

Exon Skipping Quantification by qRT-PCR in Duchenne Muscular Dystrophy Patients Treated with the Antisense Oligomer Eteplirsen

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ABSTRACT

Restoration of the open reading frame of the *DMD* gene and dystrophin protein production in Duchenne muscular dystrophy (DMD) can be achieved by exon skipping using antisense oligomers (AOs) targeted to splicing elements. Several such RNA-based gene therapy approaches are in clinical development where to date, all studies have assessed AO efficacy by semi-quantitative nested RT-PCR. Precise evaluation of dystrophin protein levels is complex and hindered by the large size and low abundance of dystrophin; thus an accurate and standardised measurement of *DMD* exon skipping at the RNA level remains important to assess and compare patient responses in DMD exon skipping clinical trials.

Here we describe the development of a Taqman qRT-PCR assay to quantify exon skipping and highlight its use to determine the levels of exon skipping in DMD patients treated intra-muscularly with a morpholino AO to skip exon 51, eteplirsen (AVI-4658). The muscle biopsies of these patients have previously been thoroughly characterised providing a valuable benchmark for the evaluation of novel methodology.

We demonstrate that levels of dystrophin protein restoration, and thus patient response, correlate accurately with the RNA level. Furthermore, this sensitive assay detects revertant exon 51 skipped fibres in untreated biopsies providing an important baseline to precisely quantify treatment success. This study represents the first quantitative assessment of exon skipping in a clinical trial setting. We present a standardised and reproducible method to assess patient response that will complement protein studies in future pre-clinical and clinical exon skipping-based gene therapy studies for DMD.

INTRODUCTION

The fatal X-linked neuromuscular disorder Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene which disrupt the open reading frame and prevent the full translation of its protein product dystrophin (Abbs et al., 2010; Muntoni et al., 2003). Dystrophin is localised to the sarcolemma where it provides a link between the cytoskeleton and the extracellular matrix via the dystrophin-associated protein complex (DAPC). The main role of dystrophin in skeletal muscle is to stabilise the muscle fibre against the mechanical forces of muscle contraction (Ervasti, 2007). An absence of dystrophin leads to progressive damage and necrosis of muscle fibres. DMD patients typically develop muscle weakness in the early years of life and lose ambulation by their early teens; affected individuals usually die from respiratory and cardiac complications in the late teens unless anticipatory care to avoid respiratory and cardiac insufficiency is put in place (Abbs et al., 2010; Bushby et al., 2010; Muntoni et al., 2003).

A milder disorder, Becker muscular dystrophy (BMD), is caused by in-frame *DMD* mutations that preserve the reading frame allowing for the translation of an internally deleted dystrophin protein (Bushby et al., 1993). Some individuals with BMD are asymptomatic and experience no functional difficulties (Melis et al., 1998) while others lose the ability to walk in their late teens or early twenties (Bushby et al., 1993). This large variation in clinical severity is thought to be due to the different functionality and expression levels of the different internally deleted dystrophin proteins (Anthony et al., 2011).

Many patients with DMD, as well as the DMD *mdx* mouse model, have rare dystrophin positive 'revertant' fibres, thought to be due to either secondary somatic mutations or naturally occurring alternative splicing of dystrophin pre-mRNA that can restore the reading frame in DMD (Klein et al., 1992; Lu et al., 2000). We recently extended this observation by characterising in detail a large number of DMD biopsies and reported that this phenomenon is present in approximately 50% of DMD patients (Arechavala-Gomez et al., 2010).

The majority of DMD mutations are deletions accounting for ~65% of cases (Abbs et al., 2010). An out of frame DMD deletion can be converted into an in-frame BMD deletion by the skipping of one or more exons using antisense oligomers (AOs) targeted to splicing elements (Wood et al., 2010). The skipping of exon 51 could rescue the largest group (13%) of DMD mutations. Following the recent demonstration of clinical safety and biochemical efficacy of both locally and systemically delivered AOs targeting exon 51 (eteplirsen and PRO051/GSK2402968) (Cirak et al., 2011; Goemans et al., 2011; Kinali et al., 2009; van Deutekom et al., 2007); this promising RNA-based gene therapy is currently undergoing phase II/III clinical trials.

Due to the low abundance of dystrophin mRNA (Chelly et al., 1990), the efficacy of AOs to induce exon skipping is typically first assessed at the transcriptional level by semi-quantitative nested RT-PCR, after up to 70 cycles of cumulative amplification. However, semi-quantitative end point detection is extremely variable, relies on imprecise size discrimination and is not reliable when the

exon skipping efficiency is low. One study using the *mdx* mouse concluded, unsurprisingly, that exon skipping was overestimated when quantified using two rounds of amplification, and therefore that nested PCR should not be used (Spitali et al., 2010). However, a quantitative assessment of dystrophin exon skipping remains lacking from clinical studies and is needed for the standardisation of methodology across different groups in order to assess and compare patient response in exon skipping clinical trials. Development of an exon skipping qPCR assay has been slow, previous studies have used only tissue from the *mdx* mouse and AO transfected patient derived fibroblasts (Ferlini and Rimessi, 2012; Spitali et al., 2010; Spitali et al., 2009) where exon skipping is readily detected. These studies do not address the challenges associated with quantifying exon skipping in DMD patients in whom muscle tissue is severely affected; in particular it remains unclear whether one round of amplification is sufficient to detect exon skipping in treated patient muscle tissue. A micro fluidic digital PCR platform, the FluiDMD, has recently been described whereby 85 TaqMan assays recognising 76 out of 78 *DMD* exon junctions are simultaneously analysed (Bovolenta et al., 2012). The FluiDMD has been successfully validated on both patient muscle tissue, cultured myoblasts and Myo-D transformed fibroblasts and provides a valuable diagnostic tool for the molecular profiling of DMD and other dystrophinopathies. Nevertheless, the use of micro fluidic technology containing 85 assays is expensive and alternative ways to quantitatively measure exon skipping are desirable, especially for the optimisation of AO target sequences and multi-centre clinical trials.

In this study we have designed and validated TaqMan assays for skipped and unskipped dystrophin targets and highlight their use to determine the levels of exon skipping in DMD patients treated intramuscularly with the morpholino AO eteplirsen. Our study provides the first quantitative assessment of exon skipping in a clinical trial setting and a simplified, standardised method to quantitatively assess patient response in future pre-clinical optimisation studies and exon skipping clinical trials for DMD.

MATERIALS AND METHODS

Patients

Skeletal muscle biopsies were obtained with informed consent during a proof of principle, dose escalation study (ClinicalTrials.gov number: NCT00159250) to test the safety and efficacy of an intramuscular (IM) administration of eteplirsen (AVI-4658) (Kinali et al., 2009). All applicable regulatory requirements were met (UK Medicines and Healthcare Products Regulatory Agency) and ethical approval was obtained from the UK Gene Therapy Advisory Committee and local research ethics committees.

Seven DMD patients with deletions amenable to exon 51 skipping were investigated (table 1): two patients received a low dose of 0.09 mg of the AO eteplirsen (AVI-4658) while 5 patients received a high dose of 0.9 mg of the same compound into the extensor digitorum brevis (EDB) muscle; the contralateral muscle received an equivalent volume of normal saline. Bilateral EDB biopsies were obtained 3-4 weeks post treatment and cut into three 6x6x7mm muscle blocks. For the purpose of this study, we analysed only the five high dose patients as the two patients in the low dose cohort showed very little exon skipping and dystrophin protein restoration (Kinali et al., 2009) (table 1).

Nested RT-PCR and molecular cloning

To generate template DNA for qPCR optimisation experiments, nested RT-PCR products from AO treated patient-derived fibroblasts were used (Arechavala-Gomez et al., 2007; Kinali et al., 2009). To sequence qPCR products and to generate plasmid DNA templates, qPCR or nested RT-PCR products were gel purified and ligated into the pGEMteasy TA cloning vector (Promega); successful cloning was confirmed by sequencing.

Quantitative real time PCR

Total RNA was extracted from treated or untreated frozen muscle sections from three different muscle blocks per patient using Trizol (Invitrogen) as per the manufactures instructions. Isolated RNA was treated with TURBO DNase (Ambion) and quality assessed using a NanoDrop (Thermo Scientific) and an Agilent bioanalyser 2100. All samples were of sufficient quality for qPCR with RNA integrity numbers (RIN) ranging between 7-8 and A260/280 ratios of at least 1.7. Reverse transcription was performed on 0.5 µg of total RNA per sample using the superscript III first strand synthesis system (Invitrogen) with random hexamers.

Custom TaqMan MGB probes and primers were designed using the primer express software (Applied Biosystems), sequences are provided in table 2. To specifically detect exon 51 skipped transcripts, a skipped assay was designed for each deletion; a universal assay for unskipped transcripts was designed to target the exon 51/52 boundary (figure 1). A single endogenous control was selected due to tissue limitations and space on the plate. 18S was chosen from a panel of six endogenous controls (18S, GAPDH, RPLPO, TBP, myotilin and α -actinin-2) using a combination of

computational analysis software (geNorm and NormFinder) and an evaluation of the standard deviation between Ct values in 10 samples.

To quantify exon skipping, skipped and unskipped transcripts were specifically and unbiasedly preamplified for 14 cycles (95°C for 10min followed by 14 cycles of 95°C for 15sec and 60°C for 4min) using the TaqMan PreAmp master mix (Applied Biosystems). Preamplification was performed in a total volume of 25µl using 5µl diluted cDNA (1 in 5), 12.5µl of PreAmp mastermix and 6.25µl of pooled assay mix (0.2X). The pooled assay mix was prepared by combining equal amounts of each TaqMan assay (figure 1) diluted in 1X TE buffer so that the final concentration of each assay was 0.2X. The final concentration of each TaqMan assay in the preamplification reaction was 0.05X. We validated, according to the manufactures instructions, that all skipped and unskipped Taqman assays used were preamplified uniformly without bias thus ensuring that the differential expression levels between samples is maintained after preamplification (figure 2B). The endogenous control, 18S, was excluded from preamplification because it is so highly expressed.

Preamplified cDNA was diluted 1 in 5 and analysed in triplicate by qPCR using the StepOnePlus real-time PCR system for 40 cycles (50°C for 2 min, 95°C for 10min followed by 40 cycles of 95°C for 15sec and 60°C for 1min). Reactions contained 6.25µl of diluted cDNA, 1.25µl of TaqMan probe and 12.5µl of TaqMan universal PCR master mix (Applied Biosystems) in a total volume of 25µl. Skipped, unskipped and 18S transcripts were detected separately by dividing the cDNA into three aliquots on the same reaction plate. Data was analysed using the LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009), which uses linear regression analysis to calculate the PCR efficiency of each individual well. To calculate percent exon skipping, the mean PCR efficiency (E) per amplicon (table 2) and the mean Ct value per sample were used to calculate the starting concentration (N0) using the equation $N0 = N_t / E^{Ct}$ where N_t is the fluorescence threshold (Ramakers et al., 2003; Ruijter et al., 2009). Samples assigned a N0 value of -999 by the LinRegPCR program failed quality control checks and were excluded from the analysis. Data were normalised to 18S and presented as percent exon skipping $[(N0 \text{ skipped} / N0 \text{ skipped} + N0 \text{ unskipped}) * 100]$.

Immunohistochemistry

Unfixed frozen serial EDB muscle sections (7 µm) were incubated with primary antibodies for 1 hour at room temperature followed by three washes in phosphate buffered saline (PBS). Sections were then incubated with anti-mouse or anti-rabbit biotinylated IgG secondary antibodies (GE Healthcare UK, 1:200) for 30 minutes at room temperature. After washing with PBS, muscle sections were incubated with streptavidin conjugated to Alexa 594 (Invitrogen UK, 1:1000) for 15 minutes at room temperature then washed and mounted using Hydromount mounting medium (National Diagnostics, USA). For the study of revertant fibres, exon specific anti-dystrophin antibodies were obtained from the MDA monoclonal antibody resource, Prof. Glenn Morris, Oswestry, UK with the exception of Dys 2 (exon 79, Novacastra, UK). The antibodies used were MANDYS106 (exon 43), MANEX44A (exon 44), MANEX45A (exon 45), MANEX46B (exon 46), MANEX47 (exon 47), MANEX4850A (exons 48-50), MANEX50 (exon 50), MANHINGE3C (exon 51), and MANEX5556A (exons 52-59). To confirm

the sarcolemmal integrity of the muscle fibres, a β -spectrin antibody (Novacastra, UK) was used. The original analysis of the biopsies was performed using MANDYS106 (Kinali et al., 2009). Sections were evaluated using a Leica DMR microscope interfaced to MetaMorph (Molecular Devices, USA).

RESULTS

Assay validation

TaqMan assays were designed to specifically detect skipped and unskipped transcripts (figure 1, table 2). Due to the high sequence similarity between exon 51 skipped and unskipped dystrophin transcripts, the specificity of the skipped and unskipped TaqMan assays (figure 1) were tested using either skipped or unskipped plasmid DNA as a template (figure 2A). In figure 2A, skipped template DNA was detected by the $\Delta 48-50$ skipped probe but not by the unskipped probe and vice versa for the unskipped template demonstrating the high specificity of the $\Delta 48-50$ skipped probe. This was also confirmed by an agarose gel of the PCR products where the skipped band is only present in the lane corresponding to the skipped template amplified with the skipped probe (figure 2A). The same procedure was followed to validate the TaqMan exon skipping assays designed for all the deletions, and the results confirmed that all assays are specific (data not shown).

To determine whether skipped and unskipped probes have similar efficiencies and to ascertain the dynamic range (the range of initial template concentrations over which accurate C_t values are obtained) of the assays, standard curves were generated using serially diluted PCR product containing both skipped and unskipped templates for a particular deletion. All assays had very similar efficiencies of at least 90% and similar dynamic ranges of between 10 and 32 cycles (figure 2B).

When using AO-treated DMD muscle tissue without any preamplification, we observed that skipped targets were infrequently detected with C_t values of >35 cycles. It is accepted that outside the dynamic range ($>30-35$ cycles) qPCR becomes less reliable and much less efficient. To overcome this problem, we performed cDNA preamplification using the TaqMan PreAmp master mix, which unbiasedly preamplifies specific cDNA targets. In order to confirm that the dystrophin targets are indeed preamplified uniformly without bias, we performed a relative quantification experiment on all skipped and unskipped TaqMan assays to compare amplification of cDNA (yielding C_t values of <35 obtained from AO transfected patient-derived fibroblasts) to the amplification of preamplified cDNA as described in the user manual. A $\Delta\Delta C_t$ between cDNA and preamplified cDNA of $0 (\pm 1.5)$ indicates preamplification uniformity. All TaqMan exon skipping assays used in this study are preamplified uniformly with $\Delta\Delta C_t$ values within ± 1.5 of zero (figure 2C).

Thus, we have designed and validated several TaqMan exon skipping assays and demonstrated their specificity, efficiency and preamplification uniformity.

Quantification of exon skipping in muscle from DMD patients treated with eteplirsen

We next used the skipped and unskipped TaqMan assays to quantify exon skipping in DMD patients treated intra-muscularly with the AO eteplirsen. The muscle biopsies of these patients have been thoroughly characterised biochemically by nested RT-PCR, semi-quantitative immunohistochemistry and western blotting (Kinali et al., 2009) thus providing a valuable benchmark from which to evaluate novel methodology. For each patient, RNA from untreated and treated biopsies were analysed with

the TaqMan probes appropriate for their deletion; biological replicates were performed on three different muscle blocks from each biopsy. Data analysis was performed using the LinRegPCR programme which calculates efficiency corrected starting concentrations (N0) using a mean PCR efficiency per amplicon (the mean efficiencies for each assay were comparable and are provided in table 2) and data are presented as percent exon skipping $\pm$ SD (figure 3A). Previous nested RT-PCR analysis confirmed exon skipping in all seven patients after 70 cycles, but this was not designed to provide any quantitative information (Kinali et al., 2009). Here we demonstrate that, using TaqMan qPCR, exon skipping among clinical trial patients can be more reliably compared. An increase in exon skipping post treatment was observed in all high dose patients tested, although patient 5 had equivalent exon skipping in both untreated and treated biopsies and the lowest number of positive fibres post treatment (table 1, 44%) as determined in the original study (Kinali et al., 2009). These results highlight the need to analyse untreated/pre-treatment biopsies; incidentally the two patients (4 and 7) with no detectable skipping in untreated samples were in the top three best responders at the protein level (table 1, patients 4, 6 and 7). The fact that we detected a range of exon skipping levels including zero percent in untreated samples demonstrates that, unlike nested RT-PCR, this assay is ideally suited to quantify the variable levels observed in response to AO-mediated exon skipping.

Figure 3A highlights substantial variability in exon skipping between muscle blocks from the same biopsy in several patients. For example, there is no exon skipping observed in block 2 of patient 4, whilst 87% skipping was observed in block 1 accompanied by 79% dystrophin positive fibres (table 1). Patient 4 is thus considered a top responder due to on average having a large post treatment increase (48%) across all three blocks. Indeed we have previously highlighted the fact that dystrophin restoration following AO administration is not homogeneous (Cirak et al., 2011). Additionally, muscle fibres close to injection sites are likely to have the highest levels of exon skipping and dystrophin restoration (Kinali et al., 2009).

We next sought to determine whether our quantification of exon skipping correlated with the restoration of sarcolemmal dystrophin protein expression. We re-evaluated the captured immunohistochemical data from all three blocks of treated and untreated muscle biopsies from the clinical trial (Kinali et al., 2009) to confirm that the variability observed by qRT-PCR also corresponds to variability in dystrophin restoration between different muscle blocks (figure 3A). It was clear from the original study that patient 4 was the best responder in terms of the intensity and percentage of dystrophin positive fibres (table 1); this analysis was performed on only one of the three available frozen-blocks. A simple comparison between the qRT-PCR analysis and immunohistochemistry of the three muscle blocks of this patient confirms that block 2, having minimal exon skipping, also had minimal dystrophin protein restoration whilst blocks 1 and 3 have high levels of exon skipping (87% and 58% respectively) and high levels of dystrophin restoration. Indeed, for all five high dose patients, the variability observed by qRT-PCR between the muscle blocks matched the variability observed with immunohistochemistry (figure 3A). Therefore we postulate that the inconsistency observed at the immunohistochemical level is entirely due to the efficiency of exon skipping; most likely dependent on uneven AO diffusion in muscle following direct injections demonstrating that the

areas of the biopsy that responded best to local administration of AV1-4658 can be reliably identified at the RNA level by qRT-PCR.

In order to rank patients according to their exon skipping levels we considered both an average of all three muscle blocks and the “best blocks” alone (figure 3B); the latter was used for previous IHC analysis (table 1) and was therefore used to compare patient ranking orders ascertained by qRT-PCR and IHC (figure 3B). We defined the best blocks as those with the highest level of exon skipping, this ranking matched with the best blocks used for the original study (table 1). Patient 4 was unequivocally the best responder. Whilst patient 3 has high exon skipping levels post treatment (best block, 70%), the intensity of dystrophin positive fibres was the lowest of the five patients (22% of control) and there was also significant exon skipping detected in the untreated biopsy. The best block of patient 5 had little increase in exon skipping post treatment and the lowest percentage of dystrophin positive fibres; however those fibres were of a high intensity.

Detection of revertant fibres by qRT-PCR

During this study, we consistently detected low levels of exon skipping in the untreated biopsies of some patients (3, 5 and 6) by qRT-PCR that were undetectable by previous nested RT-PCR analysis (Kinali et al., 2009). The skipping detected in the untreated samples of patient 6 was minimal compared to patients 3 and 5 who also had the lowest post-treatment levels of dystrophin expression (figure 3 and table 1). These natural skipping events normally result in the presence of revertant fibres. We therefore investigated whether the amplification in the untreated samples corresponds to exon 51-skipped revertant fibres. The untreated qPCR amplification plots for patient three were compared with the original immunohistochemistry pictures and pathology reports (Figure 4A). Block three had the highest level of amplification by qPCR and was also noted to contain several revertant fibres during the original immunohistochemistry analysis. In fact, we previously reported that the untreated biopsy of this patient had 5% dystrophin positive fibres compared to the <1% reported for other patients (Table 1) (Kinali et al., 2009). The qPCR product amplified by the skipped probe in the untreated biopsy of patient 3 was subsequently run on an agarose gel. A single band of the expected molecular weight was gel purified and cloned into a TA cloning vector for sequencing; its identity was confirmed as exon 51 skipped dystrophin mRNA (figure 4A). Thus the exon skipping assay we have developed is sensitive enough to detect low abundant transcripts responsible for revertant fibres.

To further assess the exon composition of the revertant fibres in this $\Delta 48-50$ patient, we performed immunohistochemistry on serial sections stained with exon specific antibodies (figure 4B). We captured images of a large cluster of revertant fibres expressing dystrophin containing exons 43 to 47, 52 to 59 and exon 79; the integrity of these fibres was demonstrated by spectrin staining. Sarcolemmal staining of exons 48-51 was absent as predicted from the patient's mutation. Thus our method of exon skipping quantification reliably detects very low levels of skipping and provides a true reflection of response taking into account the low starting levels detectable in several patients.

DISCUSSION

Nested RT-PCR is currently used to assess AO efficacy in pre-clinical studies and exon skipping clinical trials for DMD, however this method is not reliable enough to assess patient response at the RNA level in treated muscle tissue. Here, we describe a method that accurately quantifies exon skipping in samples where the template is very low. This allows us for the first time to compare the correction of the reading frame at the RNA level with the restoration of sarcolemmal dystrophin expression in AO treated human DMD muscle. A previous study on exon skipping in the *mdx* mouse reported that AOs that induce high levels of exon skipping also lead to higher dystrophin levels than less efficient AOs (Heemskerk et al., 2009). We demonstrate in AO treated patient muscle tissue from a recently completed clinical trial (Kinali et al., 2009) that, when analysed with untreated, the best responders and muscle blocks can be readily identified at the RNA level by qRT-PCR providing additional quantifiable data on the response to AO administration.

Whilst we agree with the conclusion of an earlier paper (Spitali et al., 2010) that nested RT-PCR is not a reliable method for exon skipping quantification, we report that pre-amplification of cDNA remains necessary for the accurate detection and quantification of exon skipping in DMD affected muscle tissue. Our method of pre-amplification offers several advantages over traditional nested RT-PCR: firstly pre-amplification is performed for only 14 cycles rather than the 30-35 cycles typically used in nested PCR, secondly skipped and unskipped targets are specifically pre-amplified rather than all the transcripts present in cDNA and finally, specific targets are pre-amplified uniformly without bias – an assurance which can be validated by the user. Whilst digital PCR (dPCR) can offer an absolute quantification on limited samples, the cost of performing dPCR is currently at least fourfold greater than the method we describe here. In addition, using a multiplex dPCR assay, Spitali et al (Spitali et al., 2010) observed VIC to FAM channel bleeding with both skipped and unskipped probes that can influence quantification.

In our cohort of five patients, the untreated samples of three patients had levels of skipped transcripts large enough to be identified by our quantitative assay, nevertheless two of these patients still showed a clear increase in exon skipping post treatment. In contrast to our findings in this study, our previous analysis (by nested RT-PCR) of patient 5 did not show any skipping in the untreated sample (Kinali et al., 2009). This is likely due to the improved sensitivity and specificity of the current assay as well as the fact that the current study was performed on additional blocks of the muscle biopsy than the original nested RT-PCR and immunohistochemical analysis. Thus, given the patchy nature of both revertant fibres and dystrophin restoration (Arechavala-Gomeza et al., 2010; Cirak et al., 2011; Kinali et al., 2009) it is possible that the untreated sample from the original study contained levels of skipping below the detection limit of the less sensitive nested RT-PCR.

We present an exon skipping assay that is reliable enough to easily identify responding patients among clinical trial cohorts, and sensitive enough to accurately quantify any low levels of that particular transcript present in revertant fibres – this could be especially useful for future trials involving patients that have a higher proportion of revertant fibres. Indeed, high levels of trace and

revertant dystrophin expression have been reported in patients with deletions skippable for exon 44 (Lourbakos et al., 2011). Our finding that revertant fibres can result, in some instances, from the skipping of just one additional exon rather than larger deletions is supported by a study of a patient with a deletion of exon 45; in this study they found 15 revertant fibres with a deletion of a single exon (exon 44 or 46) (Thanh et al., 1995). Thus our study highlights the need to precisely quantify the levels of pre-treatment exon skipping for each patient in future clinical trials.

The formation of rare shorter fragments due to the activation of a cryptic splice site in exon 51 has been described in control and patient myoblasts after AO treatment (Aartsma-Rus et al., 2002; Aartsma-Rus et al., 2003). It is important to note that the skipped TaqMan probes used in our method will not detect such fragments, although our previous nested RT-PCR analysis did not reveal their presence (Kinali et al., 2009) indicating that the AO eteplirsen does not induce cryptic splice site activation in exon 51. Rather, our method specifically detects only correctly skipped transcripts which, coupled with providing an accurate base line skipping level, provides a true reflection of exon skipping in treated patients.

Whilst dystrophin protein restoration is the primary biochemical outcome measure for exon skipping clinical trials, there remains a need to quantify dystrophin at the RNA level. For example, it could be of interest to compare the levels of dystrophin mRNA and regulatory miRNAs involved in translational regulation of dystrophin expression, as this may account for variability in patient response (Cacchiarelli et al., 2011a; Cacchiarelli et al., 2011b). Furthermore, the method we describe here provides a way to measure the efficiency of response in vitro (using patient fibroblast cultures) thus enabling the identification of good and poor responders' prior to treatment. Whilst numbers are low in our study, the widespread use of this method will provide further information on patient variability.

The use of eteplirsen treated muscle from a previous study (Kinali et al., 2009) provided us with valuable benchmarks from which to assess our novel exon skipping assay. Retrospective analysis confirmed matching high variability in both exon skipping and dystrophin protein restoration between different muscle blocks from the same patient. This highlights an important issue regarding the often patchy nature of dystrophin restoration (Cirak et al., 2011; Kinali et al., 2009) and the need to test several muscle blocks.

In summary, we describe a TaqMan qRT-PCR assay to reliably quantify exon skipping in muscle from AO treated DMD patients. In addition the assay is easily adaptable to assess the skipping of other target exons providing a reproducible and standardised method to quantitatively assess levels of exon skipping in future pre-clinical and clinical trials for DMD.

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AUTHOR DISCLOSURE STATEMENT

FM serves on scientific advisory boards for Acceleron Pharma, Genzyme, AVI BioPharma, Debiopharm Group, GlaxoSmithKline, Prosensa, and Santhera Pharmaceuticals, receives research support from AVI BioPharma, and has received funding for trials from AVI, Trophos, and PTC. KB has served on scientific advisory boards for Acceleron, AMT, AVI BioPharma, Debiopharm Group, Genzyme, GlaxoSmithKline, Prosensa, PTC, and Santhera Pharmaceuticals and has received funding for trials from AVI and PTC. VS has served on scientific advisory boards for Acceleron and Genzyme and has received funding for a trial from GlaxoSmithKline. All other authors declare that they have no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Design of TaqMan assays. TaqMan assays were designed to quantify AO-mediated exon 51 skipping. To specifically detect unskipped transcripts containing exon 51, forward and reverse primers in exons 51 and 52 and a TaqMan MGB probe spanning the exon boundary were designed according to the guidelines of the primer express software. To specifically detect exon 51 skipped transcripts, a skipped assay was designed for each deletion, all with reverse primers in exon 52 and forward primers and probes positioned as indicated.

Figure 2. TaqMan assay validation. **A.** The specificity of each TaqMan assay was confirmed by testing both skipped and unskipped probes on either skipped or unskipped plasmid DNA templates. Representative data is shown for the $\Delta 48-50$ assay. **B.** The efficiency of skipped and unskipped probes was assessed using a standard curve prepared from a PCR product containing both templates. Representative data are shown for the $\Delta 48-50$ assay; skipped and unskipped probes have similar efficiencies of at least 90%. Skipped slope = -3.505, $R^2 = 1$, efficiency = 92.9%; unskipped slope = -3.578, $R^2 = 1$, efficiency = 90.3%. For all assays, amplification was >90% efficient for Ct values between 10 and 32. **C.** All TaqMan exon skipping assays are uniformly preamplified without bias demonstrating that differential expression levels between samples are maintained after preamplification. The $\Delta\Delta Ct$ between cDNA and preamplified cDNA ($\Delta\Delta Ct = \Delta Ct_{(preamp)} - \Delta Ct_{(cDNA)}$) was within the recommended range (0 ± 1.5) for all probes tested $n=3$.

Figure 3. Exon skipping quantification of DMD patients treated intra-muscularly with eteplirsen. **A.** Exon skipping in the five high dose patients is presented as percent exon skipping ($[(N0 \text{ skipped}/N0 \text{ skipped} + N0 \text{ unskipped}] * 100$). For each patient, three muscle blocks were analysed and plotted individually $\pm SD$; the standard deviation between triplicates was always lower than 0.33. The average Ct value for unskipped transcripts after preamplification was 19 ± 0.85 ; for skipped transcripts (that passed quality control) the Ct values ranged between 21 and 29. Mean efficiencies used to calculate N0 are given in table 2. Retrospective immunohistochemical analysis of patient biopsies demonstrates that the variability between the muscle blocks is observed by both qRT-PCR and immunohistochemistry. Scale bar = 20 μm . **B.** Exon skipping levels are presented as an average of all three muscle blocks and the “best blocks” alone $\pm SD$. The best blocks were as follows: patient 3 (block 3), patient 4 (block 1), patient 5 (block 3), patient 6 (block 2) and patient 7 (block 3). The table compares the ranking order of the best blocks determined by qRT-PCR and IHC.

Figure 4. Detection of revertant fibres by qRT-PCR. **A.** Skipped amplification plot for untreated patient 3 and representative immunohistochemistry images (7 μm sections) for each muscle block (6x6x7mm). Sequencing of the qPCR product confirmed an exact exon 47-52 boundary. **B.** Analysis

of revertant fibres in block three using exon specific antibodies confirmed the absence of exons 48-51. Scale bar, 20 μm .

TABLE 1: Patient characteristics

Patient	<i>DMD</i> deletion	Dose	Dystrophin intensity relative to control		Percent dystrophin positive fibres	
			Untreated	Treated	Untreated	Treated
1	14 bp deletion in intron 49 including the 3' acceptor splice site	Low	9%	6%	n.d	n.d
2	Exon 50	Low	8%	6%	n.d	n.d
3	Exons 48-50	High	11%	22%	5%	49%
4	Exons 48-50	High	14%	32%	<1%	79%
5	Exons 45-50	High	10%	31%	<1%	44%
6	Exons 45-50	High	8%	25%	1%	65%
7	Exons 49-50	High	4%	22%	<1%	63%

TABLE 2: TaqMan assay sequences

Assay	Forward primer	Reverse primer	Probe	Product length (bp)	Mean PCR efficiency†
Unskipped	(exon 51) GTGATGGTGGGTGACCTTGAG	(exon 52) TTTGGGCAGCGGTAATGAG	CAAGCAGAAGGCAACAA	115	1.78
Δ45-50 skipped	(exon 44) TTGGGAACATGCTAAATACAAATGG	(exon 52) GACGCCTCTGTTCCAAATCC	ATCTTAAGGCAACAATG	65	1.78
Δ48-50 skipped	(exon 47) TGAAAATAAGCTCAAGCAGACAAATC	(exon 52) GACGCCTCTGTTCCAAATCC	CAGTGGATAAAGGCAACA	71	1.71
Δ49-50 skipped	(exon 48) CAACCAAACCAAGAAGGACCAT	(exon 52) GACGCCTCTGTTCCAAATCC	TGACGTTCAAGGCAACA	64	1.74

† Mean experimental efficiencies used to calculate starting concentration, NO. All assays had efficiencies of >90% when determined using the standard curve method on unlimited samples (Fig. 2B).