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Research paper

Methylation of *C9orf72* expansion reduces RNA foci formation and dipeptide-repeat proteins expression in cells

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HIGHLIGHTS

- Repeat methylation reduces molecular pathology in cellular model of c9FTD/ALS.
- Novel method for *C9orf72* repeat methylation levels quantification.
- This technique could potentially be used in patients as a bio- and prognostic marker.

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ABSTRACT

A hexanucleotide repeat expansion in the C9orf72 gene is the most common genetic cause of both frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), together referred to as c9FTD/ALS. It has been suggested that a loss of C9orf72 protein expression, the formation of toxic RNA foci and dipeptide-repeat proteins contribute to *C9orf72*-related diseases. Interestingly, it has been shown that trimethylation of histones and methylation of CpG islands near the repeat expansion may play a role in the pathogenesis c9FTD/ALS. Recently, methylation of expanded repeat itself has been reported. To further elucidate the mechanisms underlying these diseases, the influence of epigenetic modification in the repeat expansion on its pathogenic effect was assessed. Here, a reduced formation of toxic RNA foci and dipeptide-repeat proteins upon methylation of the GGGGCC repeat in a cellular model of c9FTD/ALS is shown. Additionally, a novel methylcytosine-capture DNA hybridization immunoassay for semi-quantitative detection of the repeat methylation levels is presented, potentially usable for methylation analysis in patients carrying *C9orf72* repeat expansion carriers as a diagnostic tool. Presented results suggest that increased level of pathogenic GGGGCC expansion methylation may be sufficient to alleviate the molecular pathology of the *C9orf72*-related diseases.

1. Introduction

Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are devastating neurodegenerative diseases with substantial genetic, neuropathological, and clinical overlap [1]. A repeat expansion in the chromosome 9 open reading frame 72 (C9orf72) gene is the most frequent genetic cause of FTD and ALS, collectively referred to as c9FTD/ALS [2,3]. This repeat expansion is believed to cause the disease by three distinct mechanisms. First, a reduction of *C9orf72* expression [2–4] could result in a loss of C9orf72 protein, impairing its potential functions such as membrane trafficking [5,6]. Second, RNA fragments containing sense or anti-sense repeats are known to accumulate and form nuclear foci in expansion carriers [2,7,8], which may lead to RNA-mediated toxicity by sequestration of RNA-binding proteins [9]. Third, repeat-associated non-ATG (RAN) translation has been reported in *C9orf72* expansion carriers, generating dipeptide-repeat proteins (DPRs), such as poly-glycine-proline (GP), that form toxic neuronal inclusions [8,10,11].

Previously, it has been shown that the region adjacent to expanded repeat in *C9orf72* binds strongly to trimethylated histones H3 and H4, which are known to repress gene expression

Abbreviations: FTD, frontotemporal dementia; ALS, amyotrophic lateral sclerosis; C9orf72, chromosome 9 open reading frame 72; c9FTD/ALS, FTD/ALS caused by expansion in C9orf72 gene; RAN translation, repeat-associated non-ATG translation; DPR, dipeptide-repeat protein; poly(GP), poly-glycine-proline; MSD, Meso Scale Discovery; M, plasmid with methylated $(GGGGCC)_{66}$ repeat; U, plasmid with unmethylated $(GGGGCC)_{66}$ repeat; HEK293T cells, human embryonic kidney cells; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TBS-T, tris–buffered saline with Triton X-100; FISH, fluorescence in situ hybridization; DEPC, diethylpyrocarbonate.

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[12]. Other reports have revealed abnormal methylation of the 5′ CpG island near the GGGGCC deoxynucleotide repeat [13–15]. In a more recent study, methylation of the *C9orf72* expansion itself has been reported [16] and we also observed this phenomenon (unpublished data). Furthermore, it has been suggested that methylation may influence disease penetrance and severity in *C9orf72* expansion carriers [13–15,17,18].

In general, most CpG islands are unmethylated in adult human cells, but methylation can occur within repetitive DNA elements and their surroundings [19]. Additionally, GC-rich DNA sequences with complex secondary structures are known to represent ideal targets for methylation [20]. Given the nature of the *C9orf72* expansion that comprises hundreds to thousands of GGGGCC repeats, it is possible that the expansion is highly susceptible to (hyper) methylation. The aim of this study was to unravel whether the repeat methylation may affect the RAN translation and nuclear RNA foci formation. Additionally, a novel technique potentially usable in expansion carriers to investigate this epigenetic phenomenon within the GGGGCC repeat is presented.

2. Materials and methods

2.1. Cell culture and transfections

Human embryonic kidney (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (both Sigma–Aldrich), 100 U/mL penicillin and 100 mg/mL streptomycin (Life Technologies) at 37 °C in an humidified atmosphere containing 5% CO_2. Transient transfections were performed when cells reached 70–80% confluence with Lipofectamine 2000 (Life Technologies), following manufacturer's instructions.

2.2. Cloning of pAG3-66R expression vector

Generation of the $(GGGGCC)_{66}$ expression vector was reported previously [8,21]. In brief, genomic DNA from muscle or spleen of a *C9orf72* expansion carrier was used as template for a nested PCR, using ThermalAce DNA Polymerase (Life Technologies) to amplify the $(GGGGCC)_{66}$ repeat region, including 113 bp of 5′ and 99 bp of 3′ flanking sequence. PCR products were cloned into the pAG3 expression vector using restriction sites HindIII and BamHI (New England Biolabs). This expression vector has a pcDNA3.0 backbone and a CMV-enhanced chicken β-actin promoter. Clones containing $(GGGGCC)_{66}$ were verified by hairpin sequence analysis.

2.3. In vitro *methylation of repeat*

For this experiment, HpaII methyltransferase (New England Biolabs) was used, which recognizes CCGG sites and is able to methylate the internal cytosine residue ($CC^{m}GG$). The methylation reaction of the pAG3-66R plasmid was performed in the presence of 1× HpaII Methylase Reaction Buffer (New England Biolabs), and 80 μM *S*-adenosylmethionine (New England Biolabs) as the methyl group donor (at 37 °C for two hours). The plasmid was then purified using the DNA Clean & Concentrator kit (Zymo Research). To ensure that only the $(GGGGCC)_{66}$ repeat was methylated, the methylated and unmodified plasmids were digested with HindIII and BamHI restriction enzymes (New England Biolabs). Resulting fragments were separated by agarose electrophoresis and purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The repeat from the methylated plasmid was then ligated to the unmethylated vector backbone (M) using T4 DNA Ligase (Life Technologies). An identical procedure was used for the unmethylated repeat (U). Ligated plasmids were purified by Qiaquick PCR Purification Kit (Qiagen) prior to subsequent use. The methylation of the repeat was confirmed by bisulfite sequencing, using a ACAATAAAAATAACACCTTCCAAAATCAAAAAAAAC primer (complementary to the anti-sense bisulfite-treated strand).

2.4. Western blot analysis

To determine whether methylation of the repeat in pAG3-66R could affect poly-glycine-proline (GP) levels, U and M plasmids were transfected into HEK293T cells (2 μg of DNA; 3.5 cm plate). After 36 h, cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). Cell pellets were lysed in a buffer consisting of 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 1% Triton-X-100, 5 mM ethylenediaminetetraacetic acid (EDTA), 2% SDS, phenylmethylsulfonyl fluoride (Life Technologies), and a protease and phosphatase inhibitor cocktail. After centrifugation at 10,000 × *g* at 4 °C for ten minutes, supernatants were prepared in Laemmli's buffer, heated at 95 °C for five minutes, and equal amounts of protein were loaded into 4–20% Tris–Glycine polyacrylamide gels (Life Technologies). The immunoblot procedure was performed as described previously [22], using purified rabbit polyclonal anti-GP antibody (1:1000; generated as previously described [8]) or mouse monoclonal β-actin antibody (1:10,000; Sigma–Aldrich).

2.5. Immunocytochemistry

Cells were grown and transfected on glass coverslips. After 36 h, cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100/PBS and blocked in 5% skim milk/tris–buffered saline with 0.05% Triton X-100 (TBS-T). Immunocytochemistry was performed as described elsewhere [23]. Samples were incubated with anti-GP antibody (1:500; 5% skim milk/TBS-T) at 4 °C overnight. Secondary anti-rabbit Alexa-488 secondary fluorescent antibody (1:1000, Life Technologies) was used, and confocal microscopy was performed with a Zeiss LSM 510 microscope.

2.6. RNA fluorescence in situ hybridization (FISH)

HEK293T cells were grown and transfected by U or M pAG3-66R plasmids on glass coverslips. To detect RNA foci, the cells were fixed 24 h post transfection and permeabilized in 4% PFA/diethylpyrocarbonate (DEPC)-treated PBS and washed twice with DEPC-PBS 36 h after transfection. Samples were then dehydrated with a series of 70% and 100% ethanol washes and air dried, followed by incubation in hybridization buffer (10% dextran sulfate, 50% formamide, 50 mM sodium phosphate buffer [pH 7], and 2× SSC) at 66 °C for 20–60 min. To ensure that our RNA FISH protocol detects RNA foci and not simply overexpressed DNA, some cells were treated with RNAse free DNase I (50 U/mL, Life Technologies) for 45 min at room temperature and RNase A (4 μM; Qiagen) for 15 min at 37 °C and washed three times with DEPC-treated PBS prior to pre-hybridization. Prior to usage, the locked nucleic acid probe (5′TYE563-CCCCGGCCCCGGCCCC-3′; Batch #612968; Exiqon) was denatured at 85 °C for 75 s and diluted to 40 nM with hybridization buffer. Hybridization with the probe was performed in a sealed, light-protected, humidified chamber at 66 °C for 16–24 h. Subsequently, coverslips were washed with 0.1% Tween-20/2× SSC for five minutes, followed by two ten-minutes stringency washes in 0.2× SSC at 66 °C. Cells were stained with Hoechst 33258 (Life Technologies), rinsed with DEPC-treated water, dehydrated through 70% and 100% ethanol and air dried. Coverslips were then mounted with Prolong Gold anti-fade reagent (Life Technologies)