Investigating the subcellular localisation and function(s) of dystrophin protein Dp71 isoforms in glioma

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Abstract

The Dp71 protein is the most expressed product of the DMD gene in the nervous system. Mutation in the region codes dystrophin protein (Dp71) linked to cognitive disturbances in Duchenne muscular dystrophy (DMD) patients. There is growing evidence that the gene is contributing to the development of Central Nervous System related cancers. The aim of this study is to characterise the role of the main 4 Dp71 isoforms by investigating its subcellular localisation and putative cellular functions in glioblastoma cells, the most aggressive and common type of glioma. By transfecting the four GFP-Dp71 constructs into a well characterised human glioblastoma cell line – U251-MG. Immunoblotting was used to assess Dp71 expression in human glioblastoma cell line. Moreover, we examined the subcellular localisation and the effect of Dp71 over expression on the nuclear Lamin B1, cell migration immunofluorescence, and scratch assay. A 71 kDa endogenous Dp71 was expressed in all glioblastoma cells and only GFP-Dp71a (99 kDa) isoform was overexpressed in the transfected cells. Lower Lamin B1 fluorescence intensity and abnormal nuclear shape was observed in cells overexpressing GFP-Dp71a. Furthermore, cytoplasmic and nuclear localisation of Dp71 isoform was found in both cytoplasm and nucleus, but higher in the nucleus. Overexpression of Dp71a transfected cells reduced the cell migration and covering the scratched tissue gaps with 5% and 6%, whilst that of the control cells were 29% and 50% at time 24 and 48 hours, respectively compared to the that at time 0. Dp71ab transfected cells showed similar cell migration to the control. We concluded that Dp71 overexpression had a clear effect on the expression of Lamin B1 and cell migration. Additionally, localisation of Dp71a was higher in the nucleus than in the cytoplasm.

Keywords: DMD; Dp71; glioblastoma; Lamin B1; cell migration
1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease, mostly affects boys who lack the dystrophin protein (Dp). In 1987, the DMD gene was identified to be the cause of a considerable disorder (Koenig et al. 1987). Depending on how the dystrophin gene expression is disrupted in case of DMD gene mutations, DMD or a milder form, known as Becker muscular dystrophy can be developed (Muntoni et al. 2003). DMD is a severe disease and affected patients usually developing the features of severe progressive skeletal muscle wasting associated with movement difficulties and reduced life expectancy (Mercuri 2019). Since the Dp71 is the most expressed product of the DMD gene in the nervous system and the mutations in the Dp71 region are linked with cognitive impairment, the relation of DMD with tumour progression needs to be further studied in different types of tumours (Ruggieri et al. 2019).

In addition to the gene responsibility for DMD, there is growing evidence that it is also contributing to the development of different types of cancer such as, but not limited to, those related to the central nervous system (CNS), including gliomas (Jones et al. 2021). Gliomas are representing a common type, constituting more than 30% of brain tumours. Glioblastoma is the most aggressive type of gliomas with a poor therapeutic response and a high mortality rate, usually between 16–21 months (Ladomersky et al. 2019). Furthermore, it was found that the DMD gene is involved in the myogenic and non-myogenic tumour progression, which showed that the majority of people with DMD gene alterations and expression had a poorer overall survival (Luce et al. 2016). In several cancers, such as leukaemia's, lymphomas, sarcomas, melanomas, tumours of the nervous system, and various carcinomas, it was found that the DMD gene is disrupted. The recurrent loss of 5’ exons might contributes to Dp427
gene product inactivation, whilst the Dp71 is maintained. Hence, it was hypothesised that 
*DMD* is implicated in tumour tumorigenesis (Jones *et al.* 2021).

The Anthony/Machado laboratory and others, propose that the ratio of Dp427 verses Dp71 
is important in tumorigenesis, whilst *DMD* alterations are uncommon in low-grade glioma 
(LGG), survival and multivariate Cox analysis of RNAseq data derived from LGG tumour 
tissue strongly implicates *DMD* gene expression in survival outcomes, and Dp71 isoforms, as 
independent prognostic markers for LGG (Wang *et al.* 2014, Mauduit *et al.* 2019, Naidoo *et al.* 2022). Dp71 is ubiquitous and strongly expressed as the predominant type of dystrophin 
found in neurones and glia.

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*Figure 1. The nomenclature of Dp71 isoforms. The isoforms are arranged in groups depending on the C-terminus (Naidoo & Anthony 2020).*

The *DMD* gene is located at position Xp21.2-p21.1 of the short arm of the X chromosome. 
It is one of the largest human genes of around 2.5 Mbp and consists 79 exons (Laing *et al.* 2011). The DP is mainly expressed in skeletal muscles with 427 kDa at its full length 
(Dp427) (Muntoni *et al.* 2003). The function of Dp427 is to connect actin cytoskeleton to the
extracellular matrix playing an important role in the support of muscle integrity. Smaller Dp, namely Dp71, are produced by independent promoters, the most predominant and ubiquitous in the brain are produced by independent promoters (Tadayoni et al. 2012). There are at least 14 isoforms of Dp71 (Fig. 1) (Naidoo & Anthony 2020). The 4 main isoforms, Dp71, Dp71a, Dp71b and Dp71ab, differ by presence or absence of exons 71 and 78 (Fig. 2) (González et al. 2000). Depending on cell type, there was a controversy over their exact function and cellular localization (Naidoo & Anthony 2020).

![Figure 2: Illustrative graph of the Dp71 cDNAs fused with the GFP gene’s C-terminus controlled by the cytomegalovirus (CMV) promoter and contain polyadenylation signal (poly A) of bovine growth hormone. The horizontal lines indicate the 71 and 78 spliced exons (González et al. 2000).](image)

Given the colocalization of Lamin B1 with Dp71, both play a role in nuclear scaffolding processes that participate in nuclear architecture and reduction in Dp71 associated with Lamin B1 overexpression that demonstrated in glioblastoma cells (Ruggieri et al. 2019). Lamin B1 is one of the nuclear lamina filament proteins, It is located adjacent to the inner nuclear membrane which are responsible of organizing and regulating the shape of the nucleus (Melcer et al. 2007, Garvalov et al. 2019). It has a key role in maintaining the cell proliferation, formation of mitotic spindle, DNA replication and gene transcription. In addition, knockout mice revealed that Lamin B has an essential involvement in neuronal survival and brain development by playing a role in the neuronal migration (Jung et al. 2013). Reduction or absence of Lamin B1 production was found to be related to lung cancer. As
well as it has an effect on the expression of some genomes that has specific regions which Lamin B1 bind to (Guelen et al. 2008).

Studies showed decrease in Lamin B1 expression correlates with several cancer types. Although it has been observed that Lamin B1 was expressed in all tumours, there was a clear reduction in the expression or presence in lung cancer and gastrointestinal tract neoplasms (Broers et al. 1993, Moss et al. 1999). On the other hand, there is an increase in the expression of Lamin B1 and relation with the tumour differentiation and high rate of mortality in liver, prostate and clear cell renal cell carcinoma cancers (Coradeghini et al. 2006, Sun et al. 2010, Radspieler et al. 2019). As well as poorer clinical outcome in pancreatic and colon cancers (Li et al. 2013, Izdebska et al. 2018).

The aim of this study is to characterise the role of Dp71, Dp71a, Dp71b and Dp71ab isoforms in glioma cells, by investigating its subcellular localisation and putative cellular functions. This is pertinent to exploring the role of Dp71 in tumorigenesis as well as better understanding the neuropathophysiology of Duchenne Muscular Dystrophy. By transfecting these constructs into a well characterised human glioma cell line – U251-MG to determine their subcellular localisation and the effect of Dp71 over expression on the nuclear Lamina B1 and cell migration.
2. Materials and Methods

In our investigation we used U251-MG cell line supplied by the American Type Culture Collection (ATCC). These cells were transfected with the four GFP-tagged Dp71 constructs, gifted from the González et al. 2000, for phenotypic and functional studies. They are:

- Dp71 = +71/+78 (contains both exons 71 and 78 sequence)
- Dp71a = -71/+78 (Lacks exon 71 and contains exon 78 sequence)
- Dp71b = +71/-78 (contains exon 71 and lacks exon 78 sequence)
- Dp71ab = -71/-78 (lacks both exons 71 and 78 sequence)

2.1 Tissue culture

U251-MG cell line have been used as a well characterised human glioblastoma. The media used for cell culture was DMEM (Dulbecco’s Modified Eagle Medium) with 10% FBS (Foetal Bovine Serum) and 1% P&S (Penicillin and Streptomycin) incubated at 37°C and 5% CO₂.

2.2 Transfection of U251-MG

Four DNA plasmids of Dp71 isoforms were used to transfect over 90% confluent tissue culture. The Dp71a, Dp71b, Dp71ab and Dp71 (González et al. 2000) were all used to transfect the glioblastoma (U251-MG) cell line.

The cells were seeded to reach high density in 6-well plates. Transfection was done using Lipofectamine 2000 and 2.5μg of DNA plasmids in Opti-mem media and incubated for 12-18 hours.
2.3 Protein harvesting, Protein acrylamide electrophoresis and Western blotting

Protein harvesting

Pre-chilled PBS on ice used to wash the cells while keeping them on ice. Using a pipet add 50 µl, on each well, of 95-100°C pre-heated 2X SDS harvest buffer (100 ml 2X SDS harvest buffer: 100 mM TRIS HCL (157.6 mw) - 1.58g, 4% SDS – 4 ml, and 20% glycerol – 20 ml). Cell scrapers were used to collect the cells while on ice, and the lysate was transferred to pre-chilled Eppendorf tubes. Then 50 µl dH₂O, 10 µl 0.5% bromophenol blue (0.1g bromophenol blue in 20ml dH₂O) and 5µl β-mercaptoethanol were added. The Eppendorf tubes were then heated at 100°C on a heat block for 5 minutes. The protein lysate can be stored at -20°C to be used after short time and -80°C if kept for long term.

Protein acrylamide electrophoresis

Samples were heated at 100°C for 5 minutes, then left on bench to cool down before loading on the gel. The tank was filled with running buffer solution, and 10% polyacrylamide precast gels (GenScript SurePAGE™) were used. The first lane was loaded with 10 µl of a 2:1 Seeblue (SeeBlue™ Plus2 Pre-stained Protein Standard) and Magicmark (MagicMark™ XP Western Protein Standard) mix. The lysate samples were then loaded as a 20µl in each precast gel. Afterward, the gel was ran at 100v for 60 minutes.

Wet transferring step, after removing the gel from the cast and placed in a transfer sandwich (two sponges, four pre-cut filter papers, and one PVDF membrane) on an assembly cassette. The membrane was activated in 100% methanol before placing the gel on it. Run the transfer sandwich in a transfer buffer for 90 minutes on 30v. The membrane was then incubated in a 5g dry milk powder in 200 ml of PBS Tween for blocking, then placed on the rocker for 60 minutes.
Wester blotting

An anti-GAPDH (Santa Cruz) mouse monoclonal 1:2000 in milk solution, and an anti-dystrophin (abcam) rabbit monoclonal 1:500 in milk solution are the primary antibodies used. Then, the milk is replaced with the primary antibody solution and incubated overnight at 4°C. The PVDF membrane were washed three times in PBS Tween for 10 minutes on the rocker each time then incubated with secondary antibodies for 1 hour on the rocker. The secondary antibodies were prepared as GAPDH anti-mouse – 1:2000 in PBS Tween and Dystrophin anti-rabbit - 1:1500 in PBS Tween. Then washed again with PBS for three times 5 minutes each. Keep the blot membranes in PBS until ready for to visualise. Add enhanced chemiluminescent substrate (ECL) solution (SuperSignal™ West Pico PLUS Chemiluminescent Substrate) on the membranes, 1 ml Luminol/Enhancer Solution and 1 ml Stable Peroxide Solution mixture on each membrane, and place in the machine to visualise using Genesys software.

2.4 Immunocytochemistry for Lamin B1

About 4x10^5 cells were seeded in each well of a 6-well plate with sterile cover slips at the bottom of the wells incubated 24 hours to reach over 90% confluency. Transfection was done next day. Cells were washed with warm PBS in the tissue hood and plates were kept in the dark all the time after transfection. Ice cold 100% methanol was added to the wells for fixation and incubated at -20 for 15 minutes. Wells then were washed 3 times with room temperature PBS. Blocking and permeabilising step was done at room temperature using a 5% FBS and 0.1% Triton X-100 in PBS solution submerging the coverslips for 30 minutes. Thereafter, incubate in a moist chamber with primary rabbit antibody (Abcam ab 16048 Rb pAb to Lamin B, 1:1000) anti-Lamin B1 for 1 hour, then washed 3 times with PBS. Incubate with secondary antibody (Alexa fluor 594 goat anti-rabbit IgG, 1:500) for 1 hour in the dark.
Using 2 drops (30-50 µl) of Mount Invitrogen™ ProLong™ Glass Antifade Mountant with NucBlue™ Stain for hard settings, incubated overnight at 4°C. Viewing with fluorescence microscopy was done next morning with x40 objective lens.

2.5 Scratch assay

A 6-well plate was seeded with ~4x10^5 cells per well with 2ml of media. Technical replicates were done by dividing the wells to 3 areas using a marker to draw at the bottom of the wells. Then were incubated at 37°C and 5% CO₂ for 24 hours and transfected them once they reached over 90% confluent by incubating with Dp71a gene fused cDNA plasmids for 5 hours then washed and starved by incubating with Opti-mem for 12-18 hours at 37°C and 5% CO₂. Using a P10 pipette tip to scratch the middle of the wells, wash the cells with Opti-mem and incubate the plates at 37°C and 5% CO₂. Images were taken at time 0, 24 and 48 hours. To analyse the wound healing, we used ImageJ MRI wound healing tool and Microsoft Excel to calculate the mean free cell area and wound healing percentage.
3. Results

3.1 expression of Dp71 transcripts in U251-MG cells

In order to determine the effect of Dp71 expression in glioma cell lines, Dp71 constructs were used. The Dp71 under the control of CMV promoter fused with Dp71 cDNAs spliced for exons 71 and/or 78 fused with GFP were used to investigate the subcellular localisation of Dp71 protein isoforms. To assess the proteins synthesised from the hybrid genes, transfection of human glioblastoma U251-MG cells with the four different plasmids was carried out to analyse the cell lysates. An anti-dystrophin monoclonal antibody and anti-GAPDH monoclonal antibody were used by western immunoblot (Fig. 3). Figure 3B shows the housekeeping protein, which is used a loading control. Clear bands were observed for all samples around 40 kDa. A clear band in the Dpa71a lane was observed (Fig. 3A) with approximately size of 100 kDa, a product of the fused GFP-Dp71a construct. However, we could see faint bands in all the lanes of approximately 70 kDa that correlates to the endogenous expressed Dp71 in all the cells. Note that the molecular weight of Dp71 is 71 kDa and the GFP is 28, which explains the molecular weight overexpressed fused GFP-Dp71 is around 100 kDa and 71 kDa the endogenous Dp71.
3.2 Expression of Lamin B1 and localisation of Dp71 in transfected cells

Following this, to study the localisation of expressed Dp71 in the cells, the transfected cells with Dp71a plasmids were stained with rabbit anti-Lamin B1 antibody. Nucleus blue
staining is used to highlight the cells’ nuclei which helps in confirming the localisation of the nuclear envelop.

The GFP-tagged Dp71a was not expressed in all the cells, however, in the cells expressed it, we can observe intense GFP in the nucleus and distributed in the cytoplasm of the cell. In other words, higher expression of Dp71a in the nucleus with presence in the cytoplasm. The control samples have not showed any GFP expression as expected, while we can visualise GFP expressed in some cells transfected with Dp71ab which we used as an empty vector control.

From the Lamin B1 antibody staining we visualise the expression of Lamin B1 proteins in the nucleus and compare the fluorescence intensity between the transfected with Dp71a, Dp71ab and un-transfected cells. In the cells where GFP-tagged Dp71a is overexpressed we were able observe that the GFP-tagged product did not colocalise with the Lamin B1 protein. However, by visualising a number of cells under the fluorescence microscopy we could see an organised distribution of Lamin B1 protein in the Dp71ab and untransfected cells.

Furthermore, lower fluorescence intesity of the red staining Lamin B1 was observed in the Dp71a transfected cells, therefore, less Lamin B expression. As a result, there is a change in nucleus shape of these cells. In contrast, fluorescence intesity or the shape of the nuclear Lamin B1 in the two controls (Control and Dp71ab) was higher with clear protein rings of Lamin B1 in the vicinity of the inner nuclear membrane.
Figure 4: Representative images of fluorescence microscopy of U251-MG transfected with GFP-Dp71a construct and two controls (Dp71ab as a not overexpressed control). Red antibody staining against Lamin B1, and blue nucleus staining were used.
3.3 U251-MG migration in cells transfected with Dp71

The scratch assay results from the starved U251-MG cells in the presence and absence of dystrophin Dp71a. The cell migration (wound closure) from the control (Control and Dp71ab) and the Dp71a samples at time 0, 24 and 48 hours after the scratch was performed (Fig. 5). We can see difference from the illustrating images of the three transfected wells that the cells migrated more distance to close the gap in the control and Dp71ab than in Dp71a tissue culture.

Figure 5: Representative images of the scratch at three different time points (24 and 48 hours) in the U-251-MG cell line scratch assay of three different transfection conditions; Dp71a, Dp71ab and control. The wound healing assay was employed to evaluate the effect of dystrophin protein Dp71 overexpression, the overexpression of Dp71a showed slower cell migration compared to the other conditions.

The ImageJ analysis of the three technical replicates photos from the three different tissue cultures in Figure 6. The mean cell free area is calculated at the same time points with the Standard Deviation error bars in the graph (Fig. 6A). There is clear reduction in the mean cell free area in the control and Dp71ab conditions at times 0, 24 and 48 hours after the scratch.
compared to the Dp71a sample. In numbers, the control sample scratch in ImageJ measured a mean cell free area of 368631 µm² at time 0 hours, 263293 µm² at time 24 hours and 182786 µm² at time 48 hours. Similarly, the in Dp71ab condition the ImageJ measured 320027 µm², 229518 µm² and 199277 µm² at 0, 24 and 48 hours, respectively. In contrast, the Dp71a scratched plate 363297 µm², 343832 µm and 340824 µm² at 0, 24, and 48 hours, respectively as shown in figure 6A.

Figure 6B shows the wound healing percentage at the three time points between the three different conditions. The cell closure percentage after 24 hours was 29% and 28% for the control and Dp71ab samples, respectively, 50% and 38% for control and Dp71ab after 48 hours, respectively. While the transfected cells with Dp71a have remarkably lower closure percentage, 5% after 24 hours, and 6% after 48 hours.
4. Discussion

In this study, for the first time, we have investigated the glioblastoma U251-MG cell line transfected with the Dp71 constructs. We demonstrated the effect on cell migration, Lamin B1 expression and localisation of dystrophin protein, and compare it to non-expressed Dp71
cDNA transfected and un-transfected cells. There was a limited number of studies investigating the role of Dp71 constructs in glioblastoma and its effect on cell characteristics.

We found a clear overexpression of Dp71a isoform in the glioblastoma cells. A similar finding was reported from Ruggieri et al. demonstrate the overexpression of Dp71a in transfected glioblastoma cells (Ruggieri et al. 2019). Additionally, this study showed low expression of endogenous 71 kDa dystrophin protein in the cell lysate of all transfected and control samples. These results were in agreement with another study showed that Dp71 protein is normally expressed in the tumour cells, while the full dystrophin protein Dp427 is inactivated (Jones et al. 2021). On the other hand, an earlier study showed that Dp71 expression was decreased in glioblastoma cells (Ruggieri et al. 2019). However, Dp71ab, Dp71b and GFP-Dp71 plasmids did not overexpress the dystrophin protein isoforms in the transfected cells.

Although the Dp71ab DNA plasmid did not express the dystrophin isoform protein, we observed GFP expression in the cytoplasm of the transfected cells. However, this could be explained as a partial expression of the Dp71ab construct missing a number of amino acids, which led to the synthesis of truncated non-functional dystrophin protein at different points. As a consequence, the partially synthesized protein lacks the sites for the binding of anti-dystrophin antibodies.

Our result was in agreement with another study showed that Dp71 protein is normally expressed in the tumour cells, while the full dystrophin protein Dp427 is inactivated (Jones et al. 2021). On the other hand, a previous study showed that Dp71 expression was decreased in glioblastoma cells (Ruggieri et al. 2019).

This study showed that the Dp71a is localised in the cell cytoplasm and most prominent in the nucleus of the U251-MG cells. Previous studies concluded that there is a variation in the
load of expression and the localisation of Dp71 protein depending on the cell type as well as the presence and absence of exon 71 and/or 78 (González et al. 2000, Radspieler et al. 2019, Ruggieri et al. 2019 p. 71).

The results of immunofluorescence staining revealed differences in the nucleus shape and decreased fluorescence intensity of Lamin B1 in transfected cells that express GFP-Dp71a compared to the control cells. In addition, there was no colocalization observed between Dp71a and Lamin B1 in glioblastoma transfected cells. This corroborated with the results that demonstrated the involvement of dystrophin Dp71 in the Lamin B1 intensity of expression and colocalisation (Ruggieri et al. 2019). Additionally, it was found that overexpression of Lamin B associated with the increased nuclear size of the tumour cells. Normally the dystrophin-associated proteins (DAPs) are linked with Dp71. In glioblastoma there was an alteration in the expression of proteins associated with Dp71 and DAP, such as aquaporin 4 (AQP4), with corresponding changes related to brain tumours, i.e., cell migration and angiogenesis (Nico et al. 2004, Papadopoulos et al. 2008, Nico & Ribatti 2011, Noell et al. 2012).

To mimic the in vivo wound healing, we used an invitro scratch assay to study the effects of Dp71 on migration by affecting cell-cell interaction and cell matrix (Liang et al. 2007). The starved cells with Opti-mem media showed decrease in the trendline of the mean cell free area overtime of control (368631 µm at time 0 hours, 263293 µm at time 24 hours and 182786 µm at time 48 hours, respectively) and Dp71ab samples (320027 µm, 229518 µm and 199277 µm at 0, 24 and 48 hours, respectively).

This result indicates there is reduction in the scratched tissue culture and hence higher cell migration. Whilst the Dp71a sample had a negligible reduction in the mean free cell area of the scratched tissue (363297 µm², 343832 µm² and 340824 µm² at 0, 24, and 48 hours,
respectively). On the other hand, longer cell extensions-like were observed. Along with that, the wound closure percentage in Dp71a transfected cells had a 5% and 6% closure after 24 and 48 hours, respectively. In contrast, cells in un-transfected condition closed a 29% and 50% of the gap at 24 and 48 hours, respectively. Similarly, the Dp71ab transfected cells showed a 28% closure after 24 hours and 38% after 48 hours. This can explain the dystrophin complex in mediating cellular signalling e.g. cell migration, adhesion and mechanical force transduction (Nico & Ribatti 2011, Gao & McNally 2015).

Despite there are a few studies conducted to assess the relation of Dp71 with glioblastoma, this is the first study carried out the transfection of Dp71 constructs in U251-MG cell line. Another point of the study strength is the performance of cell migration assay and immunocytochemistry of such cell line after transfection. However, there was certain limitations that should be mentioned. First, performance of quantitative analysis of the expressed Dp71 isoforms and analyse the effect of variation in the amount of expressed Dp71 did not implement. Second, the ability to generate a well-constructed GFP-Dp71 plasmids and express a considerable volume of isoforms in the transfected cells would be a huge advance for future studies. Third, impact of time-consuming in performing such a study is a major challenge, especially in maintaining and supporting the tissue culture and providing an ideal environment to reduce changing in the cells’ characteristics. Fourth, the availability of confocal microscopy might be a crucial tool for a high quality colocalisation result.

5. Conclusion

In conclusion, Dp71a protein synthesis effects the Lamin B1 expression in the nuclear envelop of the transfected cells. Besides that, Dp71a protein isoform results in reducing migration and wound healing in transfected cells. Further investigations need to be performed
to confirm the changes caused by the overexpression of dystrophin constructs in the U251-MG cells and the potential association of Dp71 isoforms with Lamin B1 in tumorigenesis.
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