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The pathogenic alternative splicing, caused by the common GNB3 c.825C>T allele, using a novel, antisense morpholino.

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Running Title

GNB3, hypertension, antisense morpholino

Abstract

The very common GNB3 c.825C>T polymorphism (rs5443), is present in approximately half of all human chromosomes. Significantly the presence of the GNB3 825T allele has been strongly associated, with predisposition to essential hypertension. Paradoxically the presence of the GNB3 825T allele, in exon 10, introduces a pathogenic alternative RNA splice site into the middle of exon 9. To attempt to correct this pathogenic aberrant splicing, we therefore bioinformatically designed, using a *Gene Tools*® algorithm, a GNB3 specific, antisense morpholino. It was hoped that this morpholino would behave *in vitro* as either a potential " splice blocker and/or exon skipper, to both bind and inhibit/reduce the aberrant splicing of the GNB3, 825T allele. On transfecting a human lymphoblast cell line homozygous for the 825T allele, with this antisense morpholino, we encouragingly observed both a significant reduction (from ~58% to ~5%) in the production of the aberrant smaller GNB3 transcript, and a subsequent increase in the normal GNB3 transcript (from ~42% to ~95%). Our results demonstrate the potential use of a GNB3 specific antisense morpholino, as a pharmacogenetic therapy for essential hypertension.

Introduction

The Guanine nucleotide binding protein (G protein), beta polypeptide 3 gene, commonly known as GNB3, is one of the 5 genes, in humans, that encode the 5 different β subunits involved in all heterotrimeric G protein signalling, namely Gβ1, Gβ2, Gβ3, Gβ4 and Gβ5 [1]. These subunits share between 50-80% homology and all form a very similar 7 propeller structure, which binds to different gamma and alpha subunits to which form the many and diverse heterotrimeric G proteins [2]. These different heterotrimeric G proteins, in turn specifically bind to different G-protein coupled receptors (GPCRs), which are stimulated by specific ligands or light. Following activation of these GPCRs, this subsequent change in confirmation, activates the bound heterotrimeric G protein [3]. As only one of the five β subunits, selectively binds, with the many different α (22) and γ (14) subunits, this results in a plethora of structurally different heterotrimeric G proteins. Many of these different heterotrimeric G proteins, will also have different pathway specificities, in the many different cell types of vertebrates [4], e.g. GNB3 is the only one of the 5 beta subunits that is expressed in adult cone cells, but is not expressed in rod cells where GNB1 is expressed [5]. Moreover in early development GNB3 expression is largely restricted to the eye [6]. Later in development, however, the GNB3 gene is more ubiquitously expressed throughout the vertebrate body and is often co-expressed, albeit at a lower level [6], with GNB1 and possibly the other GNB genes in many of the bodies vital organs e.g. brain, heart, liver and kidney [7-11].

Gβ3, together with particular α and γ subunits, therefore selectively binds to a significant number of the thousands of different GPCRs, which are encoded by the 800 genes in the human genome [12].

Given the wide range of tissues, that the GNB3 gene is expressed, it is therefore not surprising that both the GNB3 knockout mouse model [13] and the GNB3 p.D153del homozygous, retinopathy globe and glomerulus enlarged (rgage/rge) chicken, have been shown to suffer from both retinal defects, and other pleiotropic phenotypes [7,14]. For example in both of these homozygous GNB3 mutant organisms there has been reported a high embryonic/infant mortality, e.g. only ~10% of the homozygous GNB3 knockout mice survived for longer than 3 weeks after birth [15]. Similarly we previously reported a ~30% embryonic lethality in the homozygous p.D153del chicken [7]. In the latter organism initially only a severe retinopathy globe enlarged (*rge*) phenotype was detected [16]. Later studies, however, by our research group discovered that the p.D153del eight month old chickens, also suffered from enlargement of the glomerular capsule, causing glomerulomegaly and tubule-interstitial inflammation, which causes both a retinopathy, globe and a glomelular enlarged (rgage) phenotype [14]. These results suggest that the signalling pathways involving GNB3 are vital for both the normal development and maintenance of both the eye and the kidney's renal cortex. In contrast other GNB3 expressing selected tissues, taken from homozygote c.D153del GNB3 chickens, i.e. brain, heart, liver, pancreas, appeared to be histologically unaffected, despite the finding of significant alterations in signalling pathways in these tissues [14].

Recently, however, it has also been shown that GNB3 knockout mice as well, as suffering from cone function defects, have also been shown to suffer from bradycardia [6]. These mice surprisingly, however, showed no other differences in metabolic phenotypes e.g. blood pressure, and body weight compared to normal age matched controls. Moreover the decrease in heart rate appeared to be due to changes in neural signalling, as isolated GNB3 KO mouse hearts showed no

difference to isolated normal hearts, following standard cardiac ion channel functional tests [17].

No significant phenotypic alterations, have to date, been detected in either the heterozygous GNB3 knock out mouse or the heterozygous c.D153del chicken, suggesting that relatively “normal” signalling pathways in most tissues are possibly maintained with only 50% of GNB3 levels. Related to these findings it has recently been found that both humans and transgenic mice that possess an extra copy of the GNB3 gene have been shown to exhibit an obese phenotype [18]. Taken together these results suggest that altering GNB3 expression by either haplo-insufficiency, or by duplication, but probably not by complete ablation, is likely to change both “normal” metabolism and possibly dietary behaviour in humans.

To date, no humans have so far been reported, with an equivalent GNB3 inactivating mutation, similar to either our chicken D153del mutation or with a deletion knockout mouse like mutation. However the very common, GNB3 gene variant c.825C>T (rs5443), which is present in approximately half of all human chromosomes, has been shown to be significantly associated with an increased risk of hypertension, especially in obese patients [19-21], regardless of the patients ethnicity. Interestingly the global allele frequency distribution of the GNB3 825T allele is remarkably similar to that of common polymorphisms in salt controlling genes e.g. the Angiotensin I converting enzyme (ACE), angiotensinogen (AGT) and both the sodium channel, non-voltage-gated 1 alpha and gamma subunits (SCNN1A/ENACα and SCNN1G/ENACγ) [22-25]. The GNB3 825T allele and particular functional alleles (e.g. the ACE D allele) in these other salt regulating genes have significantly higher frequencies in Sub-Saharan tropical Africa than in any other part of the world, where

the GNB3 825T allele, appears to be strongly selected against. In contrast this data also strongly suggests that the common Sub-Saharan alleles, were strongly selected for before the migration of man-kinds ancestors migrated “Out-of-Africa”, ~100,000 years ago [24,26]. The most likely reason for the differential selection of the 825T GNB3 allele and other salt controlling alleles, in these different climes, is due to the hot and wet climate and low salt availability, endured by humans in central Africa at this time. In addition, the sub-Saharan hot climate inevitably led to the selection of humans with an increased capacity to sweat and cool down through evaporation. This selection has in turn also led to an increase in the frequency of gene alleles e.g. GNB3 825T, that increase renal sodium conservation, in order to combat this inevitable loss of both salt and water. GNB3 and the 825T allele in particular is therefore likely therefore to be involved in GPCR stimulated pathways that involve the control of urinary salt uptake e.g. through the angiotensin/NHE [21], bradykinin and/or SCNN/ENAC pathways [27]. However recent experiments using the GNB3 knockout mice, which were infused with angiotensin II, with no physiological changes in blood pressure, suggest that GNB3 is probably a peripheral player in this particular pathway [6]. Interactions, however between variant haplotypes in the obesity related FTO/IRX3 locus [28] and the 825T allele of GNB3, have recently been shown to help contribute to the varied clinical phenotypes in hypertension [29].

Significantly the GNB3 825T allele has also previously been implicated in obesity [25], cardiovascular disease [30-36], low birth weight [37], Alzheimer’s disease, cognitive decline [38], erectile dysfunction [39-43], tumour progression [44-49] and as a genetic marker for drug response [50-59].

The mechanism by which the GNB3 825T allele causes carriers to be predisposed to hypertension and other diseases, however still remains to be fully elucidated. The GNB3 825T allele, unlike the 825C allele, paradoxically introduces an alternative RNA splice site into the middle of exon 9, possibly by altering the GC ratio critical to both nucleosome methylation and splicesome binding, during gene expression [20]. The GNB3 gene with an 825T allele therefore produces two different transcripts in an approximately 50:50 ratio (see figure 1). The “normal” splicing event 8-9a, produces a transcript that is 123bp longer than the aberrant pathogenic 8-9b transcript. The former 8-9a transcript codes for the normal stable Gβ subunit, while the latter 8-9b translates into a highly unstable and possibly pathogenic isoform Gβ3s, which is deleted for 41 highly conserved, amino acids [60] . In addition as a result of this alternative splicing event, the expression of the normal 8-9a transcript is reduced by ~50%, compared to GNB3 genes carrying the 825C allele (see figure 1).

Finding a specific drug to specifically try to correct the hypertensive and possibly some of the other GNB3 825T allele associated phenotypes, is therefore an attractive proposition. One approach chosen, in an attempt to specifically correct the analogous mis-splicing, of the pathogenic common Haemaglobin (HbE) allele, HBB c.79G>A (p.E27K), was to use an antisense morpholino, to specifically target its pathogenic mis-splicing [61]. This antisense morpholino treatment of Hela cells, expressing the HbE allele, resulted in a ~70% increase in the normal splice product. More recently antisense “exon skipping” morpholinos, have also been successfully used to treat Duchenne Muscular Dystrophy patients, who suffer from out of frame forming exon deletions. Such patients demonstrated a dramatic improvement in their

clinical symptoms, with apparently little side effects following intravenous treatment, using apparently very stable and long lasting antisense morpholinos [62].

To attempt to combat either or both of these potentially pathogenic events in hypertensive patients, carrying an 825T allele, we therefore used the *Gene Tools*® algorithm to design a so called "exon skipping" morpholino, in order to try to target and correct the aberrant splice site, in the middle of exon 9, in the GNB3 gene (see figure 1). In addition we also designed an ordinary antisense DNA oligonucleotide in order to try to block the aberrant GNB3s transcript. Both of these oligonucleotides were then used to transfect two different human lymphoblastoid cell lines, with two different GNB3 genotypes, namely homozygous 825T and heterozygous 825T/825C.

Materials and Methods

Morpholino and antisense oligo design

The following aberrant splice site GNB3 gene sequence, in the middle of the Exon 9 gene, was submitted to the *Gene Tools*® algorithm (www.gene-tools.com) for exon skipping morpholino, design :

ctcttcatttcgggggcctgtgatgccagTGCCAAGCTCTGGGATGTGCG.

This algorithm then designed the following 25bp morpholino (M) sequence, which is complementary to the GNB3 aberrant splice site:

5'-CACTGGCATCACAGGCCCCCGAAAT-3', which was then ordered from *Gene Tools*®. In addition an antisense oligonucleotide (AS) 5'-GAGCTTGGCACACGTGGTGT-3' which is complementary to the last 10 bases in exon 8 and the first 10 bases after the aberrant 'AG' splice site in exon 9, was also

ordered from Sigma Aldrich. This oligonucleotide was designed to hopefully bind preferentially to the GNB3s mRNA transcript and less to the normal GNB3 transcript.

PCR Primers

The following RT-PCR primers were designed using Primer 3 software and ordered from Sigma: GNB3 exon 7 – 10 Forward GAGCTTTCTGCTCACACAGG, GNB3 exon 7 – 10 Reverse TCATGGAGTCCCAGACATTG-3', GNB3 Full coding with HindIII linker Forward GCAAGCTTGCCATGGGGGAGATGGAGC, GNB3 Full coding with Xho1linker Reverse CTCGAGTCAGTTCCAGATTTTGAGGAAGCTG

Actin Forward primer GCAAAGACCTGTACGCCAAC, and Actin Reverse primer 5'-CGTCATACTCCTGCTTGCTG-3'

Cell Culture

The GM18500 GNB3 825T/825T (TT) GM18506 GNB3 825T/825C (CT) and 825C/825C (CC) (GM19116) Nigerian Yorba tribe, transformed, lymphoblastoid cells were obtained from Coriell Cell Repositories (http://ccr.coriell.org/).

The cells were added drop by drop into a T-50 culture flask (50ml) containing 20ml of Roswell Park Memorial Institute – 1640 (RPMI 1640) culture medium (Invitrogen), which was then agitated to ensure even spreading. Each of these flasks was then incubated at 37^{o}C, for 24 hours. The cells were then removed after incubation and their confluence checked under the microscope. Once confluent, the cells were sub-cultured by removing the old medium and washing with Phosphate-Buffered Saline (PBS), to remove the serum.

After incubation the 20ml of cells in RPMI-1640 media were added to

T-75 culture flasks, and incubated overnight at 37^{o}C, 5% CO_2. 30ml of pre-heated RPMI-1640 media was added to top the cells up to 50ml, leaving them to incubate for around 48 – 72 hours until the pH of the media had decreased. The cultures were manually agitated daily to break up the cell clumps formed.

Transfection (Lipofectamine™)

When the cells had reached 50% to 70% confluence, they were shaken to break up clumps, transferred into 15ml centrifuge tubes and centrifuged at 720g for 8mins, removing the supernatant. The pellet was washed twice, by re-suspending the pellet in 10ml PBS, centrifuging the cell suspension at 720g for 8 minutes and removing the supernatant. The cells were then resuspended in 2ml of Opti-MEM®, and 30µl of the suspension was removed so the cells could be counted using a haemocytometer. Once the cells were counted, a 500µl dilution of each suspension was plated in a 6-well multiple well plate at a density of approximately $3.6x10^{6}$ cells per well, containing 2ml of Opti-MEM®. The 6-well plate was then incubated for 2 hours at 37^{o}C, 5% CO_2, in order to starve the cells of serum before transfection. 4µl of the GNB3 morpholino (1mM/µl) and 4µl antisense oligonucleotide (1mM/µl) were both initially mixed with 200µl OPTI-MEM® and left at room temperature for 10minutes. These DNA/OPTI-MEM® mixtures were then added to another mixture containing 12µl Lipofectamine™ and 200ul Opti-MEM®, which had also been pre-incubated for 10minutes.

To measure transfection efficiency 3µl of Green Fluorescent Protein (GFP) expressing Plasmid was also added to the mixture. The 2 mixed tubes were then incubated for a further 20 min, allowing the Lipofectamine™ to form a complex with the morpholino and antisense oligonucleotide. 100µl from the morpholino/Lipofectamine tube was added to the 4 morpholino wells, and 100µl from the antisense/Lipofectamine™ tube was added to the 4 Antisense wells. For the 4 mock samples 12µl Lipofectamine™ was mixed with 200µl OPTI-MEM, incubated for 10min, then 3µl of GFP was added and allowed to incubate at room temp for 20min before being added to the mock cells. The 6-well plate was gently shaken back and forth to insure distribution of the Lipofectamine™ complex. Another 1000µl of Advanced RPMI 1640 was added to each of the 12 wells. These were placed in the incubator at 37^{o}C overnight (approximately 24 hours). Afterwards the 6-well plates were removed from incubation and 3ml of RPMI 1640 was then added to each well. The 6-well plates were then incubated for a further 48 hours at 37^{o}C. The 6-well plate was shaken gently back and forth to insure distribution of the Lipofectamine™ complex. Another 1000µl of Advanced RPMI 1640 was added to each of the 12 wells. These were placed in the incubator at 37^{o}C overnight (around 24 hours). Afterwards the 6-well plates were removed from incubation and to each of the 12 wells, 3ml of RPMI 1640 was added. The 6-well plates were then incubated for a further 48 hours at 37^{o}C.

RNA Extraction

Medium containing the cells from each well was centrifuged and resuspended in 1ml of PBS and transferred to a 1.5ml and centrifuged at 425g for 10mins, before the supernatant was removed. The 1.5ml tubes were placed on ice and to each 100µl of

QIAzol® (Qiagen) was added and mixed by pipetting until the cell pellet was fully resuspended, the tubes were then incubated at room temperature for 5min, before being placed back on ice. To each 1.5ml tube 20µl of chloroform was added and vortexed for 10sec, then incubated for 3min at room temperature, before being placed back on ice. The 1.5ml tubes were then centrifuged at ~23,000g for 15min at 3^{o}C. After this each 1.5ml tube presented 3 phases: a top colourless aqueous phase, a middle white interphase and a lower red phenol-chloroform phase. The upper aqueous phase containing the RNA was removed from each 1.5ml tube without disturbing the 2 lower phases and added to a new labelled 1.5ml tube. The RNA was precipitated by adding 50µl of isopropanol and incubated for 10min at room temperature. The1.5ml tubes were centrifuged at ~23,000g for 10min at 3^{o}C. The supernatant was removed and the pellets were washed by mixing with 100µl of 75% ethanol, and being centrifuged at 239g for 10min at 3^{o}C, the supernatant was removed and this wash step was repeated. Afterwards the RNA pellet was left for the ethanol to evaporate for 7min. The RNA samples were then further purified to ensure no genomic DNA contamination using an RNeasy Mini prep kit QIAGEN, using the manufacturer's protocol. RNA was then quantified by spectrophotometry and diluted to a concentration of 100ng/ µl.

RT-PCR

cDNA synthesis was undertaken using the manufacturer's instructions using the Oligo dT method in an M-MLV Reverse Transcriptase kit (Life Technologies). PCR was carried out using a MyTaq™ Red Mix (Bioline) PCR kit in a 50µl final volume, containing 100ng cDNA and 0.2µM of forward and reverse primer. Thermo-cycling was carried out with an initial denaturation at 95^{o}C for 5 minutes, followed by 35

cycles of 94^{o}C for 30 seconds, 45^{o}C for 45secs and 72^{o}C for 45secs. A final extension was carried out at 72^{o}C for 45secs. PCR samples were loaded on a 2% gel containing, 1X TAE buffer and 10µl Save View™ ABM. Bioline Hyperladder II was used as a size marker and the gel was run at 150 volts for 90 minutes. Semi quantitative analysis of the jpeg image of the gel was carried out using imageJ software (http://imagej.nih.gov/ij/).

Protein Extraction

Coriell cell lines, possessing the following GNB3 Genotypes 825CC (GM19116), 825CT (GM18506) and 825TT (GM18500) cells, were either mock or antisense morpholino transfected. After 24 to 48 hours incubation the cell lines were checked for 70% - 90% confluence in their wells. Once this was achieved the cells were transferred into a 15ml centrifuge tube and pelleted at 720g. The supernatant was discarded and the pellet was resuspended in 5ml PBS to wash, the cells were then centrifuged at 720g to pellet, discarding supernatant, repeating once. The cells were resuspended in 150µl of ice cold RIPA Buffer), and mixed by pipetting then placed directly on ice, and carefully transferred into a labelled 1.5ml microcentrifuge tube, then incubated for 5mins. The microcentrifuge tubes were centrifuged at 28,000g for 10mins at 4^{o}C. 10µl of the supernatants containing the protein lysate were transferred to fresh microcentrifuge tubes for analysis of concentration, by the Bradford Assay. The remaining volumes were measured (~140µl) and added to fresh microcentrifuge tubes, adding 1:1 sample buffer (containing β-mercaptoethanol), and mixed by pipetting. The samples were heated at 94^{o}C for 15mins at shaking at 900rpm, the samples were centrifuged at 28,000g for 10mins.

The supernatants were transferred in to fresh microcentrifuge tubes and aliquots stored in a -80^{o}C freezer.

Western Blot (WesternBreeze® Kit)

The Ab154866 Gβ3 specific anti-body was obtained from Abcam. It is a rabbit monoclonal antibody raised against a 14 amino acid residue synthetic peptide taken from within the region 100-150 of Human Gβ3 protein. The antibody was diluted in a diluent blocking solution provided in the WesternBreeze® kit (7ml Filtered $_{d}H_{2}0$ + 2ml Blocker A + 1ml Blocker B), Ab154866 was diluted 1:1000 by adding 10µl antibody to 10ml diluent.

The thawed protein lysate samples were reheated at 94^{o}C, 900rpm for 10mins. And the centrifuged for 1 minute 5,000g. The SDS polyacrylamide gels were placed into the XCell SureLock® Mini-Cells filled with 1x tris-glycine running buffer and the wells loaded with 10µg of protein, according the Bradford assay results, 5µl of Life Technologies SeeBlue® Prestained Standard protein marker was also added to a The Lid was attached and set to 200v and 200mA, allowing the gel to run for 1 hour 30mins. Blots were then probed with either of the two antibodies, according to the manufacturer's instructions.

Results and Discussion

As can be seen from both figures 2 and 3, there appears to be significant inhibition of the formation of the aberrant 8-9b splicing, in both the homozygous 825TT cell line (reduced from ~58% to ~5%) and the heterozygous 825CT cell line, following transfection, with the GNB3 specific antisense morpholino. Moreover following morpholino transfection of the 825TT cell line there also appeared to be a significant increase in the production of the normal 8-9a transcript (increased from ~42% to 95%). In contrast from Figure 2 it can be seen that both mock transfection and transfection with the allele specific unmodified (AS) oligonucleotide, of the 825TT cell line, appears to have little effect in altering the expression, of neither the 8-9b, or the 8-9a splicing event. Encouragingly mock transfection or transfection with either the, morpholino or the allele specific (AS) morpholino affected the expression of the control actin gene.

These initial RNA results suggest that our GNB3 antisense morpholino appears to significantly correct both of the potential pathogenic effects of the GNB3 825T allele; firstly down regulation of the aberrant 8-9b and secondly up regulation of the "normal" 8-9a splicing event.

From the Gβ3 Western Blot results, shown in figures 4 and 5, however it can clearly be seen that only the normal Gβ3 splice variant and not the unstable and short lived Gβ3s protein, is detected with the Gβ3 monoclonal antibody Ab154866. This is despite this antibody being raised from a portion of the Gβ3 protein, amino acids 100-150, that is present in the truncated Gβ3s protein, which is deleted for amino acids 168-207. This result is similar to the one achieved by Sun et al. [60], who were also unable to detect the unstable Gβ3s protein in their experiments. It is therefore

impossible to determine, from this Western blot whether our antisense morpholino is actually down regulating the amount of the Gβ3s protein, as indicated in our RNA results. Moreover contrary to our RT-PCR results in figure 2, there appears to be no significant difference in the quantity of the normal Gβ3 protein, in both the 825TT and 825CC cell lines, following treatment with the antisense morpholino.

The semi–quantative RT-PCR results obtained in our experiments, however still suggest that the GNB3 antisense morpholino, may still be useful in helping lower blood pressure in both normal and obese individuals who carry the 825T allele [19]. This is because some or all of the pathogenic effects of the 825T allele are likely to be due to the presence of the unstable Gβ3s protein subunit, rather than the mere reduction of the expression of the normal Gβ3 subunit. In support of this argument it has recently been found that many different specific deleterious point mutations in the related GNB1 and GNB2 genes are able to transform cell lines from being cytokine dependant, to a cytokine independent phenotype [63]. Moreover these same mutations have been found in many different human cancers. These findings strongly suggest that many different mutations in the heterotrimeric C protein β subunit, including the GNB3 splice variant Gβ3s, are likely to cause dominant/negative effects on both the βγ and α subunit G protein, signalling pathways [64]. If this dominant/negative effect does ultimately prove to be the main predisposing hypertension disease mechanism, for the GNB3 825T allele, then antisense morpholinos, inhibiting the formation of the Gβ3s subunit, may have great therapeutic value, especially in obese hypertensive patients [19].

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Author Disclosure Statement

J.McGlinchey is an employee of NHS Scotland; T. Hemanth is an employee of Queen Mary University of London, D.Lester is an employee of The University of Abertay. None of these authors have any known conflict of interest.

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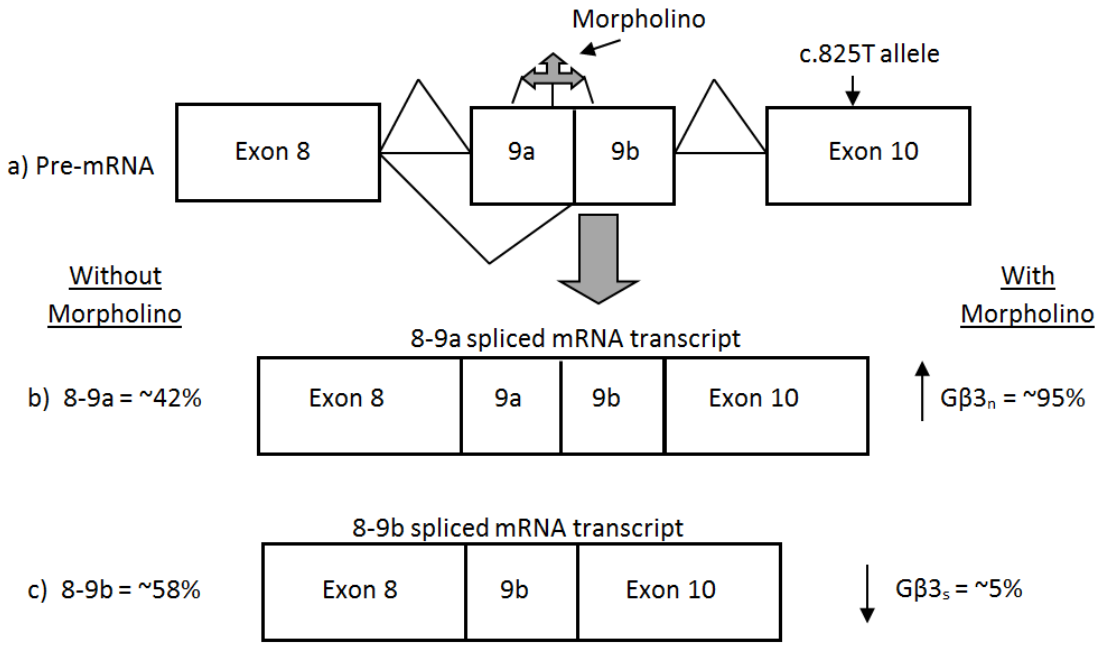

Figure 1

This diagram shows the proposed mechanism of action of the GNB3 antisense morpholino (M) and its binding site, which spans both sides (9a and 9b) of the cryptic splice site, in the middle of exon 9. Line a) represents the GNB3 pre-RNA with a c.825T allele in exon 10. The triangular shaped lines shows the two possible alternative splicing between exon 8 and exon 9 to either to exon splice junction 9a or the cryptic splice site in 9b. Line b) shows the normal GNB3 mRNA following the fusion of exon 8 to exon 9a, which translates into the normal Gβ3 protein. Line c) shows the aberrant pathogenic fusion of exon 8 to 9b, which translates into $G\beta3_s$ protein.

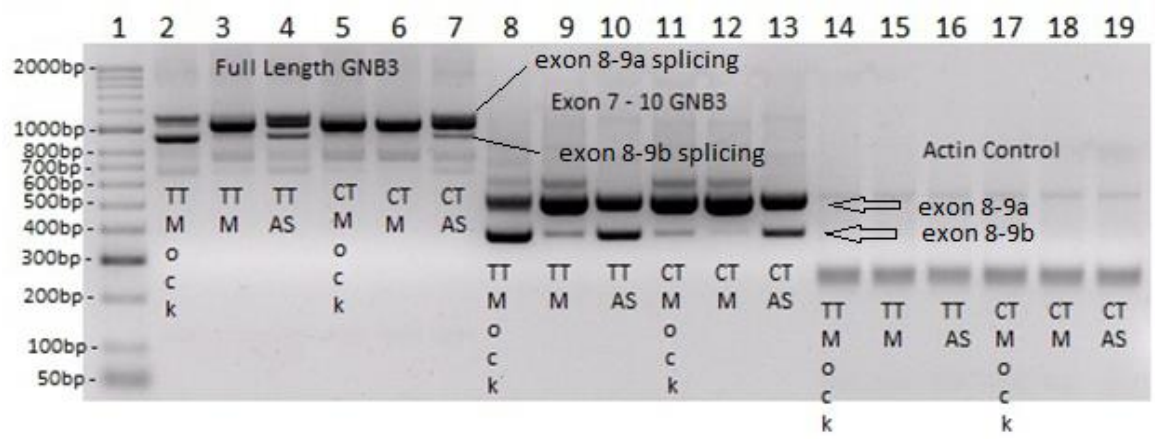

Figure 2

This diagram shows the semi-quantitative RT-PCR results for both the 825TT and 825CT cell lines. Lane 1 contains the bioline 100bp (hyperladder) marker. Lanes 2-7 are RT-PCR results generated using the “full length” GNB3 primers as described in the methods. The expected size of the full length product is 1026bp for the 8-9a normal transcript and 903bp for the alternative 8-9b pathogenic transcript. Lanes 8-13 are RT-PCR bands generated with exon 7-10 primers and generate an expected band of 490bp for the 8-9a normal transcript and 367bp for the pathogenic 8-9b transcript. Lanes 14-19 are RT-PCR products generated from the actin specific primers. Lanes 2, 5, 8, 11, 14 and 17 are RT-PCR products generated from mock transfected GNB3 825 TT or TC cell lines. Lanes 3, 6, 9, 12, 15 and 18 are RT-PCR products generated from GNB3 antisense morpholino (M) transfected GNB3 825 TT or TC cell lines. Lanes 4, 7, 10, 13, 16 and 19 are RT-PCR products generated from GNB3 antisense allele specific (AS) unmodified oligonucleotide transfected GNB3 825 TT or TC cell lines.

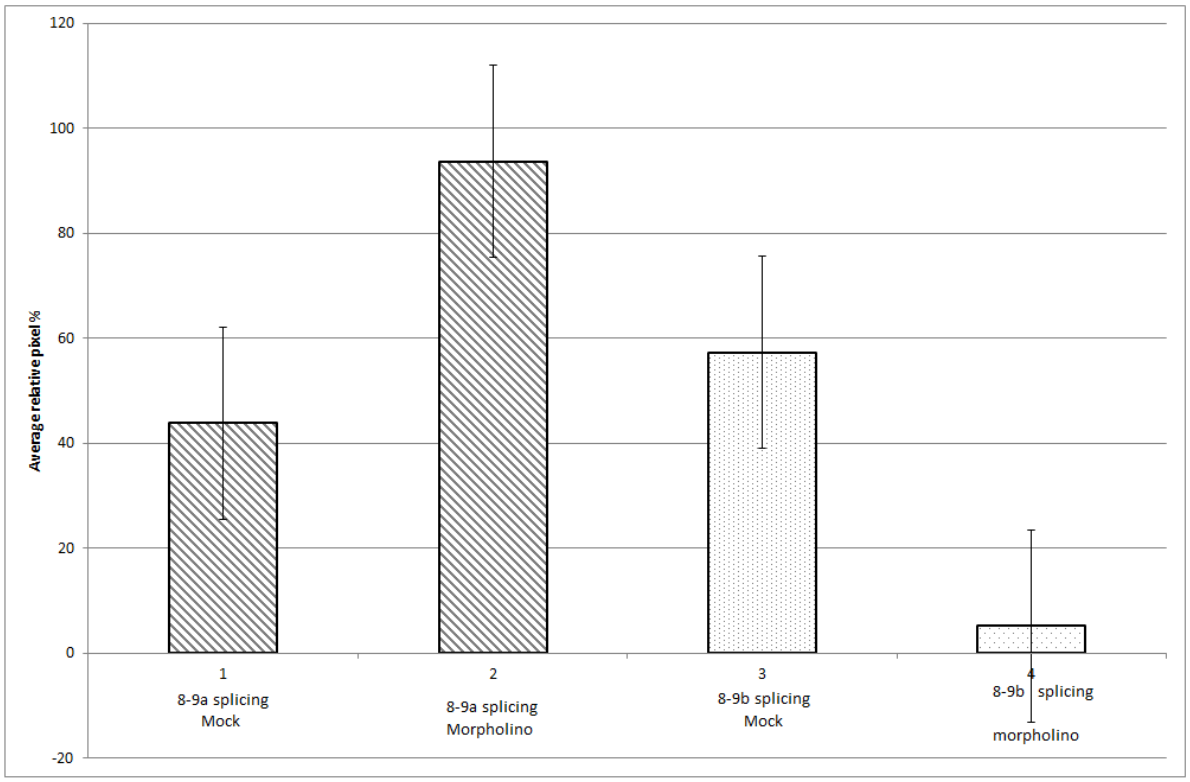

Figure 3

This diagram represents the image J relative average % pixel analysis, with standard error bars, of lanes 2 3, 8 and 9 shown in the RT-PCR semi-quantitative gel, in figure 2. Block 1 represents the average % pixels from the 8-9a splicing event with mock treatment, lanes 2 and 8 in figure 2. Block 2 represents the average % pixels from the 8-9a splicing event with GNB3 antisense morpholino (M)treatment, lanes 3 and 9 in figure 2. Block 3 represents the average % pixels from the pathogenic 8-9b splicing event with mock treatment, lanes 2 and 8 in figure 2. Block 4 represents the average % pixels from the pathogenic 8-9b splicing event with antisense morpholino (M) treatment, lanes 3 and 9 in figure 2.

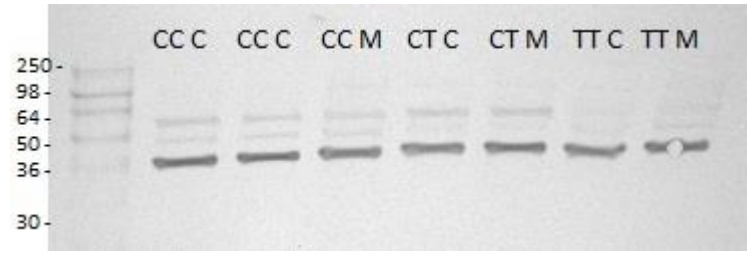

Figure 4

This Western blot shows, homozygous 825 CC, heterozygous CT and homozygous TT cell lines, which were either transfected morpholino (M) or mock-transfected control (C). The extracted protein samples from these treated cell lines were blotted and probed using the specific anti-Gβ3 antibody Ab154866.

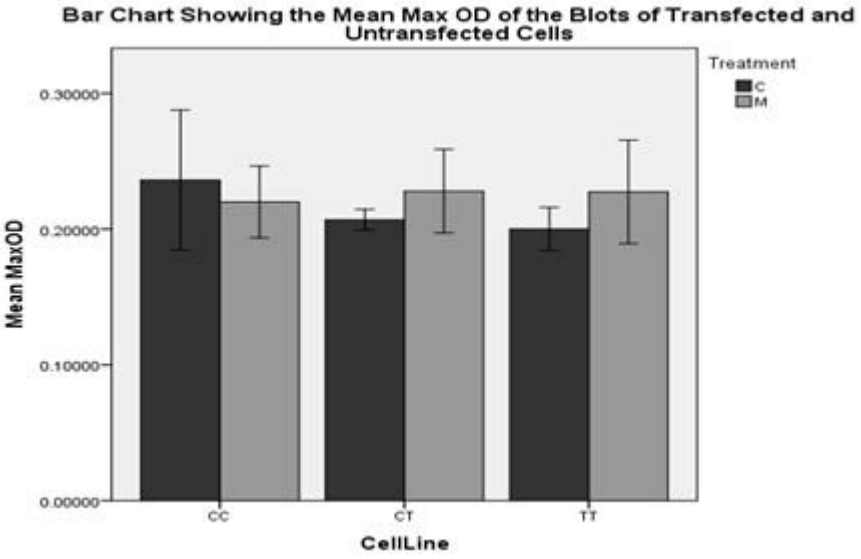

Figure 5

This bar chart shows the mean MaxOD of the Gβ3 specific bands in the Western blot shown in figure 4.