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IDENTIFICATION OF NOVEL EXON SPLICE ENHANCERS (ESEs) IN THE GROWTH HORMONE (GH) GENE MUTATED IN ISOLATED GH DEFICIENCY (IGHD) PATIENTS

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Background

Autosomal dominant GH deficiency is mainly caused by mutations influencing the correct mRNA splicing, occurring at the IVS3 donor splice site and within an ESE located in the first 7bp of exon 3 (ESE1). These mutations result in an increased level in the production of the exon 3-skipped isoform encoding the dominant negative 17.5 kDa isoform (normally present at low concentration) and variable level of the wild type 22 KDa isoform. Another ESE (ESE2) was identified by in vitro mutagenesis spanning from nt 19 to nt 32.

Results

We identified three IGHD patients carrying variations in Exon 3. One carried the non-synonymous variation E3+75G>A (Glu82Asp), the second carried two novel variations, E3+90P>P (Pro87Pro) and E3+101E>V (Glu91Val) and the third patient carried two synonymous variations at E3+90P>P (Pro87Pro) and E3+84P>P (Pro85Pro). These mutations were absent in a panel of 400 normal chromosomes. All the mutations were originated by gene conversion from one of the GH cluster homologous genes. None of them was included in the already identified ESEs. The ESE Finder software suggested the presence of two other ESEs in exon 3 from nt 83 to 89 (ESE3) and nt 98 to 104 (ESE4). The mutations at position nt 84 and nt 101 were predicted to abolish ESE3 and ESE4, respectively.

We performed *in vitro* splicing analysis with constructs bearing the entire wild type GH (GHwt) gene, and the GH gene carrying the different mutations identified in the three patients (GH82, GH85, GH87, GH91). The mRNAs from transfected GH4C1 rat pituitary cells showed the wild type 22 KDa isoform in all the constructs. GHwt and GH82 showed the full length and the exon3 skipped bands of similar intensity (the 17.5 KDa was visible as a faint band). Conversely, the GH85 and GH91 transcripts produced significantly higher level of the band corresponding to the 17.5 KDa isoform as compared to the GHwt. The 17.5kDa isoform was completely absent in the GH87 mRNA.

Conclusion

In conclusion, we identified two novel ESEs in the GH exon3 through the detection of mutations in IGHD patients. Two of these mutations E3+84P>P (GH85)and E3+101E>V (GH91) cause an increased level of the 17.5 KDa protein. Further analysis are necessary to better understand the role of these mutations. The analysis of the parents of the patients will reveal whether the mutations were inherited from the same parent or one from each parent or if they are *de novo* mutations.

1.INTRODUCTION

1.1 Human Growth Hormone

Growth hormone is a multifunctional hormone that regulates the longitudinal growth in mammals. It is a pleiotropic cytokine produced in the anterior pituitary that promotes the postnatal growth of skeletal and soft tissue [1]. It also act as an important regulator of immune function, bone turnover and muscle mass [2]. Growth hormone gets activated by binding with its receptor (GHR) molecules and enhances the proliferation and maturation in many cells [3,4]. Any genetic or acquired disorder that impairs the GH secretion or action causes a pathological phenotype characterised by GH deficiency [6].

1.2 hGH1 gene

Human growth hormone is a single chain peptide made up of 191 amino acids encoded by the GH1 gene [6]. GH1 gene is located on chromosome 17q23 within a cluster of five highly homologous (92-98%) genes consists of GH-1, CSHP (chorionic somatomammotropin pseudogene), CSH-1 (chorionic somatomammotropin gene), GH-2 and CSH-2 (Figure 1). Despite the high degree of sequence homology, they express in a tissue-specific manner. The GH-1 gene express exclusively in the somatotrophic cells of the anterior pituitary gland whereas all other express in placental level. GH1 consists of five exons and four introns for a total of 1600bp in length [6,7].

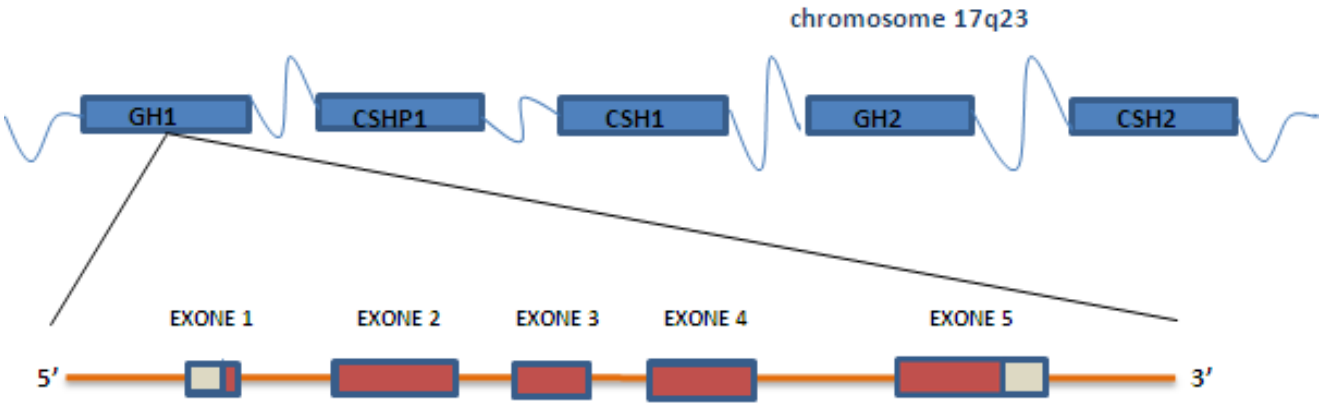


Figure 1: GH gene cluster

Approximately 75% of the circulating GH is expressed in the anterior pituitary gland as a 22kDa protein. The alternative splicing of the GH pre-mRNA gives rise to minor isoforms of both biologically active and inactive GH peptides[8].

1.3 Isolated Growth Hormone Deficiency

Isolated growth hormone Deficiency (IGHD) refers to conditions associated with childhood growth failure due to the lack of growth hormone action. The incidence of IGHD is estimated to be 1/4000 – 1/10000 births [9]. While majority of cases are sporadic, 3-30% has an affected relative. In most of the sporadic cases, no cause of IGHD can be identified and these are often referred to as Idiopathic Growth hormone deficiency or Idiopathic IGHD. It is assumed that significantly higher proportion of sporadic cases may have genetic causes. However, *de novo* mutations in the GH encoding gene (GH1) have been detected in patients with sporadic IGHD [10, 11]. Mutation in GH1 gene has been detected in 12.5% of familial and 10% sporadic cases. The mutations including the deletion of the entire gene and missense mutations produce severe growth hormone deficiency where as the splicing mutations produce milder forms [12].

Based on clinical characteristics, IGHD has been divided into three types. Based on severity and mode of inheritance they are classified as IGHD type IA, IB, II and III (Table1). IGHD Type 1A is the most severe form of IGHD with patients showing early and profound growth failure due to the lack or extremely low levels of serum GH. It has an autosomal recessive mode of inheritance and is caused by deletions removing the entire GH1 or a nonsense mutation leading to a premature stop codon [6,13]. IGHD Type IB is a milder form with low but detectable serum GH. This condition is inherited as autosomal recessive trait due to the splice site mutations of the GH gene or mutations within gene encoding the GHRH receptor. IGHD Type II, a very common form of IGHD which are inherited in an autosomal dominant pattern. They are caused by the GH1 mutations affecting the mRNA splicing [12, 14]. IGHD TypeIII is an X-linked, recessively inherited disorder. Previous studies have shown that some individuals have an associated X-linked agammaglobulinemia and multiple loci may cause IGHD III [15].

IGHD	Associated gene defects	Reported inheritance patterns	GH levels	Phenotype
Type 1a	*GH1* mutations	Autosomal recessive	Absent	Severe short stature
	Deletions, microdeletions, nonsense mutations			Neutralizing anti-GH antibodies often develop during GH replacement therapy
Type 1b	*GH1* mutations	Autosomal recessive	Low	Height ≥3 SD below the mean
	GHRHR mutations			
	Splice site mutations			
Type 2	*GH1* mutations	Autsomal dominant	Low	Short stature
	Splice site mutations, intron and exon splice enhancer, missense mutations			At least one affected parent
				Variable phenotype
				Good response to GH therapy
Type 3	None to-date	X-linked recessive	Low	Associated with X-linked aggamaglobulinemia in some individuals

Table1: Classification of IGHD

1.4 Alternative Splicing and its role in IGHD II

Alternative splicing of pre-mRNA is the process in which the non-coding regions (introns) of the primary gene transcript are removed and the coding regions (exons) are joined to produce the mRNA molecule responsible for different protein isoforms. It is well documented that autosomal dominant growth hormone deficiency (IGHDII) is mainly caused by the aberrant splicing of pre-mRNA encoding GH1 gene. This leads to the production of different protein isoforms of growth hormone [16-18]. The full length product is 22kDa isoform with the complete biological activity of growth hormone [17]. The activation of a cryptic splice site in exon 3 produces a transcript lacking first 45bp of exon 3 and encodes a shorter isoform of 20kDa. A 17.5kDa isoform is produced when Exon 3 is skipped through alternative splicing and acts as a dominant negative isoform blocking the secretion of full-length protein. However, a small amount of this isoform is present in every healthy individual [6,19]. Trace amount of transcripts with 11.3kDa and 7.4kDa have also been identified which are produced by the skipping of exones 3-4 and 2-4, respectively [17,20].

IGHD II is caused by mutations in GH1 gene that alters the mRNA splicing resulting in the increased production of the 17.5 kDa variant [14]. The mutations that alter the mRNA splicing in GH1 have been mainly identified at the splice sites bordering Exon 3. Another class of mutations (shown in the figure marked by letters a-e) are identified in the splicing enhancer elements of Exon3 and Intron3 [19,14]. Two Exon Splice enhancers (ESEs) were identified in Exon 3 of GH1 gene. ESE1 is located within the first 7 bp of exon 3 and ESE2 is a 15 nuclotide sequence spanning from 19-33 bp. The mutations in ESE1 (E3+1G→T, E3+2A→C, E3+5A→G; figure 2a-c) and ESE2 (E3+29 A→G, figure 2d) affects the splicing enhancer functions and cause exon 3 skipping. Two mutations in Intron splicing Enhancer (IVS3+28G→A;fig 2e, IVS3+del28-45) were also identified to produce transcripts encoding the 17.5kDa isoform [14,21-24]. These enhancers are necessary to maintain the exon definition to produce the full-length 22 kDa hormone and the mutations that weaken the exon definition increase the amount of transcripts encoding the 17.5 kDa isoform. The 17.5 kDa isoform exhibits the dominant negative effect in both in tissue culture and transgenic mice experiments. Moreover, these isoforms were also identified to disrupt GH packaging and destroy anterior pitutay somatotrophs [24-26].

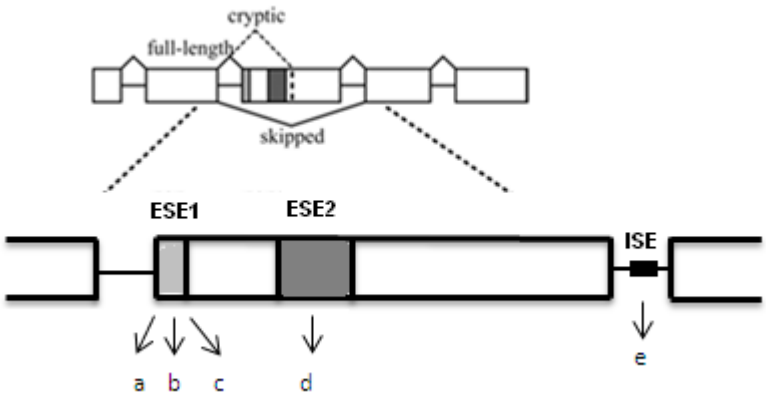


Figure2: GH1 gene structure and location of splicing enhancers. The letters a-e represents the point mutations in splicing enhancers that were already identified as a cause of dominant negative effect.

It is important to increase our knowledge about the GH1 mutational spectrum as most of the mutations causing dominant negative form of GHD may be still underreported. This points to the importance of detailed functional analysis of GH variants identified in GHD patients [16,17]. In this study we performed both computational and functional analysis of different mutations found in exon 3 of IGHD patients.

2.MATERIALS AND METHODS

2.1 DNA Extraction:

Genomic DNA was extracted from the blood samples by salting out method.

2.2 PCR Amplification of the GH1 gene:

A 2.7kb fragment including the GH1 gene was amplified from the genomic DNA using primers GH32- forward 5' CCA GCA ATG CTC AGG GAA AG 3' and GH33- reverse 5' TGT CCC ACC GGT TGG GCA TGG CCA GGT AGC C 3'. These primers were designed for the selective amplification if the GH1 gene and do not amplify other genes in the GH cluster. The PCR reaction was performed in a total volume of 25ul using 100ng template DNA, 10pM of primers, 250uM of dNTPs, 0.03U of proof reading Taq (DyNAzyme EXTTM, Finnzymes). Cycling conditions used are: denaturation at 94°C for 2 min, 30cycles consisting of 30s denaturation at94°C, 30s annealing at 58°c and 3min extension at 72°c, followed by a final extension at 72°C for 10 min and finally cooled to 4°C. The resulting 2.7kb size products were visualized in a 1.5% agarose gel.

2.3 Sequencing Analysis:

For the sequencing of the GH1 gene a series of nested PCR reactions were performed using the 2.7kb PCR product as template. Five set of primers (Gh40-Gh13, Gh14-Gh27, Gh26-Gh15, IVS4F-IVS4R, Gh16.17-Gh31) were used which cover the entire GH1 gene including the proximal promoter, introns, exons and 3' UTR regions. The PCR reaction was performed in a total volume of 25ul using GoTaq DNA polymerase and the following cycling conditions; denaturation at 94°C for 2 min, 30cycles consisting of 30s denaturation at94°C, 30s annealing at 58°c and 30 sec extension at 72°c, followed by a final extension at 72°C for 5 minutes. The PCR products were visualized on a 2% agarose gel and purified using affymerix ExoSAP-IT enzymatic PCR clean up system at 37°c for 15mins and 80°C for 15mins using a thermo cycler. The purified products were then sequenced with Big Dye Terminator kit(Applied Biosystems) and automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The Sequencing Reaction was carried out in a Thermo cycler with the following conditions: 3 minutes at 96°C 25cycles consisting of 20 seconds at 96°c 5 seconds at 50°c and 4 minutes at 60°c and finally cooled to 4°c. The sequence reaction products were purified by precipitation using ethanol and 3M Sodium Acetate (pH 5.2) and resuspended in 12ul formamide.

2.4 Site Directed Mutagenesis

pcDNA 3.1(+) expression vectors (Invitrogen, Chartsworth, CA) containing the GH1 gene was already available in the lab of genetics. The invitro mutaginized constructs (GH82, GH85, GH87, GH91, GH85+87, GH87+91) were generated from the GH(wt)-pcDNA 3.1 plasmid by the quick change site directed mutagenesis kit (Stratagene) using mismatch complementary primers containing desired mutations as indicated in Table1.

PRIMER	SEQUENCE	MUTATION
GH82Asp_for: **GH82Asp_rev:**	5'CCCTCTGTTTCTCAGACTCTATTCCGACACC 3' 5'GGTGTCGGAATAGAGTCTGAGAAACAGAGGG 3'	**Glu82Asp**
GH85Pro_for: **GH85Pro_rev:**	5'CTCAGAGTCTATTCCAACACCCTCCAACAGGG 3' 5'CCCTGTTGGAGGGTGTTGGAATAGACTCTGAG 3'	**Pro85Pro**
GH87Pro_for: **GH87Pro_rev:**	5'GTCTATTCCGACACCTTCCAACAGGGAGGAAAC3' 5'GTTTCCTCCCTGTTGGAAGGTGTCGGAATAGAC3'	**Pro87Pro**
GH91Val_for: **GH91Val_rev:**	5'GACACCCTCCAACAGGGTGGAAACACAACAG3' 5'TGTTGTGTTTCCACCCTGTTGGAGGGTGTC3'	**Glu91Val**
GH85+87for: **GH85+87rev:**	5'CTCAGAGTCTATTCCAACACCTTCCAACAGGGAGG3' 5'CCTCCCTGTTGGAAGGTGTTGGAATAGACTCTGAG3'	**Pro85+87Pro**
GH87+91for: **GH87+91rev:**	5'CTATTCCGACACCTTCCAACAGGGTGGAAACACAAC3' 5'GTTGTGTTTCCACCCTGTTGGAAGGTGTCGGAATAG3'	**Pro87+91Val**

Table 2: The primer sequences containing desired mutations

50ng of Plasmid DNA was added to a 50 ul reaction mixture of 1mM $MgSO_4$, 0.3 mM dNTPs, 0.3 uM of each primers and 2.5U Platinum Pfx polymerase (Invitrogen). Reaction mixtures were denatured at 94^0C for 5 minutes, cycled 25 times at 94^0C for 15 sec, 58^0C for 1 min, 68^0C for 8 minutes. DH5a competent cells were transformed with different constructs and grown on LB/ampicillin media. After selecting the correct clones by colony PCR, the plasmid DNA was isolated using qiagen Maxiprep Kit. The desired mutation was confirmed in plasmid by sequencing.

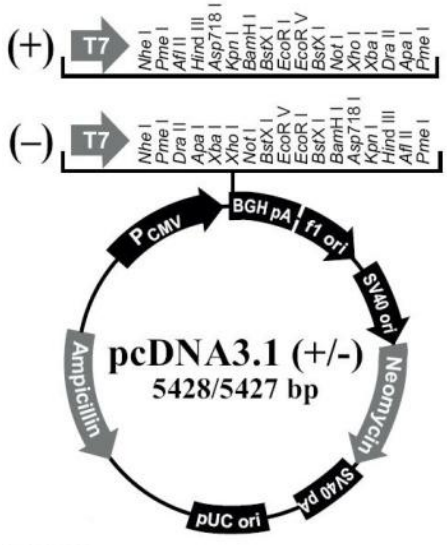


Figure 3: Schematic representation of the pcDNA vector

2.5 Cell Culture, Transfection and Isolation of RNA

GH4C1 rat pituitary cell line was used for the transfection experiments. The stock culture was grown in Ham's F10 medium (Gibco) supplemented with 15% Horse Serum, 2.5% FBS, 100U/ml penicillin, 100ug/ml streptomycin in a 5% CO_2. A day before transfection $4x10^5$ cells were seeded into each well of a six well tissue culture plate in 2.5 ml medium. The wells were previously treated with 1:10 diluted Poly-L-Lysine solution (Sigma) to allow the cells completely attach to the plate. At 50-70% confluency, the cells were transfected with 2.5ug DNA with vectors expressing GH-wt or GH-mutants using the Trans IT-LT1 transfection reagent (Mirus). A GFP control was used to analyse the efficiency of the transfection. 48h post transfection, total RNA was purified from the cells using Qiagen RNA mini Kit

2.6 Synthesis of cDNA and splicing analysis of mRNA

cDNA was synthesised from 1.5ug of RNA by High capacity cDNA Reverse transcription Kit (Applied Biosystems) following the manufacturers' instructions. The analysis of the different transcripts produced due to the alternative splicing was analysed using primers specific to GH1cDNA. These primers are designed to produce amplified products corresponding to full length (22.5 KDa), cryptic (20KDa), Exon3 skipped variant and also other splice products as indicated in figure 4. The PCR reaction mixture was denatured at 95^0C for 5 minutes and cycled 35 times (95^0C for 30s, 56^0C for 30s, 72^0C for 30s) followed by 5 minutes at 72^0C.

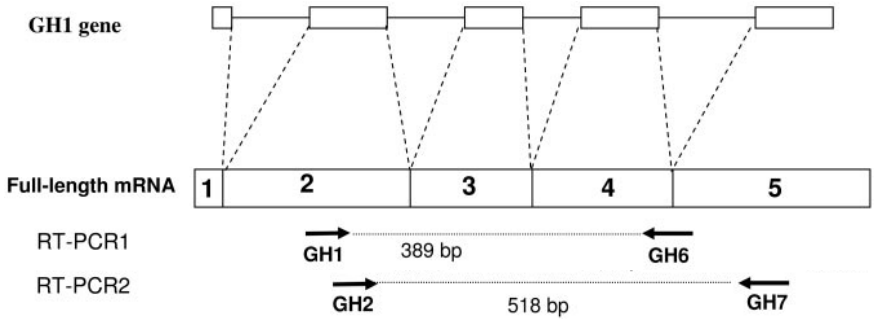


Figure4: Scheme of GH1 pre-mRNA splicing showing the full-length mRNA and the RT-PCR products.

2.7 ESE finder

The wild type and mutated exon sequences were analysed using ESE finder 3.0 web interface to search for sequences that act as binding sites for four SR family of splicing enhancer proteins. The threshold values of 1.956 for SF2/ASF, 1.867 for SF2/ASF (IgM-BRCA1), 2.383 for SC35, 2.67 for SRp40 and 2.676 for SRp55 were used for the analysis.

2.8 T/A cloning

T/A cloning (Thermo Scientific InsTAclone PCR cloning kit) has been performed to understand whether the double mutations are in the same allele or in different. The Exon 3 PCR fragments of the patients were cloned into pTZ57R/T vector. The ligated products were transformed into DH5α strains. The colonies were directly used in the PCR reaction followed by sequencing.

3.RESULTS

3.1 GH1 Exon 3 Variations

By sequencing the genomic DNA of 157 IGHD individuals, we identified 3 patients carrying different variations in GH1 exon 3. These variations are named based on their positions in exon3. First patient carried a non-synonymous variation GH-E3+75G>C (Glu82Asp), an already described SNP rs61762497. The second patient carried two novel variations GH-E3+90 C>T (Pro87Pro) and GH-E3+101A>T (Glu91Val). The third patient carried two synonymous variations GH-E3+90 C>T (Pro87Pro) and GH-E3+84G>A (Pro87Pro). The T/A cloning and sequencing revealed that the double mutations were at the same allele. These mutations were absent in 200 controls. All these mutations are originated by gene conversion from one of the GH cluster homologues genes. None of these mutations were not included in already described ESEs.

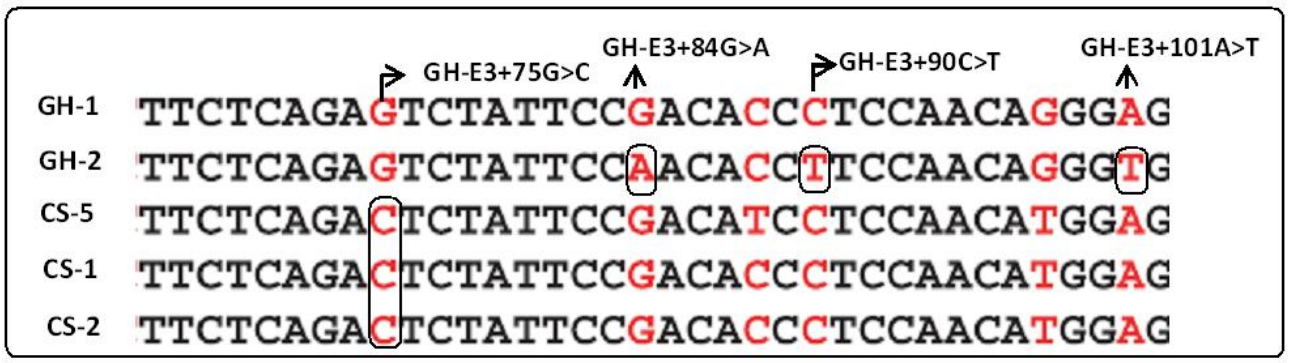


Figure5: Alignment of the GH paralogues (GH1, GH2, CS5, CS1 and CS2). The variations identified in patients are marked with arrows and the segments where the GH paralogues differ is shown in red.

3.2 ESE Finder Analysis

We Searched possible ESEs in Exon 3 using the wild type sequence. ESE finder web tool showed the presence of two high scoring ESE motifs at positions E3+83 to E3+89 (ESE3: CGACACC) and E3+98 to E+104 (ESE4: GGGAGGA) which includes E3+84 and E3+101 respectively. E3+84 is located in an ESE motif recognized by the SR protein SRp40 and the E3+101 is recognized by SF2/ASF and SF2/ASF(IgM-BRCA1). Both these ESE sequences showed an increased score compared to the threshold value (table1). The analysis of the mutated sequences showed that the presence of E3+84 G>A and E3+101A>T variations caused the loss of ESE3 and ESE4, respectively.

Tabular results

SRSF1 threshold: 1.956			SRSF1 (IgM-BRCA1) threshold: 1.867			SRSF2 threshold: 2.383			SRSF5 threshold: 2.67			SRSF6 threshold: 2.676		
Position*/Site/Score			Position*/Site/Score			Position*/Site/Score			Position*/Site/Score			Position*/Site/Score		
17 (-104)	CAAAGGA	3.67496	17 (-104)	CAAAGGA	3.42796	35 (-86)	CATTCCTG	3.26252	16 (-105)	CCAAAGG	4.39868	10 (-111)	TATATC	3.36686
25 (-96)	CAGAAGT	3.46589	25 (-96)	CAGAAGT	2.87763	46 (-75)	AACCCCCA	3.24440	24 (-97)	ACAGAAG	2.98263	41 (-80)	TGCAGA	4.34811
52 (-69)	CAGACCT	3.41188	40 (-81)	CTGCAGA	2.26826	54 (-67)	GACCTCCC	2.42696	51 (-70)	CCAGACC	3.09513			
94 (-27)	AACAGGG	2.75301	48 (-73)	CCCCCAG	2.10005				68 (-53)	TCTCAGA	2.89539			
98 (-23)	GGGAGGA	3.71421	49 (-72)	CCCCAGA	2.40075				83 (-38)	CGACACC	3.99790			
			52 (-69)	CAGACCT	3.14464				93 (-28)	CAACAGG	3.70100			
			69 (-52)	CTCAGAG	2.54670				106 (-15)	ACACAAC	3.86432			
			86 (-35)	CACCCTC	2.02741									
			98 (-23)	GGGAGGA	2.21174									

ESE3 ESE3 ESE4

Table3: Analysis of ESE sequences in Exon 3 of GH1. The potential ESE sequences showing the scores for SF2/ASF (SRSF1), SF2/ASF (IgM-BRCA1) and SRp40 (SRSF5) motifs.

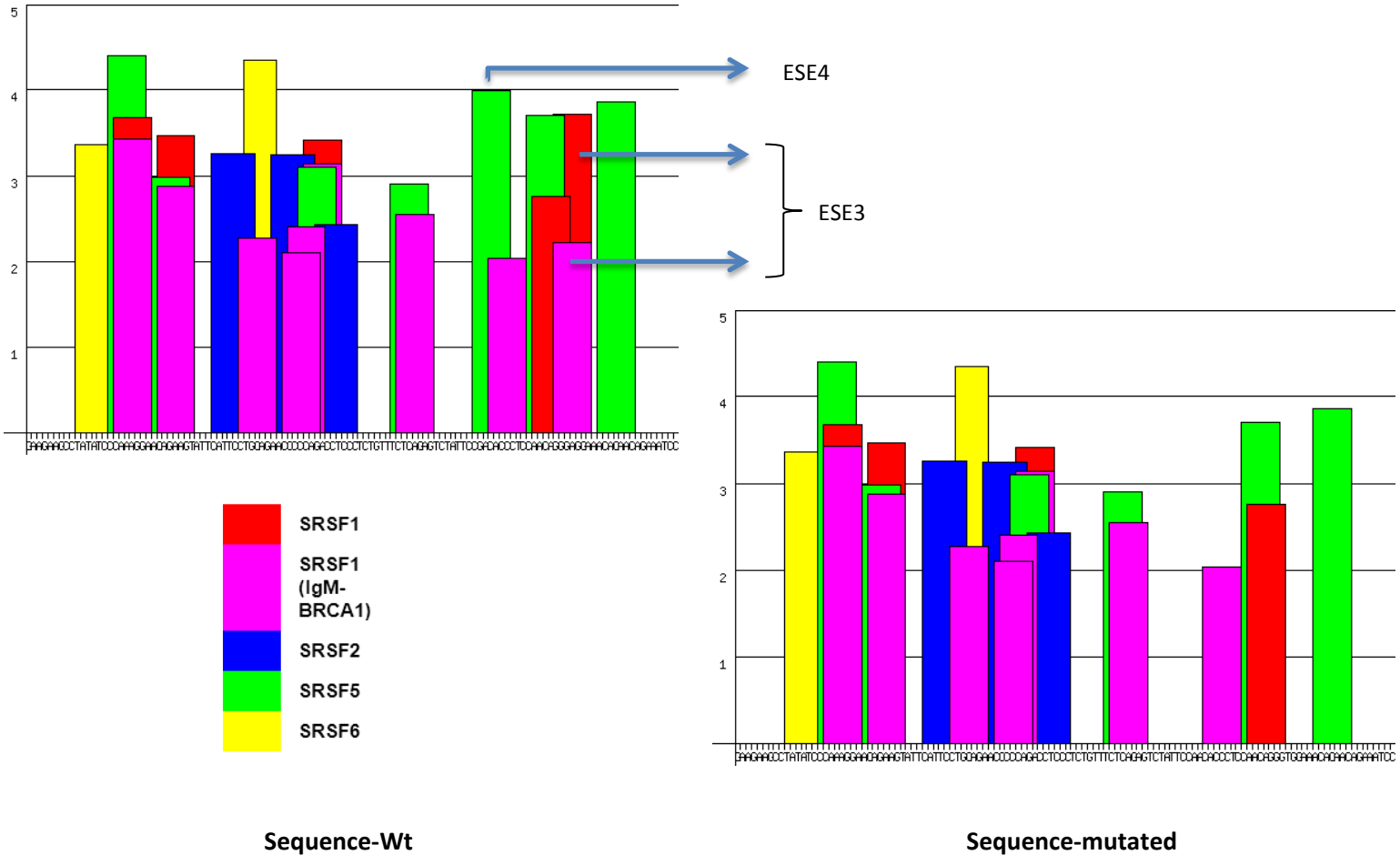


Figure6: Graphical representation of ESE finder results showing the presence of variations could completely abolish the ESEs.

3.3 mRNA Splicing Analysis

It has been previously reported that the mutations in the ESEs affect the splicing of the GH1 gene and produce the dominant negative isoform. To study the *in-vitro* splicing analysis, we transfected GH4C1 rat pituitary cells with constructs bearing the entire wild type GH (Ghwt) gene and the gene carrying different mutations. The mRNA from transfected cells were reverse transcribed and the resulting cDNA was used in two different RT-PCR experiments to analyse the splice variants.

RT-PCR1 used primers that amplifies part of exon2, exon3 and exon4 allowing the detection of Exon3 skipping. The GH-wt showed three different transcripts with a strong band of 389bp (22kDa full length) and two faint bands of sizes 344bp and 269 bp (usually detected in controls representing 20kDa and Exon skipped 17.5kDa respectively). GH-E3+75G>C showed full length and exon3 skipped bands of similar intensity as GH-wt. Conversely, GH-E3+84G>A and GH-E3+101A>T transcripts produced significantly higher level (about 35-40% of GHwt) of the exon3 skipped (17.5 kDa) band. The 17.5 kDa isoform was completely absent in GH-E3+90 C>T mRNA. The double mutation construct GH-E3 84G>A+90C>T produced a more intense band corresponding to 17.5kDa where as it was completely absent in GH-E3 90C>T + 101A>T.

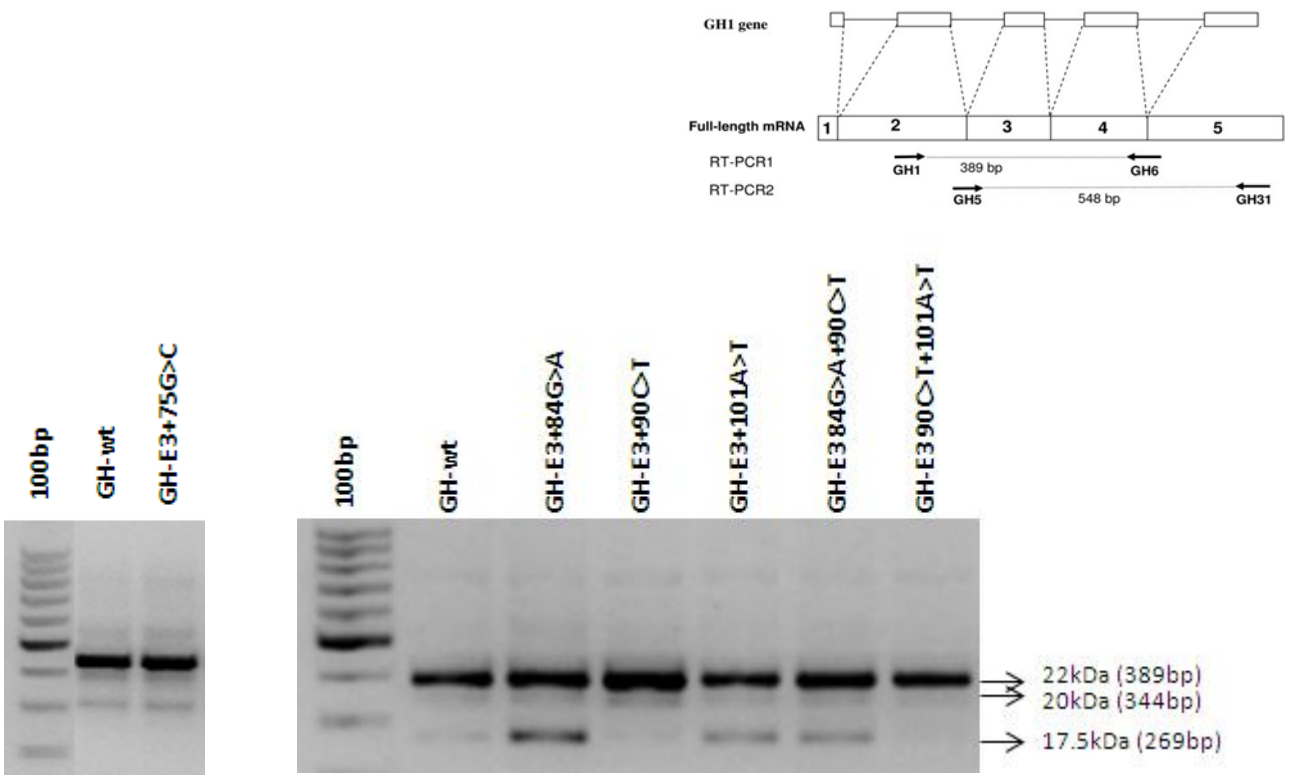
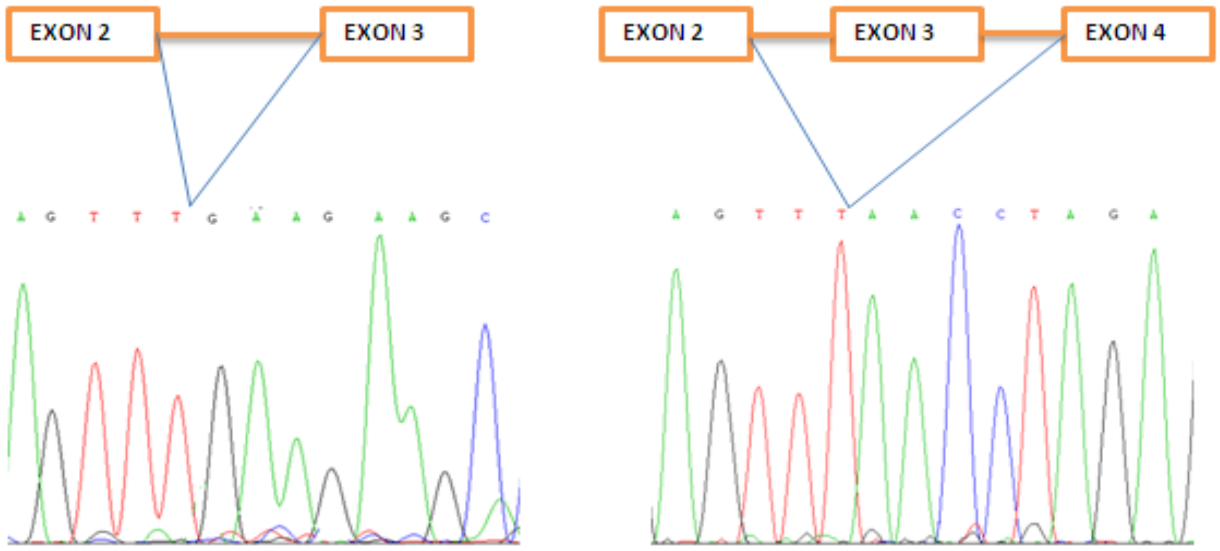


Figure1: RT-PCR1 performed on mRNA extracted from cells transfected with GH-wt and constructs harbouring different mutations.

The PCR products were extracted from the gel and sequenced. The deletion of exon 3 in the band corresponding to 269 bp was confirmed by sequencing.



(a) (b)

Figure2: The sequence result of the bands extracted from the gel corresponding to the full length (a) and the exon 3 skipped product (b)

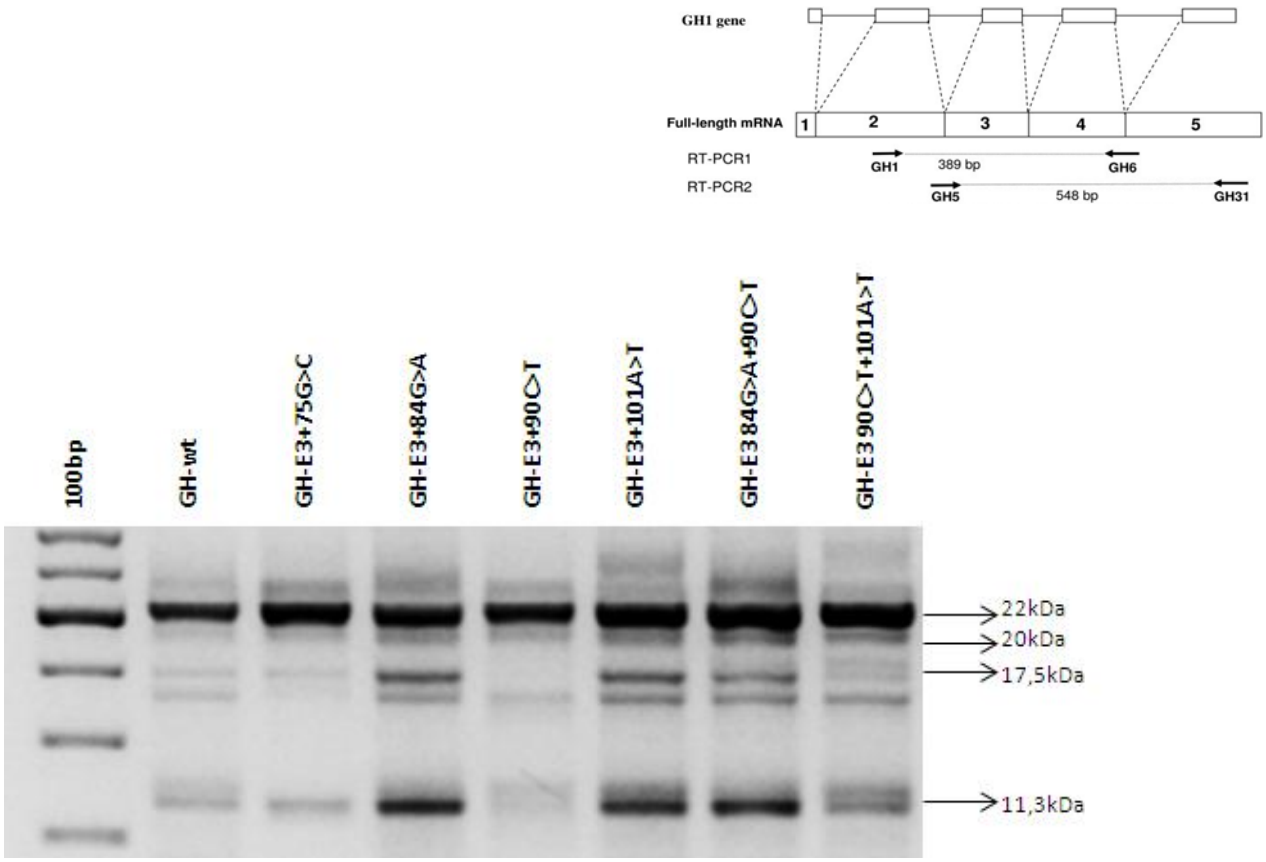


Figure: RT-PCR2 results showing different transcripts produced by alternative splicing of GH mRNA.

RT-PCR2 used primers located within exons 1-2 and exon 5 which are designed to amplify all the isoforms of the GH1 mRNA. The results showed more intense band of 17.5kDa for GHE3+84G>A and GH-E3+101A>T. The bands corresponding to 22kDa, 20kDa and smaller transcript were also visible on the gel. The band corresponding to 11.3 kDa was significantly stronger in GHE3+84G>A, GH-E3+101A>T and the constructs carrying double mutations.

4.DISCUSSION

The gene expression depends on the accurate removal of introns from the pre-mRNA molecule by alternative splicing. The splicing is primarily regulated by splice sites present at the intron-exon junctions and splicing enhancers present in exons and in introns[28]. It is well understood that both missense and synonymous mutations within exons affect the gene expression by modulating splicing [29]. In the case of alternative splicing in GH1 pre-mRNA, several studies have demonstrated that single point mutations on ESEs of exon 3 lead to the mis-splicing and the production of the dominant negative transcript [18-24].

In this study, four GH1 Exon 3 variations detected in 3 patients with IGHD have been investigated. The variations were analysed using in *silico* prediction to determine their effect on ESEs. The analysis showed that GHE3+84G>A and GH-E3+101A>T are located within the ESEs recognized by human SR proteins. The function of SR protein is to bind with the ESEs and identify the splice site [29]. The presence of these two mutations caused the loss of enhancer sequences predicted by the software. How ever, predicting the effect of these mutations purely from *in-silico* analysis of the genomic sequence is difficult due to the incomplete understanding of the multiple factors involved in splicing. It is also understood that some of these predictions gave contradictory effects in functional analysis [28, 30]. Although, computational analysis will provide useful information about the splicing enhancer motifs which are changed by mutation, functional testing is a necessity. For this purpose, we performed the functional studies of these mutations. The splicing patterns of GHE3+84G>A and GH-E3+101A>T mutations revealed the presence of more skipped transcripts. These results are analogous to those observed in most cases of IGHDII, by producing an increased level of the 17.5kDa transcript [18-24].

In conclusion, we identified two novel ESEs in the GH exon 3 through the detection of mutations in IGHD patients. The two muations, GHE3+84G>A and GH-E3+101A>T affect the pre-mRNA splicing by disrupting splicing motifs and produce an increased level of the 17.5 kDa protein. Further analyses are necessary to better understand the role of these mutations.

5.REFERENCES

1. Isaksson OG, Jansson JO, Gause IA (1982). Growth hormone stimulates longitudinal bone growth directly. Science 216:1237-9.
2. Strobe JS, ThomasMJ. 1994. Human growth hormone. Pharmacole Rev 46:1-34.
3. Wells JA (1996). Binding in the growth hormone receptor complex. Proc Natl Acad Sci USA 93:1-6.
4. Spagnoli A, Rosenfeld RG (1996). The mechanisms by which growth hormone brings about growth. The relative contributions of growth hormone and insulin-like growth factors. Endocrinol Metab Clin North Am; 25(3):615–63
5. Bona G, Paracchini R, Giordano M, Momigliano-Richiardi P (2004). Genetics defects in GH synthesis and secretion. European Journal of Endocrinology. 151: S3-S9.
6. Procter AM, Phillips JA III, Cooper DN (1998). The molecular genetics of growth hormone deficiency. Hum Genet 103:255-272.
7. Chen EY, Liao YC, Smith DH, Barrera-Saldana HA, Gelinas RE, Seeburg PH. The human growth hormone locus: nucleotide sequence, biology, and evolution. Genomics 1989;4:479-497.
8. Baumann G. Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endocr Rev 1991;12:424-449.
9. Rona RJ, Altman DG. National study of health and growth: standards of attained height, weight and triceps skinfold in English children 5 to 11 years old. Ann Hum Biol 1977;4:501-523.
10. Phillips JA III & Cogan JD. Molecular basis of familial human growth deficiency. Journal of Clinical Endocrinology and Metabolism1994;78:11–16
11. Massa CG, Binder G, Oostijik W, Ranke MB & Wit JM. De novo mutation of the growth hormone gene: an important cause of congenital isolated growth hormone deficiency? European Journal of Pediatrics 1998 157 272–275.
12. Wagner JK, Ebl A, Cogan JD, Prince MA, Phillips JA III and Mullis PE (1997). Allelic variations in the human growth hormone-1 gene promoter of growth hormone-deficient patients and normal controls. Eur. J. Endocrinol. 137, 474-81
13. Phillips JA III & Rimoin DL. Genetic disorders of the pituitary gland. In Principle and Practice of Medical Genetics, edn 3, ch 61, pp 1331–1364. Eds DL Rimoin, JM Connor & RE Pyeritz. New York: Churchill Livingstone, 1997.
14. Mullis PE. Genetic control of growth. Eur J Endocrinol 2005;152:11-31.
15. Yokoyama Y, Narahara K, Tsuji K, Moriwake T, Kanzaki S, Murakami M, Namba H, Ninomiya S, Higuchi J, Seino Y (1992) Growth hormone deficiency and empty sella syndrome in a boy with dup(X) (q13.3–q21.2). Am J Med Genet 42:660–664.
16. Mullis PE (2010). Genetics of Isolated Growth Hormone deficiency. Journal of Clinical Research in Pediatric Endocrinology;2(2):52-62.
17. Piekielko-witkowska A, Nauman A. Alternative splicing and its role in pathologies of the endocrine system. Polish Journal of endocrinology. 2011. 62:160-170.

18. Cogan JD, Prince MA, Lekhakula S, Bundey S, Futrakul A, McCarthy EMS and Philips III JA. 1982. A novel mechanism of aberrant pre-mRNA splicing in humans. Human molecular genetics. 6:6, 909-912.
19. Solis AS, Peng R, Crawford JB, PhilipsIII JA, Patton JG. 2007. Growth hormone deficiency and splicing fidelity. The journal of biological chemistry. 283(35);23619-23626.
20. Vivenza D, Guazzarotti L, Godi M, Frasca D, di Natale B, Momigliano-Richiardi P, Bona G, Giordano M. A novel deletion in the GH1 gene including the IVS3 branch site responsible for autosomal dominant isolated growth hormone deficiency. J Clin Endocrinol Metab. 2006;91:980-6.
21. Takahashi I, Takahashi T, Komatsu M, Sato T, Takada G. An exonic mutation of the GH-1 gene causing familial isolated growth hormone deficiency type II. Clin Genet 2002;61:222-225.
22. McCarthy EM, Phillips JA, 3rd. Characterization of an intron splice enhancer that regulates alternative splicing of human GH pre-mRNA. Hum Mol Genet 1998;7:1491-1496.
23. Shariat N, Holladay CD, Cleary RK, Philips III JA, Patton JG. Isolated growth hormone deficieny type II caused by a point mutation that alters both splice site strength and splicing enhancer function. Clin Genet 2008: 74: 539-545.
24. Ryther RC, Flynt AS, Harris BD, Phillips JA, 3rd, Patton JG. GH1 splicing is regulated by multiple enhancers whose mutation produces a dominant-negative GH isoform that can be degraded by allele-specific small interfering RNA (siRNA). Endocrinology 2004;145:2988-2996.
25. Lee MS, Wajnrajch MP, Kim SS, Plotnick LP, Wang J, Gertner JM, Leibel RL, Dannies PS. Autosomal dominant growth hormone (GH) deficiency type II: the Del32-71-GH deletion mutant suppresses secretion of wild-type GH. Endocrinology 2000;141:883-890.
26. McGuinness L, Magoulas C, Sesay AK, Mathers K, Carmignac D, Manneville JB, Christian H, Phillips JA 3rd, Robinson IC. Autosomal dominant growth hormone deficiency disrupts secretory vesicles in vitro and in vivo in transgenic mice. Endocrinology 2003;144:720-731.
27. Ryther RC, McGuinness LM, Phillips JA, 3rd, Moseley CT, Magoulas CB, Robinson IC, Patton JG. Disruption of exon definition produces a dominant-negative growth hormone isoform that causes somatotroph death and IGHD II. Hum Genet 2003;113:140-148.
28. Gargani M, Valentini A, Pariset L. A novel point mutation within the EDA gene causes an exon dropping in mature RNA in Holstein Friesian cattle breed affected by X-linked anhidrotic ectodermal dysplasia
29. Woolfe A, Mullikin JC, Elnitski L. 2010. Genomic features definfing exonic variants that modulate splicing. Genome Biology, 11:R20
30. Heintz C, Dobrowolski SF, Andersen HS, Demirkol, Blau N, Andersen BS. 2012. Splicing of phenylalanine hydroxylase (PAH) exon 11 is vulnerable: Molecular pathology of mutations in PAH exon 11. Molecular Genetics and Metabolism 106 (2012) 403–411.

SEMINARS ATTENDED:

- 22 March 2012 - "Molecular classification of multiple myeloma". Prof. Antonino NERI, Università degli Studi di Milano
- 23 March 2012 - "Role of Diacylglycerol kinases in the control of T cell activation and differentiation programs" Prof.ssa Isabel MERIDA, Centro Nacional de Biotecnologia, Madrid
- 28 March 2012 - "Developing strategies for tissue specific targeting" Prof Costantino Pitzalis, Barts and the London School of Medicine
- 15 May 2012 - "Microparticles as novel effectors in Inflammation" Prof. Mauro PERRETTI , William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London
- 23 May 2012 - "Numerical simulations as virtual microscope at the nanoscale: some examples with dendritic molecules" Prof. Andrea Danani, University of Applied Science of Southern Switzerland-SUPSI
- 10 July 2012 - "Next generation sequencing in T-ALL" Dr. Kim De Keersmaecker, VIB Center for the Biology of Disease, Center for Human Genetics, KU Leuven (Belgium)

ABSTRACT PUBLICATIONS:

"IDENTIFICATION OF NOVEL EXON SPLICE ENHANCERS (ESEs) IN THE GROWTH HORMONE (GH) GENE MUTATED IN ISOLATED GH DEFICIENCY (IGHD) PATIENTS"

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