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Genetics of amyotrophic lateral sclerosis in Mongolia

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Dedication

To our patients with amyotrophic lateral sclerosis

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Contents

List of abbreviations

AAV – Adeno-associated virus
ALS – Amyotrophic lateral sclerosis
ALS-FRS-R – ALS Functional Rating Scale Revised
ALS/FTD – Amyotrophic lateral sclerosis and frontotemporal dementia
BMI – Body mass index
BSA – Bovine serum albumin
C9orf72 – Chromosome 9 open reading frame 72
CNS – Central nervous system
DNA – Deoxyribonucleic acid
ECAS – Edinburgh Cognitive and Behavioral ALS screen
EDTA – Ethylenediaminetetraacetic acid
fALS – Familial amyotrophic lateral sclerosis
FAS – Flail arm syndrome
FLS – Flail leg syndrome
FTD – Frontotemporal dementia
FUS – Fused in sarcoma
GDP – Gross domestic product
HRE – Hexanucleotide repeat expansion
IQR – Interquartile range
PCR – Polymerase chain reaction
PLS – Primary lateral sclerosis
PMA – Progressive muscular atrophy
pTDP43 – Phosphorylated TAR DNA binding protein 43
p.D90A – Point mutation aspartic acid at codon 90 changed to alanine
RNA – Ribonucleic acid
sALS – Sporadic amyotrophic lateral sclerosis
SD – Standard deviation
SNP – Single nucleotide polymorphism
SOD1 – Superoxidase dismutase 1
TARDBP – TAR DNA-binding protein

I. Introduction

Amyotrophic lateral sclerosis (ALS) is a multisystem neurodegenerative disease characterized by loss of the upper and lower motor neurons in the central nervous system (CNS), with patients exhibiting progressive muscle weakness, paralysis, and eventual death due to a neuromuscular respiratory failure. This occurs within three to five years in the median from the onset of the disease. In a subset of patients, frontal lobe regions of the brain undergo degeneration with a clinical presentation of cognitive and language deficits and personality changes leading to diagnosis of amyotrophic lateral sclerosis and frontotemporal dementia (ALS/FTD) (1).

ALS is considered to be a rare disease; in Europe incidence ranges from 2-3 cases per 100.000 (2). In contrast, incidence of ALS varies based on the ancestral origin (3). A limitation of global ALS epidemiology is that 80% of studies have been conducted in Europe and the United States (4), thus data are lacking from other parts of the world.

Aetiology of the disease is still unknown, despite some cluster of studies reporting environmental causes (5,6). Epidemiologic and experimental studies attempted to find potential exogenous risk factors for ALS (7), however proof of causal associations is sparse. One study deliberately addressed some attributable risk factors for ALS (8).

Clinical presentation of ALS is broadly heterogeneous with the representation of symptoms depending on which motor neurons are affected, therefore having different clinical implications by body regions, and differing from patient to patient. Variable clinical phenotypes therefore affect disease progression and its diagnosis. In general, it is observed that early focal symptoms of ALS usually begin in the limbs (9). Associated with poorer prognosis, bulbar onset is more common in elderly patients and women (10). Clinical phenotypes also seem to vary in populations from different continents (3).

Neurological examination reveals concurring involvement of the upper (spasticity, hyperreflexia and Babinski sign) and lower motor neurons (muscle atrophy, fasciculation and cramps), which makes it hard to standardise the diagnosing criteria for ALS with the considerations of its heterogeneous nature of

symptoms. The El Escorial criteria consolidated by neurologists in the field on motor neuron diseases, was used as a main criterion of diagnosing ALS (11). It was subsequently revised and a thorough clinical classification of ALS subtypes by syndrome was sought (12). Additionally, up to half of all patients have subtle impairment of temporal and frontal lobe cognitive function, which is evidenced by the fact that 50% of ALS patients develop frontotemporal dementia (FTD) (13).

About 90% of cases are observed to be sporadic ALS (sALS), and only 5-10% of patients report family history and termed familial ALS (fALS). The first genetic mutation identified as causing ALS were *SOD1* mutations located on the longer arm of the 21st chromosome of human genome (14).

SOD1 is globally the most commonly mutated gene and is associated with approximately 20% of all fALS cases and 2% of sALS. The protein comprises 153 amino acids and, to this date, 210 mutations throughout its 5 exons have been identified in the ALS cohort, with significant and non-pathogenic variants (http://alsod.iop.kcl.ac.uk/). Depending on the location of the mutations along the *SOD1* gene, carriers have a variable phenotypic expressivity. For example, *SOD1* p.D90A (aspartic acid at codon 90 changed to alanine, in exon 4), the most commonly distributed mutation in the northern part of Europe, exhibits both in autosomal dominant and recessive patterns in different populations (15,16), and is associated with a much slower course than usual; conversely: the p.A4V, p.G41S, p.G93A and p.R115G variants of *SOD1* have consistently poor survival (17). Pathogenesis of genetic mutation in *SOD1* is explained to be induced by the overproduction of reactive oxygen species through loss of function mutations in *SOD1* (18); while the gain of function mutations results in increase of oxidative stress which impairs endogenous environment of neuronal cells (19). Accumulation of proteins functionally altered during normal proteinogenesis leads to release of excessive oxidative radicals into the cytoplasm.

Mutations in fused in sarcoma (*FUS*) gene were identified to cause about 5% of fALS (20), and rarely, some sALS cases (21). Interestingly, *de novo FUS* mutations accounted for 43% in early onset patients in Germany, genetically explaining the causes of sALS in this cohort (22).

In 2012, a major genetic breakthrough in ALS research was made with the identification of hexanucleotide repeat expansion (HRE) in a non-coding region of the open reading frame 72 of the chromosome 9 (*C9orf72*); this genetic abnormality

was frequently found in a series of families with an autosomal form of familial frontotemporal dementia (11.7%) and familial ALS (23.5%) (23,24). This discovery genetically and clinico-pathologically linked two separate diseases: ALS and FTD. Pathomechanisms associated with this expansion are reviewed in detail elsewhere (25).

Globally, the prevalence of *C9orf72* HRE predominates in the European derived populations, however, given sparse data from South and East Asia, its distributions in these regions are inconclusive. Specifically, it is recognised to be most prevalent in Finland, where it is claimed to be caused by founder effects (26). Small cluster studies indicate different founder events occuring distinct from the observed conserved haplotype (27).

A plethora of mutations identified in different cohorts, with different pathogenic implications and identification of common genes susceptible to both sporadic and familial ALS cases, complicates the definition of either sporadic or familial ALS (28). Thus, once pathogenic mutation in a disease-causing gene is found in the patient and segregates with the disease, the term hereditary or primary genetic ALS is advised (12). The search for candidate genes for this disease is accelerating, with only a few causative pathogenic variants playing a plausible role; in fact, only 15% of all ALS cases (combined sALS and fALS) are genetically explained (29). Indeed, heterogenic architecture with variants identified in several rarely mutated ALS genes was performed in a large-set cohort of ALS families in Germany, with 43% of participating families also remaining genetically unexplained (30).

Functional studies conducted to understand how these genetic implications are mechanistically altering mainly DNA/RNA processing, autophagy, vesicle transport, oxidative stress and metabolism are elucidating parts of the puzzle (31).

Pathological implications have been identified phosphorylated TAR DNA binding protein 43 (pTDP43) disposition at the cellular level of affected neurons of diseased ALS patients (32). Distribution of protein aggregates in affected neurons was observed along axonal pathways (33). Nevertheless, pTDP43 pathology is almost never observed in the cases with mutations in the *SOD1* gene, where, for instance, neuronal inclusions containing aggregated *SOD1* are recognised to be pathologic hallmarks of ALS caused by *SOD1* mutations in ALS patients (34,35).

Different protein types were identified pathologically in ALS patients with specific genetic mutations (35).

The progressive nature of the disease makes it necessary to find appropriate treatments for ALS. One treatment with a modest effect of prolonging progression from 3-6 months is Riluzole (36), a glutamate modulator that acts by restraining excitatory motor neuron firing, though the effect of the treatment depends on administration of the drug in the early stages of the disease.

Based on clinical evaluation of gene or mutation-specific carriers mediated by genes such as *SOD1* and *C9orf72*, therapies are being developed to inactivate production of toxic gene products (e.g. antisense oligonucleotides and adeno-associated virus (AAV)-delivered microRNA)(37–39). However, these therapies are for a subset of patients with specific mutation carriers, while the majority of ALS patients are unexplained genetically.

The goals of ALS research area are to find a cure, halt disease progression, or at least lengthen prognosis. Though breakthroughs in genetics, molecular biology, pathology have allowed advances in the latter goal, patients still suffer and there is no cure for this devastating disease.

Despite encouraging findings in genetics, it is also necessary to thoroughly observe how disease progresses within individuals, and whether these patients share specific phenotypic symptoms that modify the course of the disease.

Examining positive prognostic factors in patients living with disease may give us an alternative way of approaching our patients with their specific prognoses. One study described the disease to be prone to metabolic features, which had negative prognostic values for the survival of disease (40). Thus high-caloric nutrition is an established ALS therapy and has been found to prolong survival in patients with percutaneous endoscopic gastrostomy (41). Younger onset of disease is also an important positive prognostic factor for the progression of the disease.

Many studies attempt to delineate the pathogenesis of the disease, though it remains poorly understood. As in many other disease pathologies, the complex interplay between intrinsic and extrinsic factors may play a role in pathogenesis and is yet to be fully elucidated.

Reviewing the findings of ALS studies allows us to better understand the pathogenesis of the functions altered during the disease course. It is imperable to apply this understanding to evidence-based and individualized medicine globally, to

summarize the common features that ALS may encompass. Thus, narrowing our focus within populations of different backgrounds aid ascertainment of the possible disease-causing factors, whether they are genetic, environmental or population specific.

The Asian continent comprises widely diverse populations with different ethnic, social, and cultural backgrounds as well as health systems. In recent years, ALS research in Asia increased the knowledge of this disease (42–45). However, in less developed countries in the Asian region, data remain limited (46).

Developing deeper knowledge about the differences between Caucasian and Asian cohorts of ALS patients might lead to a deeper understanding of the ALS genotype/phenotype relationship and is warranted to further define ALS in different backgrounds.

The scientific sector is still at its infancy in Mongolia, comprising only 0.1% of total GDP (gross domestic product). Thus, collaborative international projects on this newly examined population may reveal distinct scientific features. Collaborative approaches such as these have made it possible to describe and reveal some genetic features of neurological diseases among Mongolians (47). Mongolia's small population size also enables us to reveal the architecture of ALS in the country more easily. Observing the findings from different population background may also differentiate the way that we explain the disease.

Mongolia, located in North Central Asia, represents a genetically distinct and relatively homogenous population compared to other parts of the world (48,49). We thus set out to characterize ALS in the Mongolian population clinically and genetically.

II. Material and methods

This was a case-control study on the genetic background of ALS in Mongolia. A collaboration initiated between two institutions (Mongolian Institute of Medical Sciences and the Department of Neurology, Ulm University) enabled this project to be carried out. The study protocol was reviewed by the Scientific Review Board of the Institute of Medical Sciences, Ulaanbaatar, Mongolia and approved by the Ethics Review Committee of the Ministry of Health of Mongolia (2015/07/III), which is recognized as following international and local ethical regulations. Written informed consent for participation in the study was obtained from all participants.

Study participants

Patients were recruited at the Department of Neurology, Institute of Medical Sciences of Ulaanbaatar, Mongolia, after the referral from local neurologists of Ulaanbaatar city's general hospitals and neurologists throughout all provinces of Mongolia. Briefly, ascertainment of cases was validated through an annual expert workshop (with 148 participating neurologists from throughout the country) and an outpatient study visit operated by German neurologists from Ulm University to Ulaanbaatar, Mongolia. About 60% of the whole Mongolian population, approximately 1.3 million inhabitants, reside in Ulaanbaatar city or its vicinity. Furthermore, neurologists of all provinces in Mongolia, from March 2015 to September 2018, participated in the study. Upon a registration of a suspected case in the countryside in Mongolia, a specialized neurologist visited immobile patients in the remotest areas of the country. We thus attempted to cover the entire population of Mongolia. Standard clinical neurological examinations were performed on a total of 74 unrelated patients. Data included age at onset, site of onset, delay between first symptoms and diagnosis, and self-reported body weight information. All patients met the revised El Escorial criteria for ALS. Clinical subtypes were examined as described previously (12,50). Electromyography testing was performed at the Reflex clinic in Ulaanbaatar, Mongolia. ALS functional rating scale revised (ALS-FRS-R) scores were collected at the first presentation. We managed inpatient clinical care for the patients residing in the remote locations; from these patients, paired scores for ALS-FRS-R were correlated with their survival. The censoring date for the survival analysis was December 2018. The patients were

contacted every three months by telephone, and when possible, hospital care was provided. Systematic mortality update was conducted once a year. The date of the last contact was used as the censoring date for those patients lost to follow up, for the others, it was the date of the last systematic mortality update.

To calculate crude incidence rates of ALS, annual reference data from the booklet of the State Office of Reference of Mongolia was used, and age-standardized rates were calculated using the world standard population database (51) (Supplementary table 1). The prevalence rate was estimated as product of the incidence rate and mean survival. We performed a descriptive statistical analysis. Quantitative data are expressed as means and standard deviations (SD) or median depending on the distribution of the values. Categorical data are described as absolute frequencies and ratios. The Kaplan-Meier method (COX regression analysis) was used to calculate survival and median survival times. All statistical analysis were performed using SPSS17 program.

Blood collection, DNA extraction and Genetic testing

Collection of human peripheral venous blood was performed in a non-fasting state using a Monovette™ blood drawing system (EDTA monovettes; Sarstedt, Germany, #01.1605.001). 8 ml EDTA blood was used for DNA extraction. Erythrocytes were lysed in 20 ml lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2 EDTA, pH=7.4) for 15 min on ice. The leukocyte pellet was re-suspended in 2.5 ml SE buffer (75 mM NaCl, 25 mM EDTA). To release the DNA, proteinase K digestion was performed using 100⌈l 20% SDS+proteinase K (10mg/ml, Roche, Switzerland) overnight at 37 °C. 900 ⌈l of saturated sodium chloride solution (6 M NaCl) was added. The DNA was precipitated in 7 ml of 100% ethanol. Then, the DNA was washed in 70% ethanol, dried at room temperature, and re-suspended in 300 ⌈l buffer (1 M Tris/HCl, 0.5 mM EDTA) (52). A total of 125 healthy control subjects, without any neurological disease at the time of sample collection provided blood samples as controls and were matched to cases for sex, residence area and age. Analysis of HRE in *C9orf72* was performed by fragment analysis and repeat-primed polymerase chain reaction (RP-PCR). Oligonucleotide primers were published previously (23,24). Fragment analysis was performed with 50ng of genomic DNA and the NEB polymerase kit One*Taq* (OneTaq 2X Master Mix with GC buffer, NEB, Germany) according to the manufacturer's protocol and a touch-

down PCR protocol (65 °C – 55 °C). RP-PCR was performed with 500 ng DN, 7-deaze-2-deoxy GTP [2.5mM], Qiagen DNA polymerase and slow-down PCR protocol (53). A *C9orf72* expansion was anticipated if alleles larger than determined by fragment analysis could be detected by RP-PCR. For Southern blot analysis 10 ⌈g DNA was digested with *HindIII* and *Xbal* overnight. DNA fragments were separated by a 0.9% TRIS-Borat-EDTA (TBE) agarose gel, transferred by alkali blotting onto Amersham Hybond NTM-XL (GE Healthcare, Fisher Scientific, Germany) and hybridized to a ^{32}P- labelled probe overnight. After washing X-ray films were exposed for 4-6 days at -80 °C. *BstE*II digested lambda DNA was used as a size marker for calculation of the repeat sizes (54,55). *C9orf72* testing was performed at the Molecular Genetics laboratories of the Institute of Human genetics for all samples.

Analysis of point mutations *SOD1* and *FUS* was tested by Sanger sequencing. We designed forward and reverse m13-tailed primers (primer sequences are available on request). Amplification PCRs were performed using U AmpliTaqGold polymerase (Life Technologies, CA, USA, #N8080247), 1.5 ⌈g BSA (PAA, Austria, #K41-001), 0.4 mM of each nucleotide, 0.5 ⌈l of each primer (10 pmol/⌈l) and 10-15 ng genomic DNA. After PCR amplification the fragments covering exons and exon-intron boundaries were treated with ExoSAP-IT (Affymetrix, CA, USA, #78201). For the sequencing reaction the BigDye Terminator v3.1 CycleSequencing kit (Life Technologies, CA, USA, #4337455) was used in accordance with the manufacturer's instructions. For haplotype analysis on *C9orf72*-HRE positive cases and respective family members we genotyped the most conserved risk haplotypes consisting of 15 single nucleotide polymorphism (SNP) (56,57). The sequencing samples were prepared using 10 ⌈l formamide. Electrophoresis was performed on an ABI PRISM→3730 DNA Analyzer (Applied Biosystems). The SNPs were genotyped using Sanger sequencing. SNPs were excluded if they had a minor allele frequency (MAF) <0.01.

Fragment analysis and Sanger sequencing products were analysed on an ABI3130 DNA Analyzer (Applied Biosystems) using Pop-7 polymer (Life technologies, CA, USA, #4352759) and dye or filter set C. Data were analysed with the PeakScanner TM (Applied Biosystems) and with Sequence Scanner v2.0 (Applied Biosystems).

III. Results

Epidemiology and Clinical demographics of ALS in Mongolia

In total, 74 patients with ALS, 37 unaffected family members, as well as 116 healthy, unrelated, age-matched and gender-matched control individuals were enrolled in the study. 3 out of the 74 index patients reported a family history of ALS (4.0% fALS) and/or a phenotype consistent with FTD. Clinical demographics and characteristics are depicted in Table 1.

Table 1. Demographics and clinical characteristics of Mongolian ALS patients diagnosed in the years from 2015 to 2018

Number of patients	74
Age of onset, median (IQR), years	52 (IQR 43.7-58.2)
Male to female ratio	1.38:1 (43/31)
Type of onset; N (%) Spinal Bulbar	 50 (67.6%) 24 (32.4%)
ALS subforms, N (%) PLS PMA FAS/FLS	 3 (4.1%) 2 (2.7%) 11 (14.9%)
BMI (kg/m^2), median	26.1 (IQR 23.8-29.2)
Riluzole use, N (%)	7 (11.1%)
Loss of ALS-FRS-R per month*	0.66 (IQR 0.35-1.29)
Survival from onset, months	56.0±6.0

Statistics given as median and interquartile range (IQR), ALS – Amyotrophic lateral sclerosis, PLS - primary lateral sclerosis, PMA - progressive muscular atrophy, FAS - flail arm syndrome, FLS - flail leg syndrome, BMI – body mass index, ALS-FRS-R – ALS functional rating scale revised * data analysed on the subset of patients with the scores at the early stages of disease.

The mean age of onset of ALS was 52 (IQR 43.7-58.2) (Table 1). Crude incidence of ALS in Mongolia was 0.61, while annual incidence adjusted for age and sex using world standardized population was estimated to be 0.74 per 100.000

people. With the mean survival of 56 months (from the date of onset), the prevalence of ALS in the Mongolian population was estimated to be 3.64 per 100.000 inhabitants. The prevalence is higher in males than females (males: prevalence 3.82/100.000; females: 2.49/100.000; ratio 1.38:1). Moreover, the age-specific prevalence peaked between 60 and 69 years of age (Figure 1).

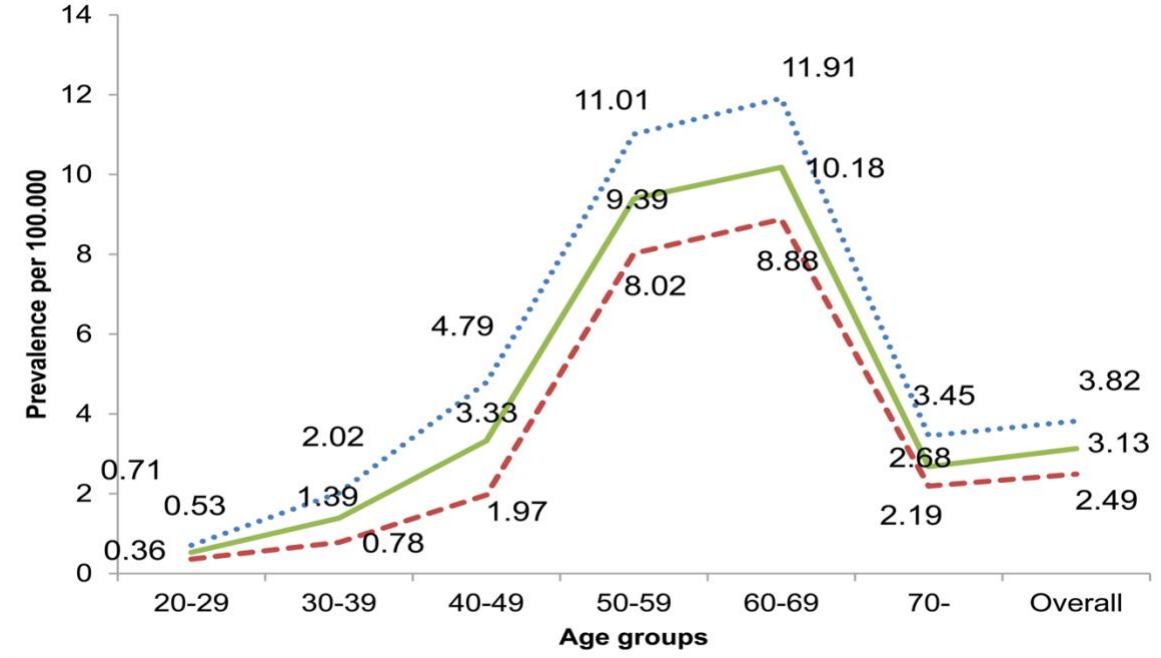

Figure 1. Age specific prevalence rates of ALS in Mongolian cohort. Green line shows estimated overall age-specific prevalence rates, the blue dotted lines are those for males, the red lined line for females.

We observed that 67.6% (n=50) of the patients had ALS with spinal onset of symptoms, while bulbar onset accounted for 32.4% (n=24). Progressive muscle atrophy (PMA), primary lateral sclerosis (PLS), flail arm syndrome (FAS) and flail leg syndrome (FLS) subforms accounted for 2.7% (n = 2), 4.1% (n = 3) and 14.9% (n=11) respectfully. The overall mean age at disease onset was 51.2 ± 11.1 years. Progressive bulbar paralysis was seen at older age, 53.6 (± 12.2) years (Table 1). Mean survival time from onset was 56.0 (± 6.0) months, with bulbar onset cases having the shortest survival with 40.1 (± 6.3) months. Classical ALS patients (upper combined with lower motor neuron disease and spinal onset) survived on average 47.2 (± 4.9) months. Positive predictive factors for survival were high body mass index, younger age at onset, lower limb onset; if all factors were present, survival reached 88.4 months.

Cox proportional hazards model analysis revealed that younger age at onset was significantly associated with longer survival (p = 0.01). Furthermore, we correlated the survival from onset of ALS by the phenotype category in our prospective cohort (Figure 2).

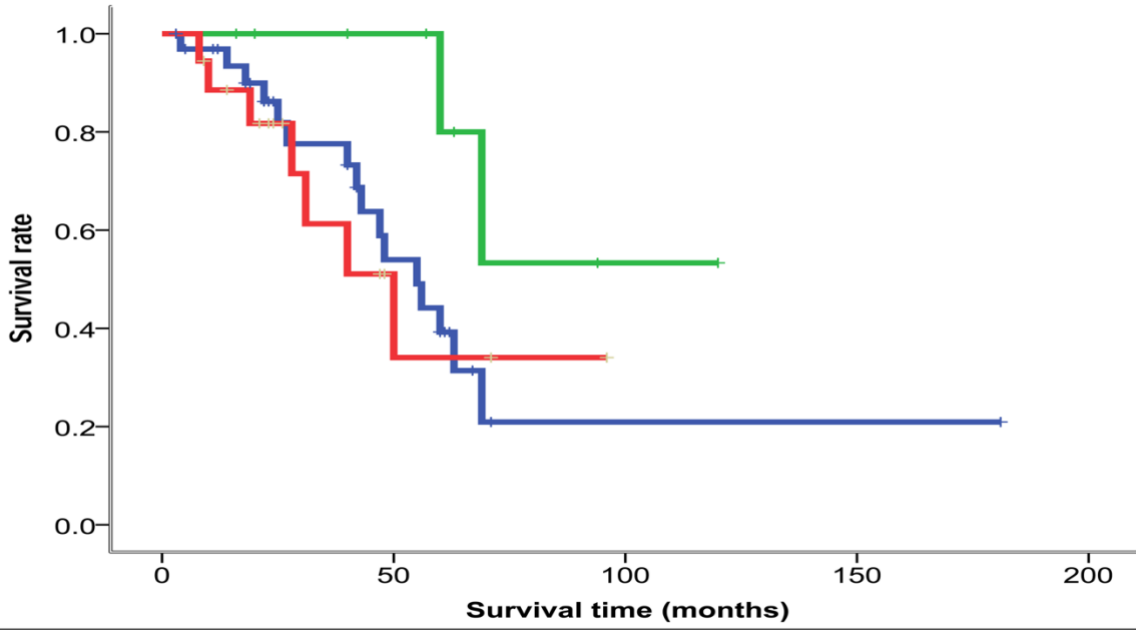

Figure 2. Survival rates according to clinical subtypes in Mongolian ALS cohort. Survival rates were highest for flail arm and leg phenotypes as illustrated with a green line. Our data also show that bulbar onset patients represented with red line have the worst prognosis. The blue line in this Kaplan-Meier curve shows survival rates of patients showing the classical ALS phenotype.

The paired ALS-FRS-R scores revealed 0.66 loss of score per month in the patient group. Seven patients (11.1%) medicated with riluzole for ALS.

Genotyping of Mongolian ALS patients

We genotyped all patients and healthy controls who were recruited for this study for mutations in the most frequent ALS genes, namely *SOD1*, *C9orf72* and *FUS.* We observed a pathogenic repeat expansion in the *C9orf72* gene in a patient with familial ALS-FTD (fALS-FTD), two patients with familial ALS (fALS) and in one apparently sALS from different families and regions of the country. The two patients without signs of FTD presented with classic bulbar onset ALS (Table 2). A patient with sALS exhibited with classic form of ALS at onset of disease on 59 years old. The length of the expansion ranged from 1200 to 2400 repeats, quantified by the

southern blotting technique. No control individuals had the *C9orf72* expansion, and all controls were tested negative for mutations in *SOD1* or *FUS*.

The case with fALS-FTD were seen in the field by an experienced ALS and FTD neurologist from the department of Neurology, Ulm University, who was always accompanied by a native speaking neurologist.

Table 2: Characteristics of Mongolian patients carrying ALS mutations. Presented are index patients of families with C9orf72 mutation, all 3 patients with SOD1 mutation have apparently sporadic ALS.

Gene	ID	Heterozygous / homozygous	Sex	Age of onset, years	Duration/ months	Site of onset	Additional features	Type of ALS
SOD1 p.D90A	M41	Heterozygous	F	52	>45	Bulbar	Slow progression	Bulbo-Spinal
	M56	Heterozygous	F	59	>53	Upper Limb	Slow progression	Spinal
	M65	Homozygous	F	56	>12	Lower Limb	Mild progression	Spinal
C9orf72 Hexanucleotide Repeat expansion	M24	Heterozygous	F	47	36	Upper limb	fALS/FTD	Bulbar
	M29	Heterozygous	M	55	18	Bulbar	fALS	Bulbar
	M54	Heterozygous	M	44	36	Bulbar	fALS	Bulbar
	M73	Heterozygous	F	59	>24	Upper limb	sALS	Spinal

fALS – familial ALS, sALS – sporadic ALS, FTD – frontotemporal dementia, F – female, M – male.

FUS mutations have been previously reported to be responsible for a large proportion of young-onset ALS cases (22). Because the Mongolian study cohort contains a relatively high number of patients with onset before 45 years, we genotyped the mutational hot-spot exons 14 to 15 of *FUS* in all cases with onset before 45 years (n=15). None of the patients examined carried a mutation in the *FUS* hot-spot exons.

Two heterozygous and one homozygous *SOD1* p.D90A mutation carriers were identified. Two of the heterozygous p.D90A mutation carriers exhibited ALS with a slow progression of disease. The phenotype of homozygous p.D90A also only moderately progressed. The family history of these patients could not be explored further since all three patients were adopted (Figure 3).

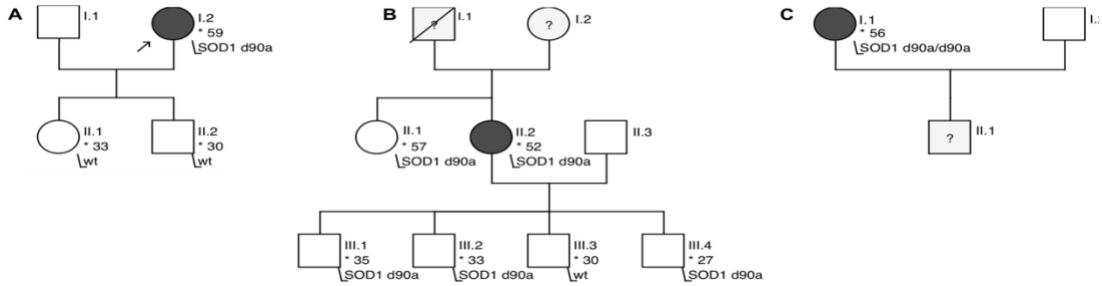

Figure 3. Pedigree of families exhibiting *SOD1* p.D90A mutation detected in our Mongolian cohort of ALS. The index patients are marked with an arrow and filled black circles, information below is given in the format: age of each subject at the time of sample collection, genotype – SOD1 d90a – heterozygous, SOD1 d90a/d90a – homozygous, wt - wildtype.

To further explore and examine our *C9orf72* patient family with fALS-FTD, we have included 18 family members for further analysis. The *C9orf72* HRE were observed in the 5 asymptomatic siblings of the index patient, and in two siblings exhibiting symptoms of FTD. Her mother died of ALS at the age of 55. The index patients´ uncle also died of ALS at a similar age of 53. The two asymptomatic uncles (both at least 10 years older than the index patient) and 4 children (all younger that the index patient) were also tested positive for a *C9orf72* HRE (Figure 4). The grandfather of the index patient had a similar disease as reported by family members. Analysis on the family member revealed an age-dependent penetrance of 57% at 55 years of age.

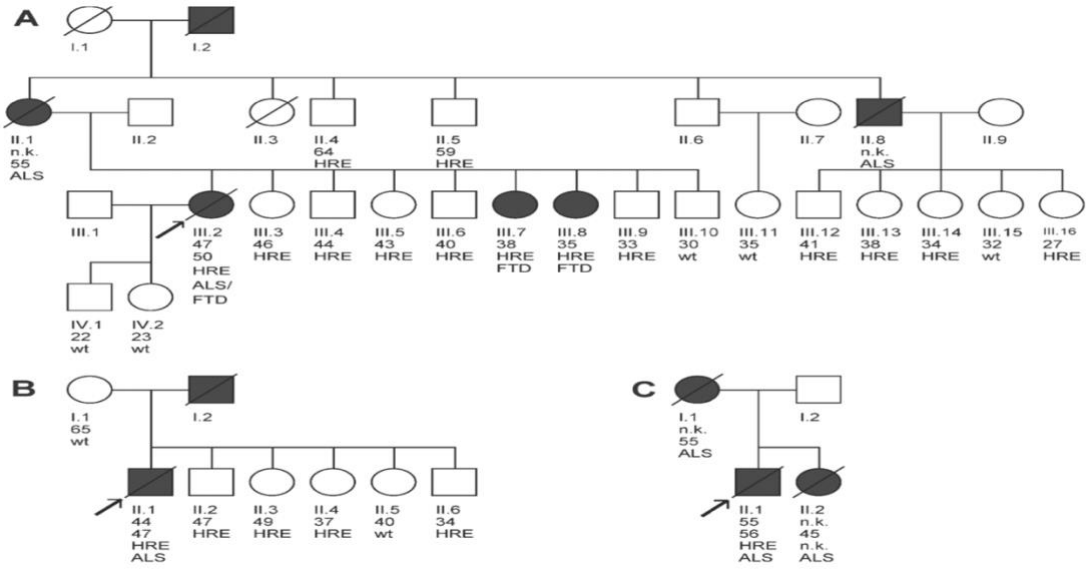

Figure 4. Pedigrees of Mongolian families with ALS/FTD or ALS and a *C9orf72* HRE. The index patients are marked with an arrow, information below is given in the format age of each subject at the time of sample collection, age at death, genotype, and phenotype (58).

Haplotype analyses of C9orf72 HRE carriers

We then investigated the most conserved *C9orf72* risk haplotypes described before, specifically the predominant European and recently published Han Chinese haplotype. To that end, we tested the genotypes of in total 15 SNPs including risk haplotype SNPs associated with the European *C9orf72* mutation haplotype and previously observed SNPs defining a Chinese *C9orf72* mutation haplotype. The common European founder haplotypes rs73440960A, rs11789520A, rs3849945C, rs2453555/5 T/A, rs774359G, rs774357T and rs774356G were inconsistent with alleles observed in Mongolian expansion carriers (Supplementary table 2). Recently published Han Chinese risk haplotypes were partially present in the Mongolian cohort. The Mongolian repeat expansion carriers did not harbor the European risk alleles at rs1982915 and rs2281241 described previously. Moreover, specifically the SNPs rs1948522T, rs1565948T and rs3849943A were strongly associated within our expansion carriers. Taken together, the Mongolian risk haplotype differs both on centromeric and telomeric side of the *C9orf72* HRE from both the European and the Chinese haplotype, indicating the presence of a Mongolian risk haplotype (58).

Linkage analysis conducted in three families with ALS or ALS/FTD revealed that the common haplotype was found in all expansion carriers (n=20) (Figure 5). Therefore, it is likely that a conserved risk haplotype underlies ALS/FTD in Mongolia. Our linkage region covers a 43 kilobase long region; this region is slightly shorter than the European predisposing haplotype which consists of 110kb region.

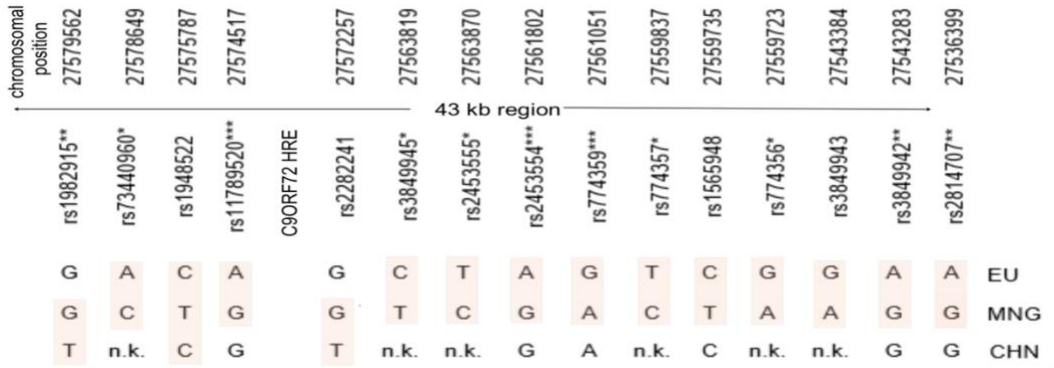

Figure 5. Schematic representation of SNP genotyping data on *C9orf72* HRE haplotypes from Mongolia compared with Europe and China. Differences between populations are shaded in light red. * = *C9orf72* HRE-associated SNP genotypes (56), ** = SNP genotypes (57) defining the Chinese *C9orf72* HRE haplotype, *** = SNP markers by (56,57); “n.k.” indicates SNPs not assessed/known in HRE carriers in the respective cohort; MNG = Mongolian, EU = European, CHN = Chinese. The markers are presented in order from telomeric (right) to centromeric (left) side (58).

IV. Discussion

In the present study, we provide the first description of ALS in the homogenous Mongolian population as one of a not yet studied rather separated Asian population. We report information on clinical phenotypes as well as a genetic analysis of the most frequently mutated ALS disease genes. We describe different clinical phenotypes when compared to Caucasian cohorts, and a surprisingly high prevalence of *C9orf72-linked* ALS/FTD cases in Mongolia when compared to other Asian populations. Moreover, we detected a distinct *C9orf72* HRE risk haplotype in the Mongolian population.

We had several methodological limitations in our study. First, the risk of incomplete ascertainment of cases might have occurred. With the help of international collaboration and neurological conference organized with even including neurologists from the countryside, we were able to raise an awareness of disease throughout the country. Another challenge was the lack of a validated neuropsychological test in Mongolian language. FTD diagnosis was established based on clinical impression by experienced neurologists from Ulm University, who have been visiting Ulaanbaatar, Mongolia annually for the last ten consecutive years. We are working on a validation of ECAS (Edinburgh Cognitive and Behavioral ALS screen) in our Mongolian language. Validation of neuropsychological screening and development of guidelines are necessary for an accurate observation and for the evaluation of cognitive status in our patients. A more sophisticated approach is now being taken for developing a proper battery for a screening of dementia in the Mongolian population.

The approach in a more detailed analysis including sequencing of over 30 other ALS disease genes, including TDP-43/*TARDBP*, must follow.

The crude incidence of ALS is 0.61 and the prevalence rate was 3.64 per 100.000 in our Mongolian prospective cohort. To supplement reports on ALS in Asia (46,59) we document a comparable incidence rate, when standardised to the world population database. This is in line with other Asian populations like China (60), but we highlight that the nature of the studies from these regions report data from the hospital-based registries, whereas we report a data on the population based prospective cohort (61).

Varying evidence from geographical and continental countries reveal lower incidences of ALS in some parts of the world (62). In Asia it can be explained by a lack of health service access for elderly people in the highly populated countries and ascertainment bias should be acknowledged. Considering the observation that more than half of the patients diagnosed with ALS in our cohort were residing in the countryside throughout Mongolia, revealing a good nationwide coverage.

People aged over 60 years old make up less than 5% of the Mongolian population, the low percentage of elderly people indicates shorter life span, which may reflect the low incidence of this late-onset disease.

The mean age of onset of the disease in our prospective cohort is 52 (IQR 43.7-58.2), which is also reported to be in the same range in other Asian cohorts (10,46,60).

We observe that ALS is more prevalent in males compared to females (1.31:1), which is in line with database online (ALS online database, ALSoD) (10). In the general characteristics of the lifestyles of our patients, smoking and alcohol usage were observed at higher level among males (data not shown). A thorough case control study needed to prove this assumption. Indeed, studies report an association of smoking playing a role as one of the risk factors for ALS in the epidemiological observational studies (63,64), though other studies did not confirm (65).

Classical ALS phenotypes (around 75%) in Asian populations does not strongly differ to those seen in patients with European origin, which is the same with our observation of spinal onset accounting for 67.5%, followed by bulbar-onset ALS. Contrary to the studies reporting clinical subtype of ALS in comparison between China and Germany, patients with FAS/FLS were diagnosed within the same range (50), where we have seen 14.9% of patients exhibiting ALS subforms.

The longer survival is observed regardless of whether patients resided in the rural or urban areas in our cohort, if considering that cases in the city would assuring have better health services. In fact, the multidisciplinary health care facilities designated for ALS patients are lacking in the community or are almost non-existent.

Several prognostic risk factors were observed in our cohort related to the longer duration of disease. We report that younger onset of disease is correlated with a longer survival of disease, supporting data reported elsewhere (46,50,60,66).

We have analysed the records with complete information on the ALS-FRS-R scores observed at the enrolment. Our patients with shorter disease duration had a faster disease progression as measured by the loss of ALS-FRS-R score per month, when comparing the scores from the early stages of disease with the later stages of disease. Given more precise consideration on the progression of the disease, reporting, and detecting prognostic indicators of short and long survival is necessary.

Only 10 percent of our patients reported the use of one of the treatments available for ALS patients. The low rate is most likely explained by the high cost of the drug in Mongolia, and probably due to unavailability and/or insurance coverage. Nevertheless, we have registered the orphan drug in the national drug registry in Mongolia, to promote the provision of the latest treatment option for our patients. We have observed a longer survival of one patient administered with riluzole, compared to a patient with the same onset with out administration of the drug.

We anticipate that the presentation of higher body mass indexes in Mongolian ALS patients could indeed act as one of the players in prolonging the disease progression, which is reported recently (50). High BMIs are reported to lower the risk of ALS in a Norwegian population-based study (67), which indeed could be reflective of the low incidence of ALS in Mongolian population. A study among healthy Mongolians, reporting natural selection on the population posing genetic features susceptible for influencing energy storage could be a genetic explanation to these obese features Mongolians exhibit (68).

The rate of familial ALS cases (4.8%) is roughly within the range of observations in other European cohorts (9). Reluctance to report hereditary diseases, adoption, and early death of family members due to other causes may have led to an underestimation of the proportion of familial ALS cases. Moreover, the reduced disease penetrance that we observed in one family with a *C9orf72* HRE could mask an even higher incidence of familial ALS in Mongolia.

A significant difference was identified previously (44), regarding the frequencies of mutations in major ALS genes between European and Asian patient cohorts. In Caucasian populations, the most common mutations were the *C9orf72* HRE (fALS 33.7%, sALS 5.1%), followed by *SOD1* (fALS 14.8%, sALS 1.2%), *TARDBP* (fALS 4.2%, sALS 0.8%) and *FUS* mutations (fALS 2.8%, sALS 0.3%), while in Asian populations the most common mutations were *SOD1* mutations (fALS

30.0%, sALS 1.5%), followed by *FUS* (fALS 6.4%, sALS 0.9%), *C9orf72* HRE (fALS 2.3%, sALS 0.3%) and *TARDBP* (fALS 1.5%, sALS 0.2%) mutation (44). While mutations in *FUS* are rarely found in the whole population of sALS patients and in less than 10% of familial cases, young onset ALS (<45 years at onset) is to a large proportion (roughly 50%) caused by *FUS de novo* mutations in respective Caucasian cohorts (22).

In Mongolian patients, we observed two major differences to Caucasians: we failed to detect a single *FUS* mutation in the 15 patients with early onset ALS and encountered an unexpectedly high number of patients with *C9orf72* HRE. Three index patients with familial ALS or ALS/FTD and one single apparently sALS case carried a *C9orf72* HRE. While several of the healthy mutation carriers in the respective families were below the age of expected onset, also several seemingly non-penetrant aged asymptomatic relatives were identified. This is in agreement with the known reduced penetrance of *C9orf72* mutations (69–72). We report an age-dependent penetrance of 57% at 55 years of age in our cohort.

The contribution of specific ALS disease genes differs between the populations, at least partially due to founder effects. For instance, *C9orf72* HRE mutations are the most frequent monogenic cause of ALS in the Western World, while only a marginal proportion of genetic ALS cases are caused by *C9orf72* mutations in most Asian populations, for example in China (42,57,73,74). This fact, strengthened by the observation of a predominant *C9orf72* risk haplotype in Europe, has fostered the hypothesis that one or very few founder events are responsible for the Caucasian *C9orf72*-linked ALS (56,75,76).

European *C9orf72* mutations linked to a specific risk haplotype (77), indicate one or few underlying mutational events arose several thousand years ago (76). However, the description of additional *C9orf72* haplotypes in non-European populations suggests independent founder events (27). To find out whether the Mongolian *C9orf72* mutation is linked to one of the known haplotypes, we further investigated the most conserved risk haplotypes described before, specifically the predominant European (56) and Han Chinese haplotype (57). Moreover, a report from another group from the Southern part of China revealed conflicting results suggesting a European founder haplotype is indeed present in their cohort of Chinese expansion carrier (78). This is partially explained by authors with inclusion

of a cohort of patients which are isolated and have a genetically homogenous nature compared with sALS patients from the north and central region of China (57,73).

Analysis of the haploblock around the *C9orf72* HRE locus revealed a common haplotype in all Mongolian *C9orf72* mutation carriers. This suggests that a founder effect is responsible for the relatively high frequency of *C9orf72* HRE in Mongolia. Moreover, we found this risk haplotype (58) to be different from the common European haplotype as well as from the haplotype previously reported in Chinese ALS patients (43,56,57,77–79). Thus, the Mongolian *C9orf72* mutation most likely originates from distinct mutational events. The previously nomadic Mongolian life could have facilitated the spread of this mutation across different parts of Mongolia, and perhaps other parts of the world. It would furthermore be interesting to see whether this haplotype is prevalent in other populations living close to Mongolia, specifically in North and Central Asia. Approximately 10 million ethnic Mongolians currently inhabit a wide geographical range that includes Mongolia, Northern China, southern Russia, and other neighboring countries (80).

SOD1 is one of the most commonly mutated genes in familial forms of ALS in European populations (30), but with varying frequencies in studies with different ethnical backgrounds (44). Consistent with these reports, we detected 3 *SOD1* mutation carriers (5.5% of the ALS patients genotyped) in this Asian cohort. The family history of all three *SOD1* mutant patients remained unclear. All three carried the p.D90A mutation in *SOD1,* two in the heterozygous and one in the homozygous state. This is also in line with several earlier reports demonstrating that the *SOD1* p.D90A mutation can be the cause of either autosomal-dominantly inherited disease or be responsible of the rarer instances of autosomal-recessively transmitted ALS (16,81–83). Autosomal-dominant and autosomal-recessive modes of *SOD1* p.D90A-linked inheritance are never observed within the same family (84). While *SOD1* is generally the most mutated gene in the studied ALS populations (44), specifically the p.D90A mutation in *SOD1* is the most frequent genetic cause of ALS in Scandinavia as well as in other parts of Europe or the US (85–87), the p.D90A SOD1 has not previously been identified in ALS patients in eastern Asia, though the mutation has been found in epidemiological study among healthy populations (88). However, the distinct mild phenotype of the Mongolian *SOD1* p.D90A-associated cases is similar to the phenotypes described before (85–87), suggesting that the phenotype linked to this mutation is largely independent of the genetic population

background. Furthermore, the phenotypic differences exhibited among only one nucleotide change, with a strong representation affecting the duration and progression of disease, is the area of interest.

Variable differences genetically and clinically among different populations may express the hidden message encompassing the modifying and protective factors affecting the phenotype and genotype of ALS. Nevertheless, physiological processes coupled with the interaction of intrinsic and exogenous factors leading to neurodegeneration, could depend on each individuals' specific vulnerability to these physiological cascades.

Exploring the genetic features of *SOD1* p.D90A mutation, would be a globally relevant study which may hinder the origin of the founder mutation. Previous deliberate reports deeply warrant studies and results from central Asian origin to link their finding to the ancestral origin (89). A key finding of this study, the high prevalence of European mutation content in Mongolian ALS cases allows us to ask further questions are: how did this European component appear in Mongolia? Where and when did it originate? In a recent population genetic study authors thoroughly revealed a shared degree of identity by descent between Finns and Mongolians, thus indicating a pattern that Northern Asian populations interacted across large geographical ranges (80).

Taken together, we observed phenotypes and a specific genotypes pattern of ALS in Mongolia when compared to Europe or other Asian regions and describe a novel *C9orf72* risk haplotype that most likely originates from a distinct mutational founder event. We demonstrate that studying ALS in homogenous, so far relatively poorly characterized non-European populations can help to improve the insight into genetics of this disease and possibly identifying modifying factors affecting some phenotypic features.

V. Summary

The clinical phenotypes and genetics of amyotrophic lateral sclerosis (ALS) vary between ethnic groups. To broaden insights into the characteristics of ALS in Asia, we studied this disease in the Mongolian population. Clinical data and DNA from 74 patients and 153 healthy control subjects (including family members of index patients with mutation carriers) were collected from 2015 to 2018 and genotyped. Mongolian ALS patients presented with an earlier onset and a more benign disease course compared to European populations. While the *C9orf72* hexanucleotide repeat expansion (*C9orf72* HRE) is reportedly rare in Asia, we identified two familial ALS index patients and one fALS/FTD patient, and one single sALS patient carrying *C9orf72* alleles expanded to 1200-2400 hexanucleotide repeats. Analysis of the haplotype surrounding the *C9orf72* HRE revealed a haplotype different from the European and Han Chinese *C9orf72* HRE risk haplotypes, suggesting a population-specific founder. Sequencing *SOD1* revealed 2 heterozygous and 1 homozygous p.D90A mutation in apparently sporadic ALS cases. In contrast to Caucasian cohorts, *de novo* mutations in *FUS* were absent in early-onset (<45 years) Mongolian ALS patients (n=15). In summary, we observed population specific genetic features of ALS, and describe its phenotypical appearances in Mongolia when compared to Europe and China. Further characterization of ALS in Mongolia may result in a better understanding of modifying factors and genotype/phenotype correlations of ALS in both Mongolia and Europe.

VI. References

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VII. Supplementary materials

Supplementary table 1. Crude incidence and standardized rates of ALS in Mongolian cohort observed by age at diagnosis using the world standard population database.

Age group	Persons living in years from 2013-2018*	ALS cases diagnosed in the years 2013-2018	Age specific incidence (per 100000)	Standard pop. (**100000 persons)	Expected events in standard pop.
20-29	3427046	2	0,06	16150	942,50267
30-39	3050584	6	0,2	14760	2903,0507
40-49	2364714	17	0,72	12630	9079,745
50-59	1640627	28	1,7	9920	16930,113
60-69	699634	13	1,86	6680	12412,204
-70	426768	5	1,2	5275	6180,1728
Crude rate:		0.61		Age standardized rate(world):	0.74
*Data from State office of reference of Mongolia, **WHO world standard population distribution based on world average population between 2000-2025					

Supplementary Table 2. Genotyping data on cases carrying the *C9orf72* HRE from Mongolia compared with European risk haplotype and Chinese haplotype (58).

#	Name	Position	Frequency of risk allele Europe	Frequency of risk allele East Asia	Frequency of risk allele South Asia	European risk allele	Mongolian haplotype	Chinese haplotype (57)	Reference / alternative allele
1	rs2814707**	27536399	0.23	0.7	0.16	A	G	G	A/G
2	rs3849942**	27543283	0.23	0.9	0.7	A	G	G	G/A
3	rs3849943*	27543384	0.23	0.9	0.9	G	A	n.k.	A/G
4	rs774356*	27559723	0.25	0.9	0.19	G	A	n.k.	A/G
5	rs1565948**	27559735	0.51	0.57	0.47	C	T	C	C/T
6	rs774357*	27559837	0.23	0.9	0.16	T	C	n.k	C/T
7	rs774359***	27561051	0.25	0.9	0.19	G	A	A	A/G
8	rs2453554***	27561802	0.23	0.9	0.16	A	G	G	G/A
9	rs2453555*	27563870	0.23	0.9	0.16	T	C	n.k.	C/T
10	rs3849945*	27563819	0.23	0.9	0.16	T	C	n.k.	C/T
11	rs2282241	27572257	0.55	0.32	0.50	G	G	T	G/T
				C9orf72 HEXANUCLEOTIDE REPEAT EXPANSION					
12	rs11789520***	27574517	0.24	0.9	0.15	A	G	G	G/A
13	rs1948522	27575787	0.81	0.91	0.89	C	T	C	C/T
14	rs73440960*	27578649	0.21	0.11	0.19	A	C	n.k.	C/A
15	rs1982915**	27579562	0.46	0.22	0.36	G	G	T	T/G

*SNPs by (56), **SNPs by (57) defined as Chinese haplotype, ***SNPs by (57) and (56), "n.k." indicates not assessed genotypes in HRE carriers in the respective cohort, the frequency of the haplotype risk alleles are from 1000 Genomes database (www.1000genomes.org/)

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Curriculum vitae