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Polymerase chain reaction and Southern blot-based analysis of the *C9orf72* hexanucleotide repeat in different motor neuron diseases

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ABSTRACT

The GGGGCC-hexanucleotide repeat expansion in *C9orf72* is the most common genetic cause of familial amyotrophic lateral sclerosis and frontotemporal dementia. This study determined the frequency of *C9orf72* repeat expansions in different motor neuron diseases (amyotrophic lateral sclerosis (ALS), motor neuron diseases affecting primarily the first or the second motor neuron and hereditary spastic paraplegia). Whereas most studies on *C9orf72* repeat expansions published so far rely on a polymerase chain reaction-based screening, we applied both polymerase chain reaction-based techniques and Southern blotting. Furthermore, we determined the sensitivity and specificity of Southern blotting of the *C9orf72* hexanucleotide repeat in DNA derived from lymphoblastoid cell lines. *C9orf72* repeat expansions were found in 27.1% out of 166 familial ALS patients, only once in 68 sporadic ALS patients, and not in 61 hereditary spastic paraplegia patients or 52 patients with motor neuron diseases affecting clinically primarily either the first or the second motor neuron. We found hints for a correlation between *C9orf72* repeat length and the age of onset. Somatic instability of the *C9orf72* repeat was observed in lymphoblastoid cell lines compared with DNA derived from whole blood from the same patient and therefore caution is warranted for repeat length determination in immortalized cell lines.

1. Introduction

Expansions of the GGGGCC hexanucleotide repeat of chromosome 9 open reading frame 72 (*C9orf72*) have been reported to be the most common pathogenic mutation in familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011). First results suggested that *C9orf72* repeat expansions are associated with an earlier age of onset, more frequent bulbar onset and a more aggressive disease course in ALS, as well as behavioral abnormalities in FTD- and FTD-ALS-patients (Brettschneider et al., 2012; Byrne et al., 2012; DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Snowden et al., 2012). Most of the genetic studies on *C9orf72*-associated diseases published so far solely rely on polymerase chain reaction (PCR)-based screening methods. The cutoff for pathogenic alleles is still unclear but it was hypothesized to be around 30 repeats (Renton et al., 2011). However, PCR-based methods cannot determine the size of larger expanded alleles accurately. Therefore, Southern blotting is the only way to determine the exact repeat length and thus necessary to establish a possible correlation between the geno- and the phenotype. Only a few and mostly smaller studies used Southern blotting to date (DeJesus-Hernandez et al., 2011; Dobson-Stone et al., 2013; Harms et al., 2013a, 2013b;

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Ishiura et al., 2012). There is only one study in which Southern blotting was performed in a larger cohort of patients with different neurodegenerative diseases (Beck et al., 2013). Interestingly, but in contrast to other repeat expansion diseases known to date, the length of expanded *C9orf72* repeats correlated positively with the age of onset (Beck et al., 2013). Furthermore, different repeat lengths in *C9orf72* have been reported in a single individual depending on the source of DNA (Beck et al., 2013). A high prevalence of *C9orf72* repeat expansions has been observed in individuals of European descent (Beck et al., 2013; Smith et al., 2013).

Although patients with different neurodegenerative and psychiatric diseases such as Alzheimer's disease, Parkinson's disease, spinocerebellar ataxias bipolar affective psychosis, and schizophrenia have been screened for expansions in the *C9orf72* hexanucleotide repeat (Beck et al., 2013; Fogel et al., 2012; Harms et al., 2013a; Huey et al., 2012; Lesage et al., 2013), little is known on *C9orf72* repeat size in other motor neuron diseases affecting clinically primarily the first or the second motor neuron (also described as primary lateral sclerosis or progressive muscular atrophy/spinal muscular atrophy, respectively) (van Rheenen et al., 2012) and hereditary spastic paraplegia (HSP).

In this study, we compared the clinical features of patients carrying a hexanucleotide repeat expansion in *C9orf72* to that of patients without a *C9orf72* repeat expansion in a German cohort of patients with familial ALS (fALS), sporadic ALS (sALS), as well as in patients with other motor neuron diseases affecting primarily the upper or lower motor neurons including genetically unselected patients with HSP. Genetic screening of the *C9orf72* hexanucleotide repeat encompassed fragment analyses, repeat-primed PCRs and Southern blotting. DNA for Southern blotting was derived from immortalized lymphoblastoid cell lines (LCLs) and from whole blood samples if possible. To determine the sensitivity and specificity of repeat length calculation using LCL-derived DNA we additionally compared *C9orf72* repeat lengths in DNA derived from whole blood and DNA derived from LCLs.

2. Methods

2.1. Patients

We studied a cohort of 166 unrelated fALS, 68 sALS, 61 HSP, 22 patients with a motor neuron disease affecting clinically primarily the lower (progressive muscular atrophy/spinal muscular atrophy) and 30 patients with a motor neuron disease affecting primarily the upper motor neuron (primary lateral sclerosis). Patients were recruited at the Department of Neurology of the University Hospital of Ulm, Germany. All patients had given written informed consent for genetic and clinical studies. The study was approved by the Ethics Committee of the University Hospital of Ulm. All tests have been conducted according to the principles expressed in the Declaration of Helsinki.

ALS was diagnosed on a clinical and electrophysiological basis when patients met the El Escorial criteria (Brooks, 1994). At least 1 index case of each family was examined by a specialist for motor neuron diseases. fALS was diagnosed according to the definition of Byrne et al. (2011). The other disease entities were diagnosed clinically and electrophysiologically. In a subgroup of 30 fALS patients, we checked whether FTD was concomitant to ALS in terms of an ALS-FTD-complex. FTD diagnosis was based on clinical features and neuropsychological examination according to the criteria of the international consensus group (Neary et al., 1998). If necessary, we carried out lumbar puncture and magnetic resonance imaging scan of the brain and spinal cord to exclude other diagnoses. For all patients, detailed clinical and demographic data were obtained by interviewing patients and/or relatives and by reviewing of medical records.

2.2. Molecular testing

DNA was derived from whole blood samples or immortalized LCLs by salting out or column extraction (DNeasy 96 Blood & Tissue Kit, Qiagen, Germany), respectively. Immortalized LCLs were established by transforming lymphocytes with Epstein-Barr virus following the procedures described previously (Ventura et al., 1988). Most fALS patients had been pre-screened for mutations in different ALS associated genes (*SOD1*, *FUS*, *OPTN*, *TARDBP*, *PFN1*, details available upon request). One fALS patient with a PCR-based hint for an expansion of the *C9orf72* hexanucleotide repeat carried the known *SOD1* gene mutation c.313A>T (p.I105F) in a heterozygous state. The sporadic ALS patients and the patients with the other disease entities were genetically unselected.

Analysis of the hexanucleotide repeat in *C9orf72* was performed by a fragment analysis and repeat-primed PCR (rpPCR). Oligonucleotide primers were published previously (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Fragment analysis was performed with 50 ng 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). rpPCR was performed with 500 ng DNA, 7-deaza-2-deoxy GTP [2.5 mM], Qiagen DNA polymerase and a slow-down PCR protocol (Frey et al., 2008). PCR products were analyzed on an ABI3730 DNA analyzer (Applied Biosystems, Germany). Data were analyzed with the PeakScanner software (v1.0, Applied Biosystems). A *C9orf72* expansion was anticipated if alleles larger than determined by fragment analysis could be detected by rpPCR. Southern blot analysis was conducted if enough and high molecular DNA was available. For Southern blot analysis 10 μg DNA was digested with *Hind*III and *Xba*I 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- labeled probe overnight. After washing X-ray films were exposed for 4–6 days at −80 °C. *Bst*EII digested lambda DNA was used as a size marker for calculation of the repeat sizes. Minimal repeat sizes were used for calculation.

2.3. Statistical analysis

Statistical analysis was performed using StatView for Windows version 5.0.1 and SAS version 9.3. Descriptive evaluation included means, standard deviations, and ranges for continuous traits. The Wilcoxon two sample test was used to compare quantitative traits between 2 patient groups whereas Fisher's exact test was applied to qualitative traits for this purpose. Spearman rank correlation coefficient R between repeat size length and age of onset was calculated and tested for the presence of an association on the population level. A p-value ≤ 0.05 is generally termed "(statistically) significant".

3. Results

In 45 out of 166 samples of the fALS cohort (27.1%) PCR-based screening indicated an expansion of the hexanucleotide repeat in *C9orf72*. In contrast, only 1 out of 68 sALS cases carried an expanded *C9orf72* allele. Neither patients with a clinical affection primarily of the upper motor neuron (n = 30) or lower motor neuron (n = 22) nor HSP patients (n = 61) showed an expanded *C9orf72* allele. Clinical and demographic data are summarized in Table 1. In our cohort of patients with a *C9orf72* repeat expansion, we found significantly more women (60.0% vs. only 39.7% women in patients without a *C9orf72* repeat expansion, $p = 0.023$). The age