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Department of Health Sciences

ILEANA FUSCO

PhD in Molecular Medicine

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IDENTIFICATION OF NOVEL GENES INVOLVED IN SHORT STATURE WITH AND WITHOUT GROWTH HORMONE DEFICIENCY.

Tutor: **Prof.ssa Mara Giordano**

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Genetic determination of Height

Height is a highly heritable and easily measurable complex continuous trait determined by a multitude of genetic, hormonal, nutritional and other environmental determinants. Resemblances in height between relatives suggest that 80% of height variation is under genetic control with the rest controlled by environmental factors such as diet and disease exposure ([1]McEvoy BP et al 2009)

In the past, several linkage genome scans have attempted to identify genetic factors involved in the determination of human height, leading to suggestive or significant linkage on many chromosomes. However, no region was consistently highlighted across scans and no gene was definitively associated to the determination of normal stature.

Recently several Genome-wide association study (GWAS) identified a subset of about 180 variations in the human genome that contribute to the determination of height. These variants, however, have small effect sizes and collectively explain approximately 10% of the heritability of height. A recent study ([2]Chan Y. et al 2011) indicated that these 180 common variants are robustly associated with stature when studied as a quantitative trait in the general population, whereas a similar strong association is not present at the extreme short tails of the height distribution. The polygenic model starts to break down in the extreme short individuals.

Short stature has been defined as height <-2 Standard Deviation (SD) below the mean for a given age, sex and population group and represents the most common cause for paediatric consultation.

In presence of “proportionate short stature”, the measurement of serum GH concentration is used to categorize two main classes of growth disorders: short stature caused by GH deficiency (GHD), and idiopathic short stature (ISS).

Growth hormone deficiency (GHD) encompasses a group of different aetiological disorders. It may occur in isolation (IGHD), or associated with other anterior and posterior pituitary hormone deficiencies. The condition may be sporadic or familial. The incidence is between 1 in 4000 and 1 in 10 000 live births ([3]Rona R.J. et al 1977), with the majority of cases being idiopathic in origin. Familial cases account for 5–20% of cases, suggesting a genetic aetiology (autosomal dominant or recessive or X-linked) for the condition.

ISS describes a heterogeneous group of children consisting of many presently unidentified causes of short stature ([4]Cohen P. et al 2008), that presents the following criteria: normal size for gestational age at birth, normal body proportions, no evidence of endocrine deficiency, no evidence of chronic organic disease, no psychiatric disease or severe emotional disturbance and normal food intake. The most frequent genetic cause of ISS is represented by mutations and deletions in the Short Stature Homeobox gene (SHOX). Different studies report mutations and deletions of this gene and microdeletions of the enhancer region located between 48 and 215 kb downstream of the SHOX gene in 7–15% of patients diagnosed with ISS ([5]Schneider K.U. et al 2005, [6]Benito-Sanz S. et al 2011, [7]Sabherwal N. et al 2007). Sixty-three sporadic ISS patients have already been screened for the presence of *SHOX* mutations through direct sequencing of all the exons and intron-exons boundaries. To date we identified causative mutations in 4 patients corresponding to a detection rate of 6.3% (data not published). Thus only a minority of the short stature patients received a precise diagnosis with the identification of the genetic defect.

The aim of my project is the identification of novel genes involved in short stature with and without growth hormone deficiency through Sanger sequencing, Array Comparative Genomic Hybridization (array-CGH) and NEXT GENERATION SEQUENCING.

1. SEARCH FOR MUTATIONS IN HMGA2 GENE

Earlier known as HMGIC, HMGA2 encodes a protein that belongs to the non-histone chromosomal high-mobility group (HMG) protein family. HMGA2 is located on chromosome 12q14 and contains 3 AT-hook motifs, involved in DNA binding, encoded by the first 3 exons; a 11-amino acid sequence characteristic of HMGA2 and absent in the other family proteins (linker domain) encoded by exon 4 and part of exon 5; and an acidic C-terminal domain encoded by the fifth exon ([8]Mari F. et al. 2009).

We investigated HMGA2 because previous GWAS studies independently confirmed that 3 SNPs (rs1042725, rs7968682 and rs7968902) in the high mobility group-A2 gene (HMGA2) are common variants influencing human height across different populations ([9]Takeshita H. et al. 2011). All of these three SNPs are located within the 3′-UTR region of HMGA2, which may directly or indirectly influence the mRNA stability of HMGA2. Moreover the gene was considered as a strong biologic candidate for height because loss of function of HMGA2 in the mouse results in the pygmy phenotype that combines pre and postnatal growth failure, with resistance to the adipogenic effect of overfeeding ([8]Mari F. et al. 2009).

In humans CGH-Array results showed that patients with a microdeletions on the chromosomal region 12q14 of different size have as common features short stature. The Critical Region in patients with severe short stature include the HMGA2 gene (Figure 1, [10]Lynch S.A. 2011).

The HMGA2 has never been screened for the presence of mutation in ISS patients. Thus this gene could be considered a good candidate for the presence of point mutations in cases in which mutations in SHOX gene have been excluded. This is the first study based on HMGA2 sequence analysis in a large cohort of ISS patients.

Materials and methods

DNA Extraction

Genomic DNA of short stature patients was extracted from the blood samples by standard salting-out method. Short stature patients were recruited by the Unit of Pediatrics of Department of Health Sciences of Novara.

Sequence analysis

Primers were designed to amplify all 5 coding exons and the 5'- and 3'-flanking regions, using NCBI database (http://www.ncbi.nih.gov/), Primer3 Input (Version 0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/). The primer pair specificity was controlled with the Basic local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

The PCR reaction was carried out with the GoTaq Flexi DNA polymerase (Promega) in a 15 ul reaction volume, at an annealing temperature of 58°C for exon 2 and 4 and 62°C for exon 1,3, 5.

The PCR products were purified using Affymerix ExoSAP-IT enzymatic PCR clean up system (37°C 15 min and 80°C 15 min) and directly sequenced with Big Dye Terminator kit (Applied Biosystems) and automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Results

All the exons and the splicing junctions of the novel candidate gene HMGA2 were sequenced in 58 short stature patients, in which mutations in SHOX were excluded.

The mutation analysis in our patients did not show any sequence changes suggesting that mutations in this gene are not a frequent cause of ISS. However we will expand the number of cases and on all the patients we will perform a multiplex ligation probe amplification (MLPA) to detect deletions of the whole HMGA2 gene and of single exons.

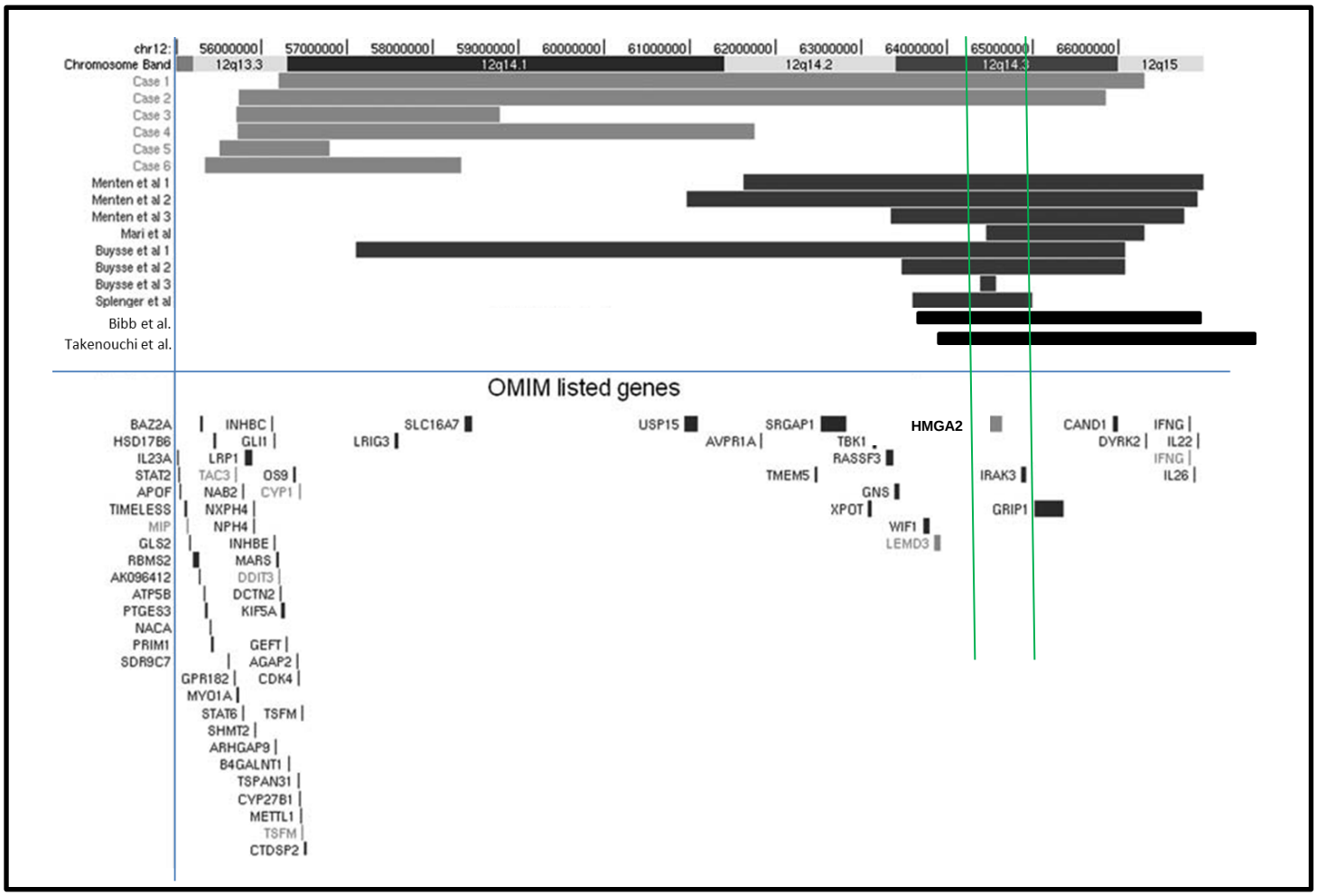

Figure 1. Schematic illustration of the patients with 12q14 deletions detected by array-CGH using the Ensembl browser.

2. IDENTIFICATION OF A NOVEL GENE (MGAT4C) WITHIN A GENOMIC REGION DELETED IN A PATIENT WITH SHORT STATURE

A Comparative Genome Hybridization array (aCGH) analysis have been performed on a 12 years old boy with severe short stature (-2.8 SDS) and no dismorphic trait. This analysis revealed the presence of two deletions that were not present in the Database of Genomic Variants (http://projects.tcag.ca/variation/): a deletion of 320 kb on chromosome 12[del(12)(q21.31-q21.32)] and a deletion of about 32 kb on chromosome 21 [del21(21)(q22.13)]. The same analysis, performed on the parents revealed that the deletion on chromosome 21 was transmitted from the father whereas deletion on chromosome 12 arose de novo in the patient. Interestingly this latter contains a gene encoding the enzyme mannosil-glycoprotein-aceylglucosamyniltransferase (MGAT4C) that have been identified as a genetic locus associated with adult height variation ([11]Yang TL et al. 2010).

CGH-Array experiment was conducted in the laboratory of Pavia that collaborate with us on this project.

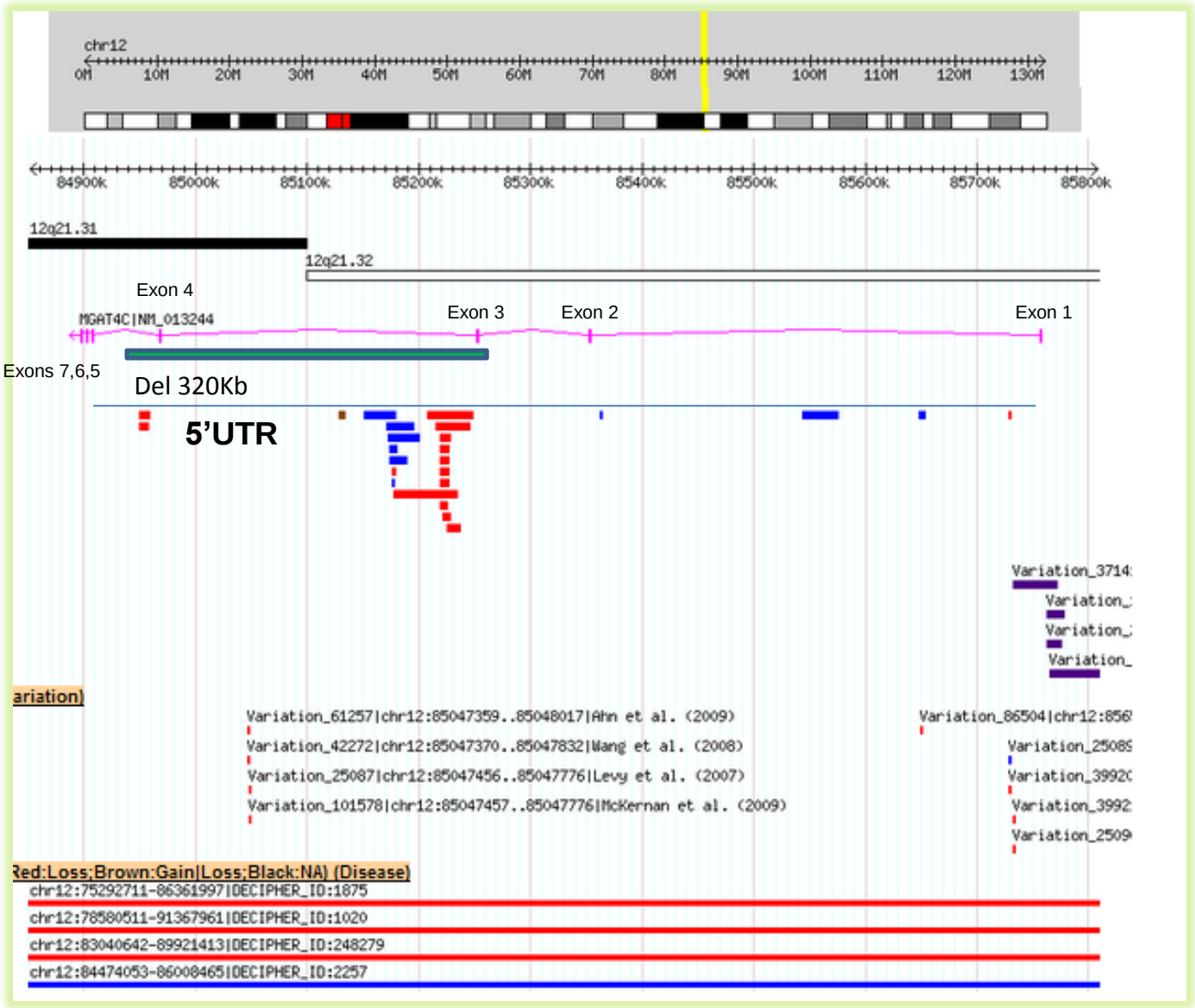

Figure 2. Schematic illustration of MGAT4C deletion that involves the exon 3 and 4 detected by array-CGH, obtained using the Database of genomic Variants (Chr12:84,945938-85,269801).

Materials and methods

DNA Extraction

Genomic DNA of patient was extracted from the blood sample by standard salting-out method.

CGH-Array

Array-CGH analysis was conducted in the laboratory of Pavia using a 105 K commercial arrays (Agilent Technologies, Santa Clara, CA, USA). This platform contains 60-mer oligonucleotide

probes spanning the entire human genome with an overall median probe spacing of 22 Kb (19 Kb in Refseq genes). A male reference DNA (NA10851- Coriell Institutes) has been used for each subject tested. Previously reported benign CNVs listed in the Database of Genomic Variants http://projects.tcag.ca/variation/ were excluded as causative for the disease.

RNA extraction and cDNA synthesis

Lymphocytes and monocytes were isolated from human peripheral blood using Lympholite-H (CEDARLANE's Lympholyte® cell separation centrifugation media). Then total RNA was purified from lymphocyte obtained with this density gradient separation method, using RNeasy®Plus Mini kit (Quiagen) and cDNA was synthesized by High capacity cDNA Reverse transcription Kit (Applied Biosystems) following the manufacturers' instructions.

MGAT4C primers

We designed primers to amplify all 3 coding exons (5,6,7) and the 5'- and 3'-flanking regions of MGAT4C (Figure 3). Then we designed primers on the MGAT4C cDNA to search for aberrant splicing (Figure 4).

Figure 3. Schematic illustration of primers designed to sequence three coding exons (5,6,7) of MGAT4C .

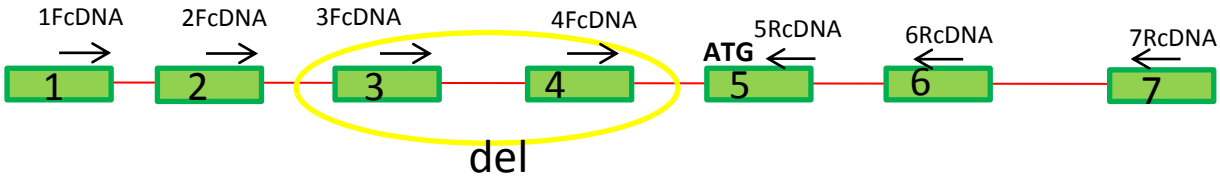

Figure 4. Schematic illustration of primers designed to search for aberrant splicing.

Results

As the deletion was present at the heterozygous state, we sequenced in the proband all three coding exons (5,6,7. Figure 3) of the other allele to see whether there were sequence changes. The sequence analysis of the remaining allele didn't reveal any sequence variations.

The deletion encompasses the exon 3 and 4 that are part of the 5′-UTR (Figure 2). It is well known that the 5'-UTRs of mRNAs contain regulatory signals for modulating mRNA stability and protein translation (12Scheper G.C et al 2007) and alternative splicing within 5'-UTRs can affect translational efficiency (13Wang G. et al 2005, 14 Shalev A. et al 2002).

We will perform an RT-PCR study to search for aberrant splicing, using primers designed on the MGAT4C cDNA (Figure 4). The Quantitative (realtime) PCR (qPCR) will be used to confirm MGAT4C deleted region.

Depending on the results, MGAT4C could be one of the genes that we are planning to screen in the short stature patients, in addition to the genes that will be identified through exome sequencing.

3. Search for novel genes involved in IGHD through Whole-Exome sequencing

Whole-Exome sequencing (WES) has, in the last three years, proven its potential in the discovery of novel Mendelian disease genes ([15]Bamshad et al, 2011), as well as in the diagnosis of rare conditions ([16]Choi et al, 2009; [17]Worthey et al, 2011), or of known genetic conditions with an unusual phenotypic presentation ([18]Majewski et al, 2011).

We collected 10 IGHD familial cases (Figure 4) that have been already screened for the presence of mutations in specific known genes involved in the disease (GH1, GHRHR), in which mutations were excluded.

In order to identify novel genes involved in IGHD, we will perform whole-exome sequencing on the affected members of the 10 families, in which the genetic causes of short stature remained unidentified by conventional approaches.

To date we sequence through the whole exome and targeted sequencing experiments the affected member of family #1 and #2 (Figure 4).

A family-based sequencing approach should actually allow an easier filtering and prioritization of the variants identified by massive parallel sequencing ([19]Robinson et al, 2011; [15]Bamshad et al, 2011), by focusing on those segregating with the condition. If the inheritance model of the disease in the family suggests a recessive condition, sequencing of the affected subjects should allow us to focus on those variants present at the homozygous state in all the individuals we sequenced, or those variants for which patients are compound heterozygotes. If the family tree suggests a dominant model of inheritance, by sequencing affected and unaffected subjects, we should be able to filter-out those variants, likely to be unrelated with the disease, found at the heterozygous state in the probands and in their healthy relatives, and to focus on those segregating with the disease. The possibility of incomplete penetrance will be taken into consideration and a re-evaluation of the family will be considered on the basis of preliminary exome data.

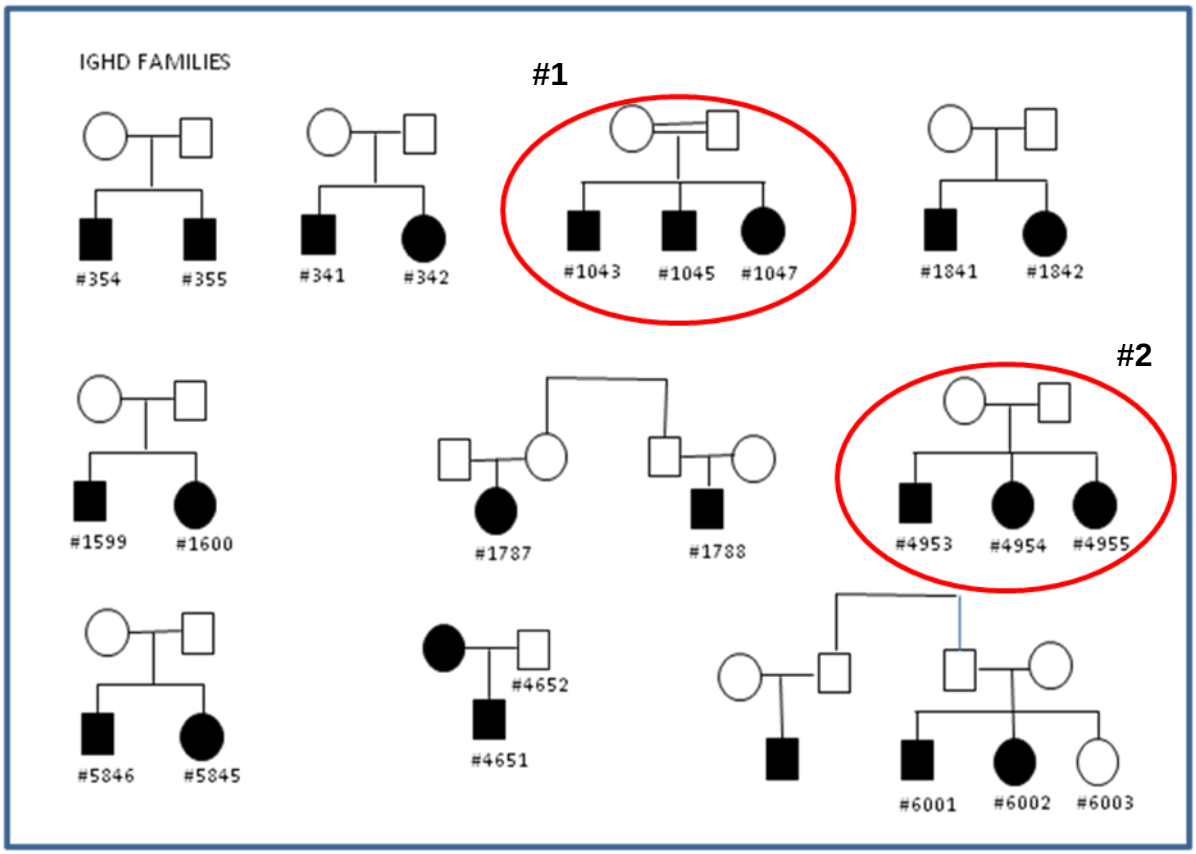

Figure 5. Pedigree of the patients that will be studied through whole exome sequencing.

3.2 Materials and methods

The whole exome and targeted sequencing experiments have been performed in the laboratory of PAVIA were a Genome Analyzer IIx, a new generation desktop sequencer MiSeq (both from Illumina, San Diego, CA, USA) and the appropriate informatics infrastructures are available. They analysed three affected individuals for each family reported in Figure 4 (#1,#2, marked by red circles).

3.3 Results

Recently we received the result of the whole-exome sequencing analysis of family #2. We are evaluating data obtained considering a recessive and dominant inheritance of the disease with incomplete penetrance.

Each variant we will identify will be considered in the light of its possible presence and allele frequency in publicly available genomic variants databases such as dbSNP and Exome Variant

Server Database (url: http://snp.gs.washington.edu/EVS/; NHLBI Exome Sequencing Project (ESP), Seattle, WA), as well as in uncorrelated samples included in our in-house database, filtering out those likely to be common in the general population.

After this selection of a group of variants, these will be validated by Sanger sequencing that will also be used to test the segregation of each mutation in other affected/unaffected family members. Moreover, by Sanger sequencing, the validated mutations will be further tested in a group of 400 normal stature individuals, matched for age, sex and geographical origin, so to reduce the number of candidate mutations, by excluding from further analysis those likely to be polymorphisms not related to short stature.

To date we selected a group of 50 candidate variants found at the heterozygous state in the probands. We are studying EGR1 gene that encodes for early growth response protein 1, that is expressed in anterior pituitary gland.

4. References

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SEMINARI

- Lunedì 19 Dicembre 2011, ore 14.00 "Alpha-MSH and the melanocortin system in inflammation". Prof. Mauro PERRETTI , William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London
- Martedì 20 Dicembre 2011, ore 10.00 "Galectins-carbohydrate binding protein: sweet or sour?". Prof. Mauro PERRETTI , William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London
- Venerdì 13 Gennaio 2012 , ore 14:00 "Hepatocellular Carcinoma, novel advances from genomics to treatment" . Dr. Rohini SHARMA Imperial College London
- Mecoledì 25 Gennaio 2012, ORE 14.00 "Next-generation DNA sequencing and target arrays in the clinics". Dr. Paolo Fortina, Department of Cancer Biology, Jefferson Genomics Laboratory, Kimmel Cancer Center, Thomas Jefferson University Jefferson Medical College, Philadelphia, PA, USA
- Venerdì 24 Febbraio, ore 12 "Exosomes Shuttle RNA" Prof. Lötvall J., Department of Internal Medicine, Sahlgrenska Academy, University of Gothenburg
- Giovedì 22 marzo 2012 alle ore 14.00 "Molecular classification of multiple myeloma". Prof. Antonino NERI, Università degli Studi di Milano
- Venerdì 23 marzo 2012 alle ore 14.00 "Role of Diacylglycerol kinases in the control of T cell activation and differentiation programs" Prof.ssa Isabel MERIDA, Centro Nacional de Biotecnologia, Madrid
- Mercoledì 28 marzo, ore 11, "Developing strategies for tissue specific targeting" Prof Costantino Pitzalis, Barts and the London School of Medicine
- Martedì 15 Maggio 2012, ore 15.00 "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
- Mercoledì 16 Maggio 2012, ore 14.00 "Resolvins and Omega-3 in Inflammation" Prof. Mauro PERRETTI , William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London
- Mercoledì 23 maggio 2012, ore 11.30 "Numerical simulations as virtual microscope at the nanoscale: some examples with dendritic molecules" Prof. Andrea Danani, Chief of the Laboratory of Applied Mathematics and Physics-LAMFI, Department of Innovative Technologies-DTI, University of Applied Science of Southern Switzerland-SUPSI, Lugano, CH
- Giovedì 21 giugno 2012, ore 11.00 "Recent Advances in Hematopoietic Stem Cell Gene Therapy: from microRNA Regulation to Targeted Gene Transfer" Prof. Luigi Naldini, San Raffaele, Telethon Institute for Gene Therapy (HSR-TIGET)
- Martedì 10 luglio 2012, ore 14.00 "Next generation sequencing in T-ALL" Dr. Kim De Keersmaecker, VIB Center for the Biology of Disease, Center for Human Genetics, KU Leuven (Belgium)

Corsi-congressi

- Torino 18-21 Aprile 2012. Golden Helix Symposium Genomic Medicine: “Translating Genes into Health”.
- Genova 13-15 Giugno 2012. Corso avanzato di citogenetica costituzionale:” Il cariotipo molecolare perché, quando e come” III edizione.

Abstract

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

D. Babu, I. Fusco., S. Mellone, M. Godi, A. Petri, F. Prodam, S.Bellone, P. Momigliano-Richiardi, G. Bona and M. Giordano**.** Department of Health Sciences, University of Eastern Piedmont, Novara, Italy

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