Correlation between ERα gene polymorphism and multiple sclerosis and neuromyelitis optica

Weifang Xing, MMed⁴, Mingfan Hong, MMed⁵, Zhisheng Wei, MD⁶, Wensheng Zhang, MMed⁴,*

Abstract

Objective: To study the polymorphism distribution of estrogen receptor (ER) α gene and the correlation between different types of polymorphism in multiple sclerosis (MS) and neuromyelitis optica (NMO) patients.

Methods: Forty-six cases of MS and NMO diagnosed from June 2018 to December 2019 were collected. Peripheral venous blood samples were collected. The patient's gender, age of onset, course of disease, and other clinical data were recorded. Fifty-eight healthy volunteers of the same age and sex were selected. By means of Pvu II and Xba I restriction fragment length polymorphism enzyme recognition sites of ER α gene, polymerase chain reaction-restriction fragment length polymorphism analysis was conducted.

Results: There was no significant difference in the frequency distribution of ER α gene’s PP, Pp, and pp genotype between MS and NMO case group and control group (P = .598). Frequency distribution of ER α gene’s XX, Xx, and xx was statistically significant between MS and NMO case group and control group (P = .021). Among them, distribution of Xx and Xx genotype frequency between patient group and the control group was statistically significant (P = .001, OR = 4.622, 95% CI: 1.803–11.852). There was no significant correlation between ER α genotypes and the onset age in patient group (P > .05). The difference was statistically significant in disease duration of XX and Xx genotype (P = .006). The comparison of Xx and xx genotype frequency distribution in gender exists a difference (P = .047, OR = 7.500, 95% CI: 1.023–54.996).

Conclusions: Xba I gene polymorphisms in the ER α gene have correlation with MS and NMO. Xba I gene could be a risk factor of MS and NMO pathogenesis, especially the women with Xx genotype are more vulnerable. Xba I gene polymorphisms in the ER α gene may impact the disease duration of MS and NMO, or rather, the disease duration of Xx genotype persists longer than Xx genotype. Pvu II gene polymorphisms in the ER α gene has no correlation with MS and NMO.

Abbreviations: ER = estrogen receptor, MS = multiple sclerosis, NMO = neuromyelitis optica, OD = optical density, PCR = polymerase chain reaction, SNP = single nucleotide polymorphism.

Keywords: correlation, estrogen receptor α gene polymorphism, multiple sclerosis, neuromyelitis optica

1. Introduction

Multiple sclerosis (MS) is just an autoimmune disease in which inflammatory demyelination of the white matter of the central nervous system is caused by the attack of the immune system. Most cases are present in 20 to 40 years of age. The incidence of MS showed a significant gender difference,[1] with females accounting for about 65% to 70%. The main clinical manifestations are limb weakness, paresthesia, visual impairment, and so on. Neuromyelitis optica (NMO) is an autoimmune disease characterized by demyelinating lesions and targeted at the optic nerve and spinal cord. Most cases are present in 35 to 40 years of age. The prevalence rate is 3 to 10 times higher in females than in males.[2] Clinical manifestations include visual impairment, limb weakness, eye pain, nausea, vomiting, defecation, and urination.[3,4] MS and NMO are the 2 most common types of demyelination of the central nervous system, and they have a lot of similarities. Demyelination and inflammation are the universal characteristics of the pathological process, and there are many similarities and overlaps in clinical manifestations. Therefore, NMO was regarded as a subtype of MS previously,[5] and the pathogenesis of both has not meant fully elucidated.

There are more women than men in multiple sclerosis, and women are more likely to relapse. For female patients during pregnancy, the symptoms often vary in degrees of relief, and often postponement of treatment results in serious complications. After childbirth, the symptoms are often more severe than before. Due to the long-term disease course, the反复ness of clinical manifestations, and the diversity of symptom expression, patients with MS/ NMO often have a lot of psychological reactions, including fatigue, nervousness, and depression. It’s necessary to do psychological intervention for multiple sclerosis/NMO patients and their family members.

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* Correspondence: Wensheng Zhang, Department of Neurology, Heyuan People’s Hospital, Guangdong Provincial People’s Hospital Heyuan Hospital, Guangdong Province, China (e-mail: 494172988@qq.com).

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aggravate or relapse in postpartum period. Studies have confirmed that estrogen is closely related to the onset of MS, and the fluctuation of estrogen intracorporal can significantly affect the change of MS condition.\(^6\)\(^-\)\(^8\) Estrogen develops its biological effect by binding to the estrogen receptor (ER).\(^9\) We believe that the differences in the biological effects of estrogen caused by single nucleotide polymorphism of the ER gene may also be related to morbidity, curative effect, recurrence and prognosis of MS and NMO. In this study, MS and NMO were selected as the correlation between ER gene polymorphism and demyelinating disease of the central nervous system. By means of Pvu II and Xba I restriction fragment length polymorphism enzyme recognition sites of ER\(\alpha\) gene, polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis was conducted. To investigate the distribution of ER gene polymorphisms in MS and NMO patients and healthy volunteers and to explore their relationship with MS and NMO.

2. Materials and Methods

2.1. General information

MS and NMO diagnosed in the Department of Neurology, the first affiliated hospital of Guangdong Pharmaceutical University from June 2018 to December 2019 were collected. Peripheral venous blood samples (3 mL) were gathered and put into the ethylene diamine tetraacetie acid anticoagulation tube in case of using. The patient's gender, age of onset, course of disease, and other clinical data was recorded. Healthy volunteers of the same age and sex were selected, and 3 mL peripheral venous blood was collected with ethylene diamine tetraacetie Acid acid anticoagulant tube, and the sample number was observed in future use. The blood samples are stored in a cryogenic refrigerator at \(-80^\circ\)C.

As of 31 December 2019, a total of 46 cases have been collected in the MS and NMO case groups (including 15 MS patients and 31 NMO patients). There were 5 males and 41 females who range from 17 to 67 years old. The mean age was (46.20 ± 14.44) years.

2.2. Criteria for case diagnosis, inclusion, and exclusion

2.2.1. MS diagnostic criteria.\(^10\)

1. The medical history and physical examination of the nervous system indicated that the presence of more than 2 lesions in the white matter of the central nervous system.
2. The course of disease was remission-relapse, or it may deteriorate slowly over 6 months.
3. Cerebrospinal fluid-oligoclonal band is positive/IgG index increased.
4. Magnetic resonance imaging showed that multiple lesions in space and/or in time.

2.2.2. NMO diagnostic criteria.\(^11\)

1. Signs of optic nerve and spinal cord impairment appear simultaneously or successively.
2. Serum NMO-IgG (that is AQP4 antibody) is positive.
3. Magnetic resonance imaging shows leukodystrophy.

2.2.3. Inclusion criteria.

1. Conform to MS or NMO diagnostic criteria, and the clinical diagnosis is definite.
2. With an ordinarily healthy body, without special physical discomfort.
3. Those who comprehend the purpose, process, and significance of this research and who give informed consent.

2.2.4. Exclusion criteria.

1. Those who are merged with other autoimmune diseases (e.g., Myasthenia gravis, myositis, dermatomyositis, systemic lupus erythematosus and rheumatoid arthritis, etc.).
2. Those who are merged with tumor (e.g., breast cancer, cervical cancer, endometrial cancer and ovarian cancer, etc.).
3. Those who are merged with craniocerebral infectious diseases (e.g., encephalitis, meningitis, and brain abscess, etc.).
4. Inability to cooperate with blood collection, extreme physical weakness, insufficient blood collection, and excessive lack of clinical data.

2.3. Methods

Ethics committee of Guangdong Pharmaceutical University approved the study.

2.3.1. Experimental methods.

2.3.1.1. The DNA extraction.\(^12\)

1. To fetch 250 μL NaI with concentration of 6 mol/L was added to the 250 μL whole blood, then mix them gently for 15 seconds.
2. Add 250 μL chloroform, shake, and mix for 15 seconds. Centrifuge at 12000 r/min for 7 minutes.
3. The supernatant was 400 μL and isopropanol was added. Centrifuge at 12000 r/min for 5 minutes.
4. The supernatant discarded and the precipitation was washed with 400 μL isopropanol with concentration of 37%. Centrifuge at 10000 r/min for 3 minutes.
5. Dry and add 50 μL TE buffers to dissolve the precipitate.

2.3.1.2. Determine the DNA concentration. Using the strong absorption peak of the benzene ring structure of the base on the DNA chain in the ultraviolet region, 15 μL DNA samples were collected and mixed with 1485 μL double-distilled water. UV spectrophotometry was used. The photometer measures the optical density (OD) at the wavelength of 260 nm, 280 nm, and 330 nm and counts them as OD260, OD280, and OD330 respectively. Concentration and ratio are calculated to determine DNA concentration and purity.

2.3.1.3. The PCR amplification.

1. Design and synthesize primers

ER alpha gene Pvu II, Xba I polymorphisms primer sequence is as follows:

Upstream: 5’-CTGCCACCTATCTGTATCTTTCCTATATTCTC-3’
Downstream: 5’-TCTTTTCTGCCCACCTGCGTGATATCTCGA-3’

2. The samples to be tested are carried out according to the following reaction system

Qualitative amplification using 2 μL DNA as template, and the apparatus is C1000 heat circulator. Reaction conditions of PCR primer system: 3 minutes at 95°C, then 15 cycles at 95°C, 5 seconds at 58°C, 60 seconds at 72°C, 10 cycles in total, 10 minutes at 72°C.

2.3.1.4. 2% agarose gel was used to detect PCR amplification products by electrophoresis.

1. Pour the 1.6 g agarose into a conical flask. Pour 80 mL TBE (1×) buffer, heat it and dissolve it in the microwave oven for 1 minute. Shake it gently and allow it cool at room temperature.
2. Cool down to 60°C, add 10 uL nucleic acid dyes and shake well.
3. Pour into the gel plate inserted into the comb without bubbles.
4. It was placed at room temperature for about 30 minutes until the gel was completely cured. Moving it from the gel plate to the electrophoresis tank and set aside.

2.3.1.4.2. Electrophoresis.
1. An electrophoresis buffer (1 × TBE) is added to the tank until it is submerged outside the gel.
2. Sample preparation: Take 8 ul amplified PCR products (including the sample buffer) and mix them gently.
3. Spot sample: The sample of the previous step is aspirated with the pipette head and then gently injected into the electrophoresis hole with 100 bp for 1/2 holes. Electrophoresis conditions: 120 V, 40 minutes.

2.3.1.5. Restriction enzyme digestion of amplification products.
1. According to the preparation of the enzyme system, using restriction enzymes Pvu II and Xba I, respectively, incubation 8 h at 37°C, and digestion of PCR products.
2. The enzyme digestion products were detected by 2% agarose gel electrophoresis: Firstly, 10 ul of each enzyme digestion product was taken and 1 uL of sample buffer was added. Then, add it to the 2% agarose gel and electrophoresis (1 × TBE buffer, 75 V, 15 mA, 60 minutes). Finally, the results were observed by ultraviolet transmission instrument.
3. The genotypes of enzyme digestion was analyzed according to electrophoresis bands.

2.2.3.2. Interpretation of ER genotype\(^{[13,14]}\). The ER alpha PCR specific amplification fragment was 1300 bp. ER alpha gene using Pvu II endonuclease enzyme reaction can distinguish between 3 genotypes: PP genotype (single strip of 1300 bp), Pp genotype (3 strips of 1300 bp, 850 bp, and 450 bp), pp genotype (2 strips of 850 bp and 450 bp). ER alpha gene using Xba I endonuclease enzyme reaction can distinguish between 3 genotypes: XX genotype (single strip of 1300 bp), Xx genotype (3 strips of 1300 bp, 910 bp, and 390 bp), xx genotype (2 strips of 910 bp and 390 bp). Combined with enzyme digestion results of Pvu II and Xba I site, it will get 9 genotypes of ER alpha such as PPXX, PpXx, and ppxx, etc.

2.4. Statistical approach
The software SPSS version 20 was used for analysis. The measurement data were described by mean ± standard deviation. \( t \) test was applied for 2 groups, 1-way analysis of variance was used to contrast the parameters between groups, and least significant difference-\( t \) test was applied for the pairwise comparison of multigroups. Pearson correlation analysis was applied for the variables with correlative trends. The chi-square goodness-of-fit test was applied for whether each genotype distribution of 2 sets abides by Hardy–Weinberg equilibrium\(^{[15]}\) and employing Chi-square test or Fisher exact test to contrast difference that shown by the genotype frequency and allele frequency distribution of 2 groups, respectively. By virtue of the Logistic regression’s odds ratio and 95% confidence interval to analyze the relative allele risk of age and gender.

3. Experimental results
3.1. The distribution of ER\(\alpha\) gene polymorphisms in MS and NMO case group and healthy control group
1. Electrophoretic result: The target genes were all positive. ER alpha PCR product: 1300 bp. An example is shown in Figure 1.

2. Example of ER\(\alpha\) genotype interpretation as shown in the Figures 2 and 3.
3. The distribution of ER\(\alpha\) gene polymorphism in MS and NMO patients as well as healthy volunteers.

By the Hardy–Weinberg test, MS and NMO case group and the control group of ER alpha Pvu II and Xba I allele frequency were consistent with genetic balance law (\(P > .05\)). This indicates that the genotype selected in this study is representative of the population.

There was no significant difference in the frequency distribution of ER\(\alpha\) gene’s PP, Pp, and pp genotype between MS and

![Figure 1. The agarose gel electrophoresis graph of PCR amplification results.](image)
Note: 1 and 9 are electrophoresis results of MS and NMO case specimens, 18 and 23 are electrophoresis results of normal control specimens, and M is marker. MS = multiple sclerosis, NMO = neuromyelitis optic, PCR = polymerase chain reaction.

![Figure 2. The agarose gel electrophoresis graph of restriction enzyme sites of Pvu II gene in ER\(\alpha\) gene.](image)
Note: From the left to the right are marker, Pp, Pp, Pp, Pp, pp, Pp, pp, PP, Pp, and PP genotype.

![Figure 3. The agarose gel electrophoresis graph of restriction enzyme sites of Xba I gene in ER\(\alpha\) gene.](image)
Note: From the left to the right are marker, Xx, xx, Xx, xx, xx, xx, xx, xX, xx, and xx genotype.
NMO case group and control group ($\chi^2 = 1.029, P = .598$). The distribution of P and p allele frequency between 2 groups was not statistically significant ($\chi^2 = 0.553$, $P = .436$). The difference between case group and control group was statistically significant in men ($\chi^2 = 6.310, P = .043$). To be specific, the frequency of P and p allele were uniform in case group of MS and NMO. The majority were Pp genotype, and the minority were PP and pp genotype. In control group, the frequency of P allele was higher than p allele. The majority were PP genotype, and the minority were pp genotype, and Pp genotype could not be detected. However, the difference between the case group and control group was not statistically significant in women ($\chi^2 = 1.762, P = .414$).

Frequency distribution of ERα gene’s XX, Xx, and xx was statistically significant between MS and NMO case group and control group ($\chi^2 = 7.686, P = .021$). It can be assumed that the frequency distribution of the 3 genotypes is not identical in the 2 groups. The results indicate that: There was no statistically significant difference in the frequency distribution of XX and Xx genotypes between the MS and NMO case group and the control group ($\chi^2 = 0.351, P = .553$). There was no statistically significant difference in genotype frequency distribution of XX and xx between the 2 groups ($\chi^2 = 1.540, P = .215$). Further pairwise comparison showed that the frequency distribution of Xx and xx genotype was statistically significant between the case group and control group ($\chi^2 = 7.684, P = .006$). It can be considered that the frequency distribution of 2 genotypes is not equal in the 2 groups.

By means of Logistic regression analysis, suggesting Xba I genes were independent factors affecting MS and NMO morbidity ($P = .002 \leq .05$). Among them, distribution of Xx and Xx gene frequency between patient group and the control group was statistically significant ($P = .001, OR = 4.622, 95\% CI: 1.803–11.852$). It is suggested that the prevalence rate of XX genotype is higher than that of Xx genotype, and the odds ratio is 4.622. There was no statistically significant difference in the frequency distribution of X and x alleles between the MS and NMO case group and the control group ($\chi^2 = 1.802, P = .179$). There was no statistically significant difference between the case group and control group of men ($\chi^2 = 10.408, P = .005$). That is to say, the frequency of X and x allele were analogous in MS and NMO case group. The majority were XX genotype, and the minority were Xx and xx genotype. In control group, the frequency of x allele was higher than X allele obviously. The majority were Xx and xx genotype, and the minority were XX genotype.

PpXX genotype was not detected in the case group, and PPxx as well as ppXX genotypes were rarely found. However, ppXX genotype was not detected in healthy control group. The genotype frequency of PXXx, PpXx, and ppXX was higher in both groups. Specific distribution of ERα gene polymorphism is shown in Table 1 and Figure 4.

### Table 1

| Group   | Case | Pvu II genotype | Xba I genotype |
|---------|------|----------------|----------------|
|         |      | PvuII allele   | PvuII allele   |
| Patient | 5     | P  12          | P  1  20       |
| Male    | 41    | PP  10         | PP  11         |
| Female  | 5     | Pp  12         | Pp  13         |
| Control | 5     | P  12          | P  5           |
| Male    | 41    | PP  10         | PP  11         |
| Female  | 5     | Pp  12         | Pp  13         |

#### 3.2. The correlation between ERα genes and MS and NMO

##### 3.2.1. Correlation between genotypes and onset age in patient group

As illustrated in Table 2. The results showed that there was no significant correlation between ERα genotypes and the onset age in patient group.

##### 3.2.1.1. Comparison of onset age of Pvu II each genotype. PP and Pp ($t = −0.499, P = .622$); Pp and pp ($t = 0.545, P = .590$).

##### 3.2.1.2. Comparison of onset age of Xba I each genotype. XX and Xx ($t = −0.311, P = .761$); Xx and xx ($t = −0.364, P = .717$).

##### 3.2.2. Correlation between genotypes and disease duration in the patient group as illustrated in Table 2.

##### 3.2.2.1. The difference was not statistically significant in disease duration of Pvu II each genotype. PP and Pp ($t = 0.843, P = .406$); PP and pp ($t = −0.495, P = .625$); Pp and pp ($t = 0.555, P = .583$).

##### 3.2.2.2. Comparison of disease duration of Xba I each genotype. The difference was statistically significant in disease duration of XX and Xx genotype, and the disease duration of Xx genotype is longer than that of XX genotype ($t = −2.947, P = .006$). Whereas, there was no significant difference in disease duration of XX and xx genotype as well as Xx and xx genotype ($t = 0.555, P = .583$).

##### 3.2.3. Correlation between genotypes and gender. Logistic regression analysis showed the comparison of XX and xx genotype frequency distribution in gender exists a difference ($P = .047, OR = 7.500, 95\% CI: 1.023–54.996$). It is suggested that patients with Xx genotype are more likely to be female than those with xx genotype, and the odds ratio is 7.500.

### 4. Discussion

The incidence of MS is the same as that of autoimmune diseases such as systemic lupus erythematosus, myasthenia gravis, and rheumatoid arthritis. Women are more frequent, especially women of childbearing age between 20 and 40 years old. Experiments have shown that MS is related to genes associated with estrogen and its receptor. The human ER comes in 2 forms, including ERα and ERβ. ERα gene is located at 6q25.1, with a length of 140 kb and a relative molecular weight of 64 Kda. It contains 8 exons and 7 introns. The gene in the first intron often contains 2 point mutations cause ER receptor polymorphism: Pvu II and Xba I enzyme loci. There are promoter, enhancer and other important DNA sequences in intron one. If point mutation occurs in intron one, the expression and function of ERα are likely to be affected.
In MS-related experiments, activation of ERα and ERβ can reduce demyelination, axonal loss, and neuropathy in experimental autoimmune encephalomyelitis. In previous studies, we have found that ICA can increase the expression of ERα and ERβ in peripheral blood mononuclear cells of MS patients, thus playing an estrogen-like role. The expression level and function difference of ER among different individuals may be determined by different genotypes; therefore, different genotypes may affect the biological effect of estrogen in vivo. Studies have shown that gender differences, age of onset, clinical manifestations, and other diseases such as breast cancer and systemic lupus erythematosus may be correlated with some ER subtypes. Polymorphic ER gene may also be one of the genetic factors that cause significant gender difference and clinical diversity between MS and NMO.

Through the analysis of some literature, it can be concluded that the distribution of ERα genotypes in healthy Han population in China is dominated by pp, xx, and ppXX genotype, while PP and XX genotype are in the minority. The ppXX genotype have not been detected in China, Japan, the United States, and other countries, and PpXX and ppXx genotype are also very rare. This study found that MS and NMO case group and the healthy control group of ER alpha gene Pvu II enzyme polymorphism distribution have no difference. However, the frequencies of PP, Pp, and pp genotype were different in men in the MS and NMO case group and the control group. Pp genotype was predominant in men in the MS and NMO case group, while PP genotype was predominant in men in the healthy control group. Pp genotype was not detected. MS and NMO and ER alpha gene Xba I enzyme polymorphisms exist correlation, Xba I gene polymorphisms should be as MS and NMO risk factors of the disease, especially Xx genotype women are more susceptible. The frequency of XX, Xx, and xx genotype were different in women in the MS and NMO case group and the control group. Xx genotype was more frequent in women in the MS and NMO case group, while Xx and xx genotype were more common in women in the healthy control group. The results of our study were roughly the same as those of Japan and the United States. PpXX genotype was not detected in the case group, and rarely appeared in the PPXX and ppXX genotype, while ppXX genotype was not detected in the healthy control group, and the genotype of ppXX, PpXx, and PPXX were relatively high in the 2 groups.

Research on ERα gene polymorphism and MS has a lot of progress in recent years. Although the results reported at home and abroad are different, they have enriched our understanding of the correlation between ER gene polymorphism and the pathogenesis and efficacy of MS. Niino et al. in Japan, PP or Pp genotype of Pvu II are associated with susceptibility to MS, and the susceptibility of MS may be determined by the weakening of estrogen immunomodulatory effect. Xba I polymorphism is associated with the onset age of MS. Xx genotype may be associated with the early onset of MS. Our research conclusion is also that Xba I enzyme polymorphisms is associated with MS and NMO, and it influences the progression of disease. Xx genotype may result in extending disease progression, data displays that XX genotype of patients have a significantly shorter duration. Domestic Sun Qingli et al. [27] the pathogenesis of MS has correlation with Pvu II polymorphism. With P allele may be a risk factor in the pathogenesis of MS, and women are more likely to suffer MS in particular. The occurrence of MS has nothing to do with Xba I polymorphism, but PpXX genotype in MS patients in majority. There is a great difference between Sun Qingli study and ours that our data show 3 kinds of enzyme genotypes distribution of Pvu II gene have no difference in 2 groups. It was concluded that the distribution of P and p allele was different between the 2 groups of men, that is, healthy men carried more P genes which may be due to the small total sample size of the men included in this study. The reports of Italy and Australia have suggested that the onset of MS has no irrelevance with Pvu II and Xba I enzyme polymorphisms of ERα gene. Domestic Wang Qingsong et al. [29] meta-analysis on 511 patients of MS and 508 cases of normal people found that MS susceptibility has no irrelevance with Pvu II polymorphism. In this study, the correlation between the polymorphism of ERα gene and MS as well as NMO was investigated. Research found that Xba I gene could be a risk factor of MS and NMO pathogenesis, especially the women with Xx genotype are more vulnerable. What’s more, the disease duration of Xx genotype persists longer than XX genotype. These may have clinical guiding significance in the diagnosis of disease screening and prognosis in the future. At present, considerable progress has been made in the study on the correlation between MS and ER gene polymorphism, but no definite and unified opinions have been reached. This may be linked to a variety of factors such as different research methods, number of samples, genetic differences, geographical, and environmental factors. We have attempted to conduct statistical analysis of ER polymorphism in MS group and NMO group and the control group, respectively, but the different trend was found in both groups without statistically significant results. This may be linked to the small sample size of the study. If the sample size of the study can be extended, especially the patient sample size, more convincing and representative conclusions can be drawn. Further efforts should be made to conduct large sample size and multicenter studies, especially considering the effects of different ethnic genetic backgrounds and different sample sizes on the study results. It is hoped that the correlation between ER polymorphism and MS and NMO can be further clarified in the near future, so as to provide more objective biological experimental basis for the study on the susceptibility of MS and NMO and the influencing factors of the curative effect, as well as the feasibility of phytoestrogen therapy.

### Table 2

| genotype | Onset age | Disease course |
|----------|-----------|----------------|
| PP       | 40.5 ± 14.7 | 1.83 ± 3.43 |
| Pp       | 45.8 ± 14.0 | 3.22 ± 5.05 |
| pp       | 43.2 ± 13.0 | 2.41 ± 2.52 |
| XX       | 42.4 ± 19.0 | 0.56 ± 0.84 |
| Xx       | 43.4 ± 13.1 | 3.14 ± 4.49 |
| xx       | 45.2 ± 14.7 | 1.84 ± 2.44 |

Note: Unit: onset age (year); disease course (year).

### Author contributions

Data curation: W.X. and W.Z.
Investigation: W.X., W.Z., Z.W.
Methodology: Z.W.
Project administration: W.X., W.Z.
Supervision: M.H.
Writing – original draft: W.X., M.H., W.Z.
Writing – review and editing: W.X., M.H., W.Z.
Data curation: Weifang Xing, Wensheng Zhang.
Investigation: Weifang Xing, Wensheng Zhang.
Methodology: Zhiheng Wei.
Project administration: Weifang Xing, Wensheng Zhang.
Supervision: Mingfan Hong.
Writing – original draft: Mingfan Hong, Weifang Xing, Wensheng Zhang.
Writing – review & editing: Mingfan Hong, Weifang Xing, Wensheng Zhang.
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