | M-spike position in SPE (n) | | | | | |
|---|---|---|---|---|---|---|
| | $\alpha_2$ | β | Fast γ | Medium γ | Slow γ | Total |
| IgG | 0 | 54 | 235 | 253 | 324 | 866 |
| IgA | 0 | 245 | 164 | 16 | 0 | 425 |
| IgM | 0 | 0 | 40 | 105 | 18 | 163 |
| IgD | 0 | 0 | 65 | 0 | 0 | 65 |
| Free κ | 0 | 56 | 23 | 9 | 23 | 111 |
| Free λ | 10 | 59 | 79 | 29 | 0 | 177 |
| Total | 10 | 414 | 626 | 402 | 355 | 1807 |

Among the intact Ig M protein, subtype IgG was 945/2007 (47.1%), IgA was 461/2007 (23.0%), IgM was 175/2007 (8.7%), and IgD was 106/2007 (5.3%). Type of free light chain monoclonal gammopathies was account for 15.9% (Figure 1). In all positive cases, 1,807 cases (90%) showed M-spike in SPE, for most cases, the position of the M-spike was mainly in γ (fast, medium, slow) region with exception of that in IgA type, in which the M-spike was partially (245/425, 57.6%) seen in β region. For type of free light chain, half of the free κ chain located in β region. None except 10 cases of free λ chain migrated in $\alpha_2$ region (Table 1).

*Quantitative analysis of M proteins*

All the M proteins, though different in Ig isotypes, were elevated to different degree upon recognition compared to normal reference values ($p < 0.01$, Table 2). The non-affected isotypes of Igs were decreased, most evidently in positive IgD or IgA type, or kept in normal range such as in some positive IgM and IgG types. In cases of free light chain types, all the non-involved Ig levels were decreased significantly ($p < 0.01$). The quantitation results of light chain in both type of intact Ig molecules or free light chain type were shown in Table 3, indicating that abnormal κ/λ ratio is an important laboratory feature in monoclonal gammopathies.

**Table 2.** Serum Ig quantitation in monoclonal gammopathies

| M protein isotype | n | IgG (g/L) | IgA (g/L) | IgM (g/L) |
|---|---|---|---|---|
| IgG | 945 | 47.36 ± 31.73* | 1.24 ± 0.85 | 1.29 ± 0.72 |
| IgA | 461 | 5.64 ± 3.52# | 39.56 ± 28.45* | 0.52 ± 0.43 |
| IgM | 175 | 11.65 ± 7.24 | 1.83 ± 1.54 | 29.62 ± 10.25* |
| IgD | 106 | 5.32 ± 1.94# | 0.47 ± 0.36# | 0.30 ± 0.18# |
| Free κ | 123 | 6.64 ± 3.51# | 0.75 ± 0.23# | 0.41 ± 0.13# |
| Free λ | 197 | 6.68 ± 3.97# | 0.55 ± 0.45# | 0.38 ± 0.36# |

The reference values for various Igs were: IgG, 8.00~15.00 g/L; IgA, 0.85~3.00 g/L; IgM, 0.50~2.50 g/L. *Statistically higher than reference values, $p < 0.01$; #significantly lower than reference values, $p < 0.01$.

**Table 3.** Light chain determination in different types of monoclonal gammopathies

| M protein type | | n | κ (g/L) | λ (g/L) | κ/λ |
|---|---|---|---|---|---|
| IgG | κ | 506 | 48.72 ± 43.75* | 1.67 ± 2.91 | 94.42 ± 91.00* |
| | λ | 439 | 3.27 ± 3.03 | 34.69 ± 34.06* | 0.31 ± 0.43# |
| IgA | κ | 223 | 32.22 ± 27.62* | 1.81 ± 1.52 | 32.43 ± 30.91* |
| | λ | 238 | 3.64 ± 2.78 | 18.12 ± 4.21* | 0.45 ± 0.46# |
| IgM | κ | 122 | 25.61 ± 21.04* | 3.66 ± 3.08 | 13.82 ± 16.44* |
| | λ | 53 | 8.98 ± 8.04 | 16.79 ± 10.73* | 0.73 ± 0.0.61# |
| IgD | κ | 10 | 9.89 ± 8.46* | 1.41 ± 0.78 | 7.17 ± 7.21* |
| | λ | 96 | 2.84 ± 1.85 | 9.73 ± 8.16* | 0.56 ± 0.46# |
| Free | κ | 123 | 12.51 ± 12.90 | 1.74 ± 1.19# | 8.48 ± 8.90* |
| | λ | 197 | 3.83 ± 2.58# | 12.59 ± 12.46* | 0.64 ± 0.54# |

Reference values: κ, 5.74~12.76 g/L; λ, 2.69~6.38 g/L; κ/λ, 1.47~2.9. *Statistically higher than reference values, $p < 0.01$; #Significantly lower than reference values, $p < 0.01$.

*The coherency of IFE with SPE, serum Ig or light chain measurement and urine light chain assay in diagnosis*

Taking IFE as the confirmation experiment for M protein and then comparing IFE with the other laboratory methods, we got the coherency of these method for diagnosis rank from high to low as follows: light chain assay ratio (κ/λ, 94.4%), quantitation of Igs (83%), serum light chain quantitation (80.9%), and urine light chain ratio (κ/λ) (993/1714, 58.0%).

*TGFβ1 genotyping and plasma TGFβ1 determination*

No genetic polymorphism at codon 25 and codon 263 was found in monoclonal gammopathies or normal control, but in codon 10 there existed three genotyping forms such as CC, CT and TT. The gene frequency for TGFβ1 codon 10CT was the highest among the three genotyping in all diseased cases and normal control (Tables 4 and 5). Although no significant differences of the genotype frequencies were observed between individuals in control group and patients suffering

**Table 4.** Genotypes of TGFβ1 gene codon 10 in different types of M protein

| Genotype | Control | IgG | IgA | IgM | IgD | Free light chain | Diseased total |
|---|---|---|---|---|---|---|---|
| Codon 10TT | 41 | 19 | 5 | 2 | 2 | 1 | 29 |
| Codon 10CT | 83 | 51 | 9 | 3 | 6 | 14 | 83 |
| Codon 10CC | 40 | 18 | 8 | 0 | 2 | 6 | 34 |
| Total | 164 | 88 | 22 | 5 | 10 | 21 | 146 |

Totally 146 cases of monoclonal gammopathies and 164 normal controls were included in the TGFβ1 gene codon 10 polymorphism analysis. The number listed here was classified according to both TGFβ1 codon 10 genotyping and M protein subtype. No significant correlation between TGFβ1 codon 10 genotyping and incidence/subtype of monoclonal gammopathies was found.

**Table 5.** Genotype and allelic frequencies of TGFβ1 gene codon 10 in different types of light chain involved in monoclonal gammopathies

| Genotype and allelic frequencies | Control (n = 164) | Monoclonal gammopaties (n = 146) | | |
|---|---|---|---|---|
| | | κ | λ | Total |
| Codon 10 T > C | | | | |
| Genotype CC | 40 (0.244) | 18 (0.225) | 16 (0.242) | 34 (0.233) |
| CT | 83 (0.506) | 45 (0.563) | 38 (0.576) | 83 (0.568) |
| TT | 41 (0.250) | 17 (0.212) | 12 (0.182) | 29 (0.199) |
| Allelic frequencies | | | | |
| C | 163 (0.497) | 81 (0.506) | 70 (0.530) | 151 (0.517) |
| T | 165 (0.503) | 79 (0.494) | 62 (0.470) | 141 (0.483) |

Totally 146 cases of monoclonal gammopathies and 164 normal controls were included in the TGFβ1 gene codon 10 polymorphism analysis. The number and percentage in parenthesis listed here were classified according to both TGFβ1 codon 10 genotyping and M protein light chain subtype. No significant correlation between TGFβ1 codon 10 genotyping or allelic frequencies and incidence/subtype of light chain in monoclonal gammopathies was found.

**Table 6.** Plasma TGFβ1 level and TGFβ1 codon 10 polymorphism

| Genotype | Control | | Monoclonal gammopathies | |
|---|---|---|---|---|
| | n | TGFβ1 (ng/ml) | n | TGFβ1 (ng/ml) |
| Codon 10TT | 41 | 12.5 ± 10.6 | 29 | 19.1 ± 12.2* |
| Codon 10CT | 83 | 11.0 ± 8.1 | 83 | 16.3 ± 9.9# |
| Codon 10CC | 40 | 9.4 ± 5.2 | 34 | 14.5 ± 6.71# |
| Total | 164 | 10.9 ± 6.1 | 146 | 16.3 ± 10.5# |

Plasma TGFβ1 was measured with double antibodies sandwich ELISA. The detection result of TGFβ1 was listed here according to the different genotyping of TGFβ1. Plasma TGFβ1 in diseased group was higher than that in consensus control. No difference of plasma TGFβ1 was observed among different TGFβ1 genotyping both in diseased and control groups. *Statistically higher than consensus control ($p < 0.05$); #Statistically higher than consensus control ($p < 0.01$).

from monoclonal gammopathies, there was a slightly, but not significant increase of the codon 10CT in monoclonal gammopathies compared to normal control (83/146 *vs* 83/164).

Plasma TGFβ1 level was elevated in diseased group compared to normal control (Table 6). If the diseased group was further subdivided according to the three genotypes of TGFβ1 (TT, TC, CC), the elevated TGFβ1 levels were still observed compared to the control. The summarized TGFβ1 genotyping data and TGFβ1 level both in monoclonal gammopathies as well as in normal control are presented in Table 6. Associating the genotyping with plasma TGFβ1 concentration, the highest level of TGFβ1 appeared in codon 10TT both in diseased and control groups. But no significant correlation was found between TGFβ1 level and its genotyping in both groups (Table 6).

## Discussion

The monoclonal gammopathies or called dysproteinemias, paraproteinemias are a group of disorders characterized by the proliferation of a single clone of plasma cells that produces an immunologically homogeneous monoclonal protein, called M protein or paraprotein (18). Each M protein consists of two heavy polypeptide chains of the same class, designated γ in IgG, α in IgA, μ in IgM, δ in IgD and ε in IgE, and two associated light chains of the same type (κ or λ) (1). Monoclonal gammopathies may be found in malignant, benign, or transient disorders. The malignant processes include multiple myeloma, Waldenstrom's macroglobulinemia, the heavy-chain diseases, and primary amyloidosis. Occasionally, M protein is associated with malignant epithelial tumors (lungs, gastrointestinal tract, and genitourinary tract) and other diseases (1). One third of M protein positive cases demonstrated a monoclonal Ig in the absence of a progressive disease, called monoclonal gammopathy of undetermined significance (MGUS) (19).

Though epidemiologically, monoclonal gammopathies are uncommonly seen in China and Asia, with the improvement of diagnostic methods as well as availability of medical facilities to elderly patients, the incidence of this group of diseases seems to increase these years. In our lab, we have collected 10,682 cases of suspected samples from East China region during the past 10 years, and the M protein positive rate for these samples was 18.8% (2,007/10,682). The median age for these positive cases was about 62, which was exactly the same as that reported by the Mayo Clinic 13 years ago (1). As to the subtype and the molecular structure of the M protein, the case distribution of M protein is following the normal Ig concentration from high to low: IgG > IgA > IgM. But M protein of free light chain type is as high as 15%, which is much higher than that reported by Mayo Clinic in 1992 (1). For the type of free light chain, free λ M protein was higher than that of κ (9.8% versus 6.1%), which is contradictory to the normal situation that there are twice as many κ as λ-producing plasma cells in human. Our result was in accordance with the report years ago by Bradwell (2001) that approximately twice the concentration of serum λ levels appeared compared to that of κ (20). The explanation for the higher incidence of λ free light might be: 1) κ molecules usually are monomeric (25 kDa), while λ molecules trend to form dimeric structure, thus renal clearance is faster for κ than for λ (20); 2) dimeric structure of λ free light chain may lead to overestimated antigen concentration due to multi-antigenic targets in immunoprecipitation quantitation (21). The definite mechanism for the reversed κ/λ free light chain M protein incidence remains to be elucidated further. Generally, the un-affected Ig levels will be decreased in M-protein positive cases when diagnosed, while the quantitation of unrelated Igs can be in the normal limits. This might reflect the degree of bone

marrow involvement, and the former situation might indicate the strong involvement of bone marrow which causes the paucity in the development of normal B lymphocytes.

SPE is regarded as the screening test for monoclonal gammopathies (18). M-spike in SPE is the characteristic change of the diseases group. In our study, M spike in SPE existed in 1807/2007 (90%) cases, but immunofixation electrophoresis is necessary for the confirmation as well as isotype identification. IFE is approximately 10-fold more sensitive than SPE according to the published data (18). Free light chain in urine called Bence-Jones protein is the first tumor marker described more than 150 years ago (21). Urine free light chain can be detected with urine electrophoresis, nephelometry quantitation. In this study, we used ELISA to detect the whole κ/λ ratio of the suspected samples for cost-effective reason, among 2,007 positive cases, 1,714 cases provided urine sample together with serum sample, abnormal urine light chain κ/λ ratio was found in 992 cases (992/1714, 58%). In reference to IFE, the diagnostic coherency from high to low in our study is serum light chain ratio (κ/λ) > quantitation of Igs > light chain quantitation > urine light chain ratio (κ/λ). Since the amounts of light chain, especially free light chain, entering the urine are usually strongly influenced by renal tubular function (22), this might lower the sensitivity of urine light chain assay in diagnosis. On the other hand, urine light chain detection with ELISA, though cost-saving, was time-consuming and less sensitive. Now we have switched the urine free light chain qualitative detection to quantitative nephelometry, which seems more sensitive for recognition in parallel study (data not shown). Although IFE was regarded as confirmation experiment in this study and was more sensitive than SPE, it was less sensitive to detect small amounts of monoclonal free light chain according to the report from Katzmann (2002) (22), the dimeric or polymeric structure of free λ chain might smear the sharp monoclonal band and then caused false-negative in IFE (22, 23). So combination of all the above detection methods is necessary to improve the identification of M protein (18, 20, 22, 23).

Though monoclonal gammopathies have been recognized for several decades, the etiology of monoclonal gammopathies is unknown until now. A better understanding of any defects in the immune response in these patients is required before effective therapeutic strategies can be developed (24). Several studies supported that myeloma tumor cells in MM, the most common disease type in monoclonal gammopathies, evaded the immune system and could induce immunosuppression by producing immunoregulatory factors such as TGFβ1 (25). Myeloma cells increase Th3 cytokine response by secreting TGFβ1, which causes defective Th1 and Th2 cytokine responses, therefore a significant suppression of the immune system is seen (26). TGFβ1 is present in the bone marrow (BM) environment and constitutes a pivotal molecule controlling BM cell proliferation and differentiation (27). Study in MM showed that IL-2 and IL-4 levels in patients were lower than those in the normal controls, whereas TGFβ1 level were higher than that in controls (3-5). TGFβ1 stimulates IL-6 transcripts in a time- and dose-dependent manner in the *in vitro* study (28). Some studies revealed that a strong correlation was found between TGFβ1 serum levels and immunoparesis in MM patients (5, 29). Levels of TGFβ1 were correlated with the phase and stage of the diseases as well as the most important clinical and laboratory parameters associated with diseases activity. In our study we confirmed that TGFβ1 level was significantly higher than that in control, which is similar to the most reported studies.

Based on the elevated plasma TGFβ1 level in monoclonal gammopathies in this study and other related etiological studies (30-34), we further probed the correlation between genetic structure of TGFβ1 gene and monoclonal gammopathies, and three SNPs of TGFβ1 gene at the coding region were studied. Both subjects (diseased or normal) showed the G-allele at codon 25 encoding Arg. This finding along with the recent demonstration about the variability at this site is also absent in populations from Korea and Japan (35, 36) suggests that this polymorphism is generally not found in populations of Asian ancestry. Further, we also observed no genetic alteration at position 263 in all subjects. The allelic frequency of codon 10 (C > T) was neither associated with the existence of M protein nor with the M protein subtype and TGFβ1 concentration. But only 146 cases of positive M protein were included in the study, investigation on large scale collaboration is required for further conclusion.

In summary, we identified 2,007 cases of M protein in 10,682 suspected samples from East China region with Ig nephelometry, SPE, IEF and urine light chain ELISA. Though TGFβ1, an important cytokine in immune regulation was elevated in monoclonal gammopathies, the SNPs in coding region of TGFβ1 gene did not confer susceptibility to the development of monoclonal gammopathies in this study. With the improvement of diagnostic methods, the availability for medical facilities as well as the prolongation of lifespan, we believe that monoclonal gammopathies, a group of diseases prevalent in elderly population, though not so common in China and the other Asia region, will be increased in future. Further elucidation of genetic, environmental predisposing factors and thus better application in the clinical diagnosis and treatment will be of significant effects on improvement of life quality for the elder generation.

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