Whole Genome SNP Genotyping and Exome Sequencing Reveal Novel Genetic Variants and Putative Causative Genes in Congenital Hyperinsulinism

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Abstract
Congenital hyperinsulinism of infancy (CHI) is a rare disorder characterized by severe hypoglycemia due to inappropriate insulin secretion. The genetic causes of CHI have been found in genes regulating insulin secretion from pancreatic β-cells; recessive inactivating mutations in the ABCC8 and KCNJ11 genes represent the most common events. Despite the advances in understanding the molecular pathogenesis of CHI, specific genetic determinants in about 50% of the CHI patients remain unknown, suggesting additional locus heterogeneity. In order to search for novel loci contributing to the pathogenesis of CHI, we combined a family-based association study, using the transmission disequilibrium test on 17 CHI patients lacking mutations in ABCC8/KCNJ11, with a whole-exome sequencing analysis performed on 10 probands. This strategy allowed the identification of the potential causative mutations in genes implicated in the regulation of insulin secretion such as transmembrane proteins (CACNA1A, KCNHI6, KCNJ10, NOTCH2, RYR3, SCN8A, TRPV3, TRPC5), cytosolic (ACACB, CAMK2D, CDAK1, GNAS, NOS2, PDE4C, PIK3R3) and mitochondrial enzymes (PC, SLC2A4, and in four genes (CSMD1, SLC37A3, SULF1, TLL1) suggested by TDT family-based association study. Moreover, the exome-sequencing approach resulted to be an efficient diagnostic tool for CHI, allowing the identification of mutations in three causative CHI genes (ABCC8, GLUD1, and HNF1A) in four out of 10 patients. Overall, the present study should be considered as a starting point to design further investigations: our results might indeed contribute to meta-analysis studies, aimed at the identification/confirmation of novel causative or modifier genes.

Citation: Proverbio MC, Mangano E, Gessi A, Bordoni R, Spinelli R, et al. (2013) Whole Genome SNP Genotyping and Exome Sequencing Reveal Novel Genetic Variants and Putative Causative Genes in Congenital Hyperinsulinism. PLoS ONE 8(7): e68740. doi:10.1371/journal.pone.0068740

Editor: Degui Zhi, University of Alabama at Birmingham, United States of America

Received January 7, 2013; Accepted May 31, 2013; Published July 15, 2013

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Funding: The present study was supported by grants of MIUR PRIN2008WY2TY9. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction
Congenital hyperinsulinism (CHI), previously known as persistent hyperinsulinemic hypoglycemia of infancy (PHHI, MIM256450), is characterized by severe hypoglycemia due to inappropriate insulin secretion from pancreatic β-cells. If improperly managed, hypoglycemia can cause brain damage, learning disability, and even death [1]. This condition affects at least 1/50,000 children of European descent, and it has been reported in nearly all major ethnic groups [2].

Histologically, CHI can be associated either with diffuse insulin secretion or with focal adenomatous hyperplasia. These two forms share a similar clinical presentation, but result from different molecular mechanisms. Recently, a positron emission tomography scan using Fluorine-18 L-3,4-dihydroxyphenyalanine (18-fluoro DOPA-TC-PET-scan) has been used to distinguish focal from diffuse forms. Diffuse CHI (Di-CHI) is characterized by autosomal recessive or (less frequently) dominant inheritance, whereas focal CHI (Fo-CHI) is due to a germline paternal mutation (in the ABCC8 or KCNJ11 gene) in addition to a somatic loss of the maternally-derived chromosome 11p15.1 region in pancreatic β-cells [2]. According to the molecular defects, CHI has been classified as a channelopathy (KATP channel), as a metabolic enzyme/transporter anomaly, or as a transcription factor defect [1]. Medical treatments of CHI include different drugs such as oral diazoxide (channel activator), somatostatin analogue (octreotide) injections, and appropriate diet. The surgical treatment with subtotal pancreatectomy is required in Di-CHI when medical treatment and dietary therapies are ineffective, whereas Fo-CHI can be cured with resection of the focal area of adenomatous hyperplasia. In the first case, the long-term outcome is characterized by the risk of developing diabetes.
Overall, CHI causative mutations have been documented in 10 genes, allowing a molecular diagnosis for nearly 50% of CHI patients [3,4]. The most common molecular cause of CHI is the dysfunction of the pancreatic K\(^{+}\)-ATP channel encoded by the sulfonylurea receptor gene (SUR1, alias ABCC8) and the inward-rectifying potassium channel gene (KIR6.2, alias KCNJ11). Less frequently, recessive mutations in the HADH gene (3-hydroxyacyl-Coenzyme A dehydrogenase) have been reported. Dominant forms of CHI are due to either activating mutations in mitochondrial matrix enzymes, GLUD1 (glutamate dehydrogenase 1) and UCP2 (mitochondrial uncoupling protein 2), or to mutations in HNF4A (hepatocyte nuclear factor 4 alpha) and SLC16A1 (monocarboxylate transporter 1). Activating mutations in the GCK (glucokinase) may also lead to CHI, and an autosomal dominant mutation in INSR (insulin receptor gene) has been described in a large Danish pedigree [5]. Very recently, mutations in the HNF1A (hepatocyte nuclear factor 1 alpha) gene have been reported in CHI patients [6,7].

Insulin secretion is regulated by a complex network of proteins [8] acting at the cellular membrane level (i.e. ATP sensitive K\(^{+}\) channels, Ca\(^{2+}\), Na\(^{+}\) voltage-gated channels, glucose transporters, and transient receptors) [9], at the intracellular level (i.e. endoplasmic reticulum ion channels), and at the mitochondrial level (i.e. enzymes) [10]. Since CHI is characterized by severe hypoglycemia due to inappropriate insulin secretion from pancreatic β-cells, we hypothesized that mutations in genes involved in this process might be responsible for the disease. Moreover, considering the phenotypic variability observed within CHI families, “phenotypic modifier” genes might contribute to the global genetic picture of this disorder as described for other Mendelian disorders [11].

The mutational screening of CHI causative genes is usually carried out by PCR amplifications and direct Sanger sequencing, using an iterative approach based on clinical features and/or on medical treatment response [2].

High-density single nucleotide polymorphism (SNP)-array technology has been widely used to perform linkage analysis, including the transmission-disequilibrium test (TDT). Moreover, the combination of linkage analysis with haplotype information (applied to trios) has been successful applied to map Mendelian traits [12] and has led to the identification of gene variants causing diseases and birth defects [13]. Recent developments in high-throughput sequence capture methods and next-generation sequencing (NGS) approaches have made feasible the analysis of the whole human exome. The exome deep sequencing, called whole-exome sequencing (WES) analysis, has already provided the opportunity to detect causal gene variants in dominant and recessive disorders [14,15] and it has been recently proposed as a powerful tool to improve the molecular diagnostic of neonatal diabetes and maturity onset diabetes of the young (MODY) [16,17].

Here, we applied a multi-step screening strategy aimed at identifying genes putatively related to CHI. This strategy included a TDT study on 17 patients and their families and exome sequencing in 10 CHI probands. We have also evaluated if NGS is a suitable alternative diagnostic approach to classical Sanger sequencing for CHI.

Results and Discussion

To highlight novel CHI-associated genetic loci, we performed a multi-step screening strategy (Figure S1). First we carried out a genetic screening of ABCC8/KCNJ11 genes on 33 CHI probands by classic sequencing approach; then, applying a genome wide SNP genotyping analysis on 17 non-consanguineous CHI patients (lacking ABCC8/KCNJ11 mutations) and their families, we performed a TDT family-based association study, and finally we applied WES on 10 selected CHI probands (five from the TDT analysis group and five not subjected to previous molecular screening). To prioritize the coding variants identified by WES, we crossed the list of filtered WES variants with that comprising the TDT associated genes (51 genes), the known CHI-causative ones (10 genes), and a refined list of 211 CHI-functionally-related genes (see Methods for details).

Whole-genome SNP Genotyping and TDT Analysis

The whole-genome SNP genotyping was performed on the 17 nuclear families. The overall results of the TDT association analysis are presented in the Manhattan plot (Figure S2). We obtained 144 SNPs associated at P<0.0005 (Table S1). None of these SNPs reached the stringent genome-wide significance of P<4.4⋅10^{-7}; however, we considered any empirical P-value at 4.9⋅10^{-5} as “suggestive” (i.e. a potential reflection of an association signal) [18]. After genome annotation, we found that a subset of these SNPs map within/close to 51 genes (Table S1). Interestingly, we identified two SNPs located in the region of the TTI1 (tollid-like1) gene: rs3775321 (P = 0.000049; OR = 7.3, 95%CI = 2.2–24.5) and rs4691229 (P = 0.00043; OR = 0.2, 95%CI = 0.1–0.5). Using haplotype analysis we were able to confirm and to refine the potential association of the TTI1 locus to the CHI (Table S2).

Applying the copy-number and homozygosity mapping analyses on 17 probands, we confirmed the absence of deletion or amplification events (data not shown); conversely, in two probands we found runs of homozygosity (ROH) longer than 2 Mb. The ROH segments were located on chromosome 4q24 (4.2 Mb containing 14 genes) in patient HI06, and on chromosome 6p36.33-q13.33 (7 Mb containing 26 genes) in patient HI18. By the subsequent WES analysis, we verified that all genes located in ROH segments were lacking mutations.

WES Analysis and Gene Variants Identification

WES was performed on 10 CHI probands (Table 1). By sequencing the exome DNA libraries, we obtained 2.8–5.4 Gb of mapped sequence achieving a mean depth of target coverage between 27 and 50×, and an average of 19,181 variations per patient, including substitutions and indels (Table 2). Overall, the results of the percentage of target coverage at 10× were fairly uniform, reaching 90, 83, 80, and 75% for INSR, ABCC8, GCK, HNF4A, respectively. Only for HNF1A the percentage was low (55%). By focusing on non-synonymous single nucleotide variants (SNV, missense and nonsense) we counted an average of 9,395 variants per patient. After the filtering procedure, we counted an average of 430 variants (ranging from 368 to 494) that were not predicted to have any impact on the encoded protein by predictor tools (Table S3). Using this candidate gene list, we identified an average of 55% of the target variants predicted to have any impact on the encoded protein by predictor tools.

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resulting in a final list of 27 single base variants distributed in 24 genes (Table 3). Among them, we found 24 missense mutations causing amino-acid changes predicted to be potentially damaging with high confidence by at least one prediction tool; we also identified two nonsense mutations and one splice site mutation. The mutations have been all confirmed by direct Sanger sequencing (data not shown).

Biological Relevance of Known and Novel CHI Gene Variants
By WES in 10 CHI patients we were able to identify four patients carrying mutations in three CHI causative genes (ABCC8, GLUD1, and HNF1A), seven patients showed genetic lesions in 17 genes implicated in the regulation of insulin secretion (KCNH6, GNAS, ACA1B, NOTCH2, RYR3, TRP3, TRPC5, CAMK2D, PHGR3, CDEAL1, SCN8A, KCNJ10, PDE4C, NOS2, SLC24A6, CACNA1A, PC) and four patients carrying mutations in four genes suggested by the TDT analysis (SLC37A3, CSMD1, SULF1, TLL1). Overall, we identified: one patient displaying a unique variant in one gene, two patients are displaying mutations in two genes, and the remaining carrying multiple variants in more than two genes.

For known genes, we found a compound heterozygous patient carrying two mutations in ABCC8: a nonsense mutation p.Q953X (CM981878) previously described [19] and a novel c.3989-2A>G splicing variant (patient HI39) abolishing the canonical acceptor splice site. We also identified a homozygous ABCC8 variant (p.A390E) in exon 7 within a ROH on chromosome 11 (probandHI01).

Table 1. Clinical characteristics of the ten CHI probands.

| Proband | HI01P | HI06P | HI18P | HI26P | HI31P | HI37P | HI39P | HI42P | HI43P | HI44P |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Sex      | M     | M     | F     | M     | M     | M     | M     | M     | M     | M     |
| Macrosomia | No    | No    | No    | No    | Yes   | No    | Yes   | No    | No    | No    |
| Consanguinity | No    | No    | No    | No    | No    | No    | Yes   | No    | No    | No    |
| Onset    | 16 m  | 4 m   | 5 m   | 2 d   | 2 d   | 1 y   | 3 d   | 2 d   | 1 y   | 14 m  |
| Clinical form | mild  | severe| severe| mild  | mild  | severe| mild  | severe| mild  | severe|
| Pancreatectomy | No    | No    | Yes   | No    | No    | No    | No    | No    | No    | No    |
| Histology | No    | No    | Di-CHI| No    | No    | No    | No    | Di-CHI| No    | No    |
| TC PET   | No    | Fo-CHI| Di-CHI| No    | No    | No    | No    | Di-CHI| No    | No    |
| Response to Diazoxide | Yes*  | Yes*  | No    | Yes   | Yes*  | Yes   | Yes*  | No    | Yes   | Yes   |
| Other metabolic features | Leucine sensitive | No    | No    | Yes   | No    | No    | No    | No    | No    | No    |
| Hyperammonemia | No    | No    | No    | No    | No    | No    | Yes   | No    | No    | No    |
| Mental retardation | No    | No    | No    | No    | No    | No    | Yes   | No    | No    | No    |
| Epilepsy  | No    | No    | No    | No    | No    | No    | Yes   | No    | No    | No    |
| SNP array analysis | Yes   | Yes   | Yes   | Yes   | Yes   | Yes   | Yes   | No    | No    | No    |

*Patient in Remission.
1Medical Treatment with Diazoxide and Octreotide.
Abbreviations: m, month; d, day; y, year; Di, Diffuse; Fo Focal.
doi:10.1371/journal.pone.0068740.t001

Table 2. Exome coverage and target coverage statistics of ten CHI probands.

| CHI patients | HI01P | HI06P | HI18P | HI26P | HI31P | HI37P | HI39P | HI42P | HI43P | HI44P |
|--------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Whole exome sequencing results | | | | | | | | | | |
| Total number of raw reads (10^9) | 81.87 | 62.75 | 68.38 | 52.75 | 65.58 | 80.60 | 84.95 | 45.53 | 68.11 | 88.79 |
| Total number of mapped reads (10^9) | 67.28 | 49.14 | 50.77 | 41.70 | 52.64 | 66.24 | 70.46 | 36.74 | 54.61 | 71.53 |
| Target coverage (10^6) (%) | 88.67 | 87.38 | 88.00 | 86.46 | 88.40 | 88.51 | 88.43 | 88.16 | | |
| Target coverage (20x) (%) | 77.84 | 73.82 | 74.90 | 70.14 | 76.15 | 77.20 | 77.48 | 62.06 | 76.02 | 77.08 |
| Mean read depth (x) | 50.19 | 39.26 | 40.29 | 33.24 | 42.06 | 47.52 | 49.48 | 27.04 | 41.33 | 49.78 |
| Exonic SNV variants analysis | | | | | | | | | | |
| Total exonic SNP (N') | 19208 | 18751 | 19361 | 18959 | 19200 | 19697 | 19287 | 17808 | 20252 | 19293 |
| Total exonic Indels (N') | 281 | 258 | 262 | 240 | 265 | 247 | 257 | 223 | 266 | 282 |
| Missense and Nonsense SNPs (N') | 9436 | 9187 | 9461 | 9318 | 9456 | 9679 | 9433 | 8660 | 9890 | 9434 |
| Filtering (dbSNP) | 722 | 794 | 863 | 830 | 846 | 883 | 905 | 685 | 952 | 765 |
| Filtering (1000g MAF<0.005) | 618 | 705 | 761 | 721 | 737 | 778 | 800 | 608 | 825 | 652 |
| Filtering (internal DB) | 369 | 415 | 480 | 414 | 438 | 423 | 457 | 405 | 495 | 414 |

doi:10.1371/journal.pone.0068740.t002
The closure of the KATP channels stimulates insulin secretion, which is regulated by the calcium channel. The protein is participating in the complex of the alpha 1A subunit of the voltage-dependent (VDCC), P/Q type calcium channel. Recently, a NOTCH2 variant has been reported in one MODY affected patient [16], therefore similarly to other MODY causative genes [29–31], that are resulted also CHI causative, we propose NOTCH2 as a good candidate for further investigations. The RYR3 gene encodes a member of the 6-TM family of potassium channels; interestingly; it has been suggested to have a role in diabetes [28].

H142) which has been previously reported in a case report [20]. The GLUD1 mutation (pS498L) was found at the heterozygous state in patient (H143) who was affected by a mild CHI-HA (Hyperinsulinism-Hyperammonemia) form and epilepsy. This mutation is associated to CHI-HA in the Human Gene Mutation Database (CM980942). Finally, we identified a HNF1A gene mutation (proband H101) that has been previously reported in MODY3 patients [21]. Interestingly, the phenotype of our patient was similar to the one described by Stanescu, who recently reported mutations in the HNF1A gene in two cases of CHI [22].

For novel genes, by literature mining and database queries we have obtained indications about their biological relevance for pancreatic β-cell (Figure S3) and by gene ontology (GO) classification we group them in three main categories of cellular components such as transmembrane, cytosolic and mitochondrial proteins (Table 4).

**Transmembrane proteins.** The CACNA1A gene encodes the alpha 1A subunit of the voltage-dependent (VDCC), P/Q type calcium channel. The protein is participating in the complex regulation of insulin secretion; the mechanism implicates that the closure of the KATP channels stimulates insulin secretion depolarizing the plasma membrane; the consequent opening of VDCC results in influx of extracellular Ca2+ which then triggers insulin exocytosis in pancreatic β-cells [23,24]. CSMD1 gene (belonging to the TDT analysis list) encodes an integral membrane protein with unknown molecular function. The KCN10 gene encodes a type 1 transmembrane receptor that is expressed in the ductal cells during pancreatic organogenesis [27], and is belonging to the Notch genes [29–31], that are resulted also CHI causative, we propose NOTCH2 as a good candidate for further investigations. The RYR3 gene encodes a voltage-gated K+ channel (KV) belonging to the 6-TM family of potassium channels; interestingly; it has been described that HERG channels have a crucial role in regulating insulin secretion, raising the possibility that HERG mutations might be involved in some hyperinsulinemic diseases of unknown origin [25]. The KCNJ10 gene encodes a member of the inward-rectifier potassium channel family (also known as 2-TM family that has been suggested to have a role in diabetes [28].

**Table 3.** Whole-exome sequencing identification of non-synonymous SNP variants of 10 CHI patients.

| Sample ID | Gene symbol | Chr | Position | RefSeq ID | Amino Acid change | PolyPhen/SIFT prediction |
|-----------|-------------|-----|----------|-----------|-------------------|--------------------------|
| H101      | HNF1A       | chr12| 121426790| NM_000545 | p.A161T*           | ++/+                     |
|           | KCNH6       | chr17| 61615518  | NM_030779 | p.V532F           | −/−                     |
| H106      | GNAT        | chr20| 57428464  | NM_080425 | p.E48D            | ++/−                    |
|           | ACAB        | chr12| 109683331 | NM_001093 | p.N1760S          | −/+                     |
| H118      | NOTCH2      | chr1  | 120464961 | NM_024408 | p.R1704H          | ++/−                    |
|           | SLC37A3     | chr7 | 140035229 | NM_032295 | p.S389L           | −/+                     |
|           | CSMD1       | chr8 | 3889535   | NM_033225 | p.R168W           | ++/−                    |
|           | RYR3        | chr15| 33938601  | NM_001036 | p.T1272M          | ++/−                    |
| H126      | TRPV3       | chr7 | 3417258   | NM_145068 | p.L776F           | ++/−                    |
|           | TRPC5       | chrX | 20882080  | NM_012471 | p.G789R           | −/+                     |
| H131      | CAMK2D      | chr4 | 114435066 | NM_172115 | p.R275C           | ++/−                    |
|           | CDKAL1      | chr6 | 21231212  | NM_017774 | p.S561F           | −/+                     |
|           | SCN8A       | chr12| 21263721  | NM_014191 | p.V1148M          | −/+                     |
| H132      | KCNJ10      | chr1  | 160011280 | NM_002241 | p.R348H           | −/+                     |
|           | PIK3R3      | chr1  | 46521492  | NM_003629 | p.R306X           | Nonsense                |
| H139      | ABCC8       | chr11| 17428964  | NM_000352 | p.Q953X*          | Nonsense                |
|           | GLUD1       | chr10| 88817449  | NM_005271 | p.S498L*          | −/+                     |
|           | SULF1       | chr8 | 70501306  | NM_015170 | p.A222T           | −/+                     |
| H140      | TLL1        | chr4  | 166981332 | NM_012464 | p.G667S           | −/+                     |
|           | PC          | chr11| 66619274  | NM_001040716 | p.N657Y        | ++/−                    |
|           | TLL1        | chr4  | 166986893 | NM_012464 | p.H689P           | ++/−                    |

*Mutation reported in Human Genome Mutation Database (HGMD).

PolyPhen/SIFT: (−) benign or tolerated; (+) possibly damaging/DAMAGING Low confidence;

(++) probably damaging/DAMAGING.

In bold known causative genes.

![Table 3. Whole-exome sequencing identification of non-synonymous SNP variants of 10 CHI patients.](http://www.plosone.org)

PLOS ONE | www.plosone.org | 4 | July 2013 | Volume 8 | Issue 7 | e68740
Table 4. Functional annotation of novel variants.

| GO cellular component         | Relevant pathways (KEGG)                      | Biological process in pancreas [ref] |
|-------------------------------|----------------------------------------------|--------------------------------------|
| Transmembrane proteins        |                                              |                                      |
| CACNA1A                       | Calcium signaling pathway, Type II diabetes   | insulin exocytosis [23,24]           |
| C5MD1                         | undetermined                                | unknown                              |
| KCNH6                         | undetermined                                | regulating insulin secretion [25]    |
| KCNQ10                        | Gastric acid secretion                       | unknown                              |
| NOTCH2                        | Notch signaling pathway                      | role in diabetes, MODY [16,28]       |
| RYR3                          | Calcium signaling pathway,                   | insulin secretion [32]               |
| SCN8A                         | undetermined                                | ion permeability, insulin secretion  [8,34] |
| SLC37A3                       | undetermined                                | unknown                              |
| TRPC5,TRPV3                   | undetermined                                | ion permeability [9]                 |
| Cytosolic proteins            |                                              |                                      |
| ACACB                         | Fatty acid biosynthesis, Metabolic pathways,  | insulin secretion [35,36]            |
| CAMK2D                        | Calcium signaling pathway,Wnt signaling       | insulin gene expression regulation   [37] |
| CDKAL1                        | undetermined                                | insulin gene expression, insulin exocytosis [39] |
| GNAS                          | Calcium signaling pathway                    | β-cell proliferation, diabetes       [41] |
| NOS2                          | Metabolic pathways, Calcium signaling pathway | β-cell proliferation, diabetes [43]  |
| PDE4C                         | Purine metabolism                            | insulin secretion [45]               |
| PIK3R3                        | Insulin signaling pathway, Type II diabetes   | insulin signaling [47]               |
| SULF1                         | undetermined                                | unknown                              |
| TLL1                          | undetermined                                | glucose regulation [49]              |
| Mitochondrial proteins        |                                              |                                      |
| PC                            | Citrate cycle (TCA cycle), Pyruvate metabolism, | insulin secretion [10]               |
| SL2C24A6                      | undetermined                                | calcium homeostasis [51]             |

doi:10.1371/journal.pone.0068740.t004

gene encodes for receptors that are intracellular ion channels expressed in human pancreatic β-cell. Interestingly, the activation of these receptors by ryanodine stimulates the insulin secretion at low glucose concentrations [32]. The SLC37A3 gene (belonging to the TDT analysis list) encodes a transmembrane sugar transporters family, SLC37; to date, only the SLC37A4 gene has been found mutated in the glycerogen storage disease non-1A type [33]. The SCN8A gene encodes a member of the sodium channel alpha subunit gene family (Na, 1.6), and it forms the ion pore region of the voltage-gated sodium channel and mediates the voltage-dependent sodium ion permeability of excitable membranes. Na, 1.6 together with Na, 1.7 (the other α subunit of human β-cells) is highly expressed in human islets [8,34]. The TRPV3 and TRPC5 genes encode proteins belonging to the transient receptor potential (TRP) cation channels group, they provide the background membrane conductance required for β-cells to depolarize upon KATP channel closure [9], but their role in human pancreas islets has not been studied.

**Cytosolic proteins.** The ACACB gene encodes a complex multifunctional enzyme system that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA (the rate-limiting step in fatty acid synthesis); the malonyl-CoA can act as a coupling factor that regulates insulin secretion [35]. Interestingly, β-cell islets produce high quantity of ACACB enzyme [36]. The CAMK2D encodes a protein Ca²⁺ sensing serine/threonine kinases (CaMK2) that it is involved in the regulation of Ca²⁺ mediated glucose-sensitivity of β-cells; in particular CaMK2 delta play a role as a feedback sensor, linking Ca²⁺ induced exocytosis to the re-synthesis of insulin acting on transcriptional regulation of genes related to the metabolic control of insulin secretion [37]. In humans, the CaMK2 beta and delta subtypes represent the major isoforms that are highly expressed in β-cells [38]. The CDE1LI gene, encodes a methylthiontransferase that modifies tRNA to enhance the translational fidelity of proinsulin transcript [39]. Moreover, a study using knockout mice for the CDE1LI gene revealed its possible role in controlling the first phase of insulin exocytosis in β-cells, through the KATP channel responsiveness [40]. The GNAS gene encodes the Gαζ subunit of the G protein that is ubiquitously expressed. The encoded protein stimulates adenylyl cyclase (AC), thereby generating the second messenger cAMP. It has been shown that mice with β-cell-specific Gαζ deficiency develop severe defect in β-cell proliferation and early onset insulin deficient diabetes [41]. The GNAS locus has a highly complex imprinted expression pattern and encodes four main transcripts: Gα-alpha, XLz5, NESP55, and the A/B transcript. Specifically, we found that our mutation is referred to the XLz5 transcript, whose promoter is methylated on the maternal allele and transcriptionally active only on the paternal allele [42]. Therefore, for our proband, we hypothesize that a post-zygotic somatic event (such as methylation) and imprinting defect, resulting in the absence of
expression of the maternal Gs-alpha isoform, might lead to the expression of mutated paternal allele in some affected focal area of the pancreas. The NOS2 gene, has been implicated in β-cell damage and death in both type 1 and type 2 diabetes [43]; moreover, it mediates the IRS-2 expression contributing to β-cell failure [44]. The PDE4C gene encodes a protein belonging to the cyclic nucleotide phosphodiesterase subfamily PDE4. This protein hydrolyzes the second messenger, cAMP, thus playing a key role in many important physiological processes such as those mediated by hormones and neurotransmitters. It has been demonstrated that PDE1–4 enzymes are expressed in rodent pancreatic islets and β-cells [45] while PDE4C is the major isoform in human islets [46]. Furthermore, it has been shown that family-selective inhibition of PDE (PDE1, PDE3 and to some extent also PDE4) potentiates glucose-stimulated insulin secretion (GSIS); in particular, siRNA-mediated knockdown of PDE4C significantly enhanced GSIS in rat INS-1 (832/13) cells [45]. The PIK3R1 encodes a family of lipid kinases that binds to activated (phosphorylated) protein-tyro sine kinases through the SH2 domain, regulates their kinase activity and triggers a plethora of intracellular events involving also the insulin signaling [47]. Notably, we found a mutation in the exon 7 that causes the complete loss of the second SH2 domain that may be deleterious to the protein function (Table 3). The SULF1 gene (belonging to the TDT analysis list) encodes an enzyme with arylsulphatase activity acting on cell-surface heparan sulphate proteoglycans. It has been suggested an important role of this enzyme in pancreatic cancer progression [48]. The TLL1 gene (belonging to the TDT analysis list) encodes an astatin-like zinc-dependent metalloprotease and is a subfamily member of the metzincin family; interestingly, it has been described as a gene responsive to a member of the NR4A subgroup of nuclear receptors that have been implicated in the regulation of glucose and lipid metabolism in insulin-sensitive tissues [49].

Mitochondrial proteins. The PC gene encodes the enzyme pyruvate carboxylase, which is a mitochondrial enzyme involved in gluconeogenesis, lipogenesis, insulin secretion, and synthesis of the neurotransmitter glutamate. PC seems to play an important role in the “amplifying signal” responsible for insulin secretion through the K_{ATP} independent pathway [10]. Interestingly, Pineda et al. [50] reported an infant with what they termed the “French” type of pyruvate carboxylase deficiency, presenting initial neonatal symptoms such as respiratory distress, severe metabolic acidosis, and a tendency to hypoglycaemia. The SLC2A4G6 gene encodes a mitochondrial Na+/Ca2+ exchanger (NCKX6) that maintain cellular calcium homeostasis and it plays a critical role in the mitochondrial Ca2+ transport in skeletal muscle, stomach, and pancreas [51].

Conclusions

In the present investigation, we demonstrated that exome sequencing is useful as a diagnostic tool for CHI because identifies mutations in known genes but also gives the opportunity to discover potential damaging mutations affecting several genes combined in a complex fashion. WES, associated with family-based association analysis, revealed that CHI patients, lacking mutation in known causative genes, carry multiple exonic mutations. Due to the small number of patients of this study, some cautions should be taken into account to interpret the pathological role of identified variants. However, due to difficulties to collect large samples for such a rare disease, our results may be useful for future meta-analyses that will combine genetic data provided by other individual studies. The present study should be regarded as a starting point to design further investigations aimed from one side to the molecular characterization of the identified mutations, and from the other to investigate possible modifier genes, which might be relevant for the CHI pathogenesis. Increasing the number of well-characterized clinical CHI patients will allow the evaluation of the mutation frequency of novel gene variants and the identification of a clearer genotype-phenotype correlation.

Finally, we indicate that exome sequencing should be considered a valuable, time- and cost-effective diagnostic tool providing a fine molecular classification of CHI patient in alternative to Sanger sequencing, offering also the opportunity to better understand the genetic basis of CHI.

Materials and Methods

Study Sample and Strategy

The study was approved by the Ethical Committee of the San Raffaele Scientific Institute and performed according to the principles of the Declaration of Helsinki. Written informed consent was obtained from the next of kin on the behalf of the minors/children participants involved in this study. A detailed description of the study design is available through the Italian Registry of Congenital Hyperinsulinism (http://www.progettotorici.it).

The diagnosis of CHI was carried out according to the following criteria: a fasting and post-prandial hypoglycemia with unsuppressed insulin secretion, a positive response to the administration of glucagon, negative ketone bodies in urine and plasma, and a prolonged dependence to treatment to prevent hypoglycemia. From the study we excluded infants from diabetic mothers, children with transient neonatal hyperinsulinism, with insulinoma, or Beckwith-Wiedemann Syndrome. Patients were considered to be diazoxide responsive if satisfactory glycemic control was achieved with doses of oral diazoxide not exceeding 20 mg/kg per day and were considered to be in remission if good glycemic control was maintain without further medical or surgical therapy. Patients were considered to be unresponsive to medical treatment if recurrent hypoglycemia episodes (<3 mmol/L) occurred during diet treatment (milk enriched with maltodextrine or continuous enteral feeding) and treatment with diazoxide and octreotide at maximum dosage.

The study design is shown in Figure S1. We enrolled a total of 38 probands and their families; 33 probands were subjected to mutational screening of two “major” CHI genes (ABCC8, KCNJ11) by Sanger sequencing, following the procedure previously described [52]. After the mutational screening, 17 non consanguineous probands remained without a genetic diagnosis: together with their parents, they were hence processed for SNP genotyping and family-based TDT association analysis. Finally, we used WES analysis in 10 probands (for five of them, no mutational pre-screening was performed), either to find novel causative gene mutations or to evaluate WES as an alternative approach to classical Sanger sequencing. The clinical features of these patients are reported in Table 1.

Whole-genome SNP Genotyping and Linkage Analysis

The whole-genome scan was performed by the Affymetrix GeneChip Human Mapping 250 K Nsp Array Set (Affymetrix, Santa Clara, CA, USA), using standard protocols as described in the GeneChip® Mapping 500 K Assay Manual. Genotypes were called from cell intensity data by the BRLMM (Bayesian Robust Linear Model with Mahalanobis distance) algorithm implemented in the GeneChip Analysis Software v.4.0 (Affymetrix) using default parameter settings. SNP array raw data are available on Array Express repository (E-TABM-861). Copy number analysis and
homozygosity mapping was carried out using CNAG 3.3, as previously described [53]. Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium (HWE) considering only parents. For SNP quality control (QC), we employed predetermined QC inclusion criteria [minor allele frequency (MAF)>1%, SNP call rate >90%, HWE P=0.01]. The QC procedure also included a check for Mendelian errors (predetermined threshold: 1%), as well as sex and pedigree check. All procedures were performed by using the PLINK software [54], obtaining a set of 221,605 high-performing SNP markers for further analysis. A subset of SNPs that are in approximate linkage equilibrium with each other were hence selected by linkage disequilibrium (LD)-based pruning: 112,740 SNPs remained for family-based TDT association analysis.

We proceeded further performing the TDT association analysis on the 17 trios to search for association signals highlighting novel CHI candidate/modifier genes. Adjusted P-values were assessed by gene-dropping permutation, flipping the allele transmitted from parent to affected offspring, and using the default mode with the adaptive permutation approach. The maximum number of permutations per SNPs was 1,000,000. Considering the number of tests conducted, any empirical P-value at 4.4 would have been considered significant [53]; however, none of the SNPs reached the genome-wide significance. Hence, considering that: 1) the study cohort is obviously underpowered by its size; 2) it is possible to make use of these suggestive P-values through the incorporation of prior biological knowledge; we decided to adopt P = 4.9 as significance threshold for building a SNP list to be used in all the subsequent analyses. All P-values are hence presented as not corrected.

Finally, we refined the association analysis on the TLL1 gene performing a haplotype analysis. It was performed using all SNPs mapping in the TLL1 genomic region and the sliding window specification option of the PLINK program (with the window: 5 SNPs; shifting: 1 SNP at a time).

WES Analysis

Three micrograms of genomic DNA was fragmented using the Covaris shearing system (Covaris inc., MA, USA). Exome capture was performed with the SureSelect Human All Exon Kit (v3, target size 50 Mb) according to the manufacturer’s instructions (Agilent technologies, Santa Clara, CA, USA). The platform design covers 1.22% of human genomic regions corresponding to the NCBI Consensus CDS Database (CCDS), including more than 700 human miRNAs from the Sanger v13 database and more than 300 additional human non-coding RNAs in a single tube. The selected coding regions were then massively sequenced by the Illumina GAIIx technology (Illumina, San Diego, CA, USA); following proprietary reversible terminator-based method and producing 2×76 bp read lengths. The paired-end sequence reads were mapped to the reference genome (UCSC NCBI37/hg19) using the Burrows-Wheeler Aligner (BWA) software [56] and the variant calling was performed using SAMTools [57]. The entire dataset of mapped reads (.bam files) is available on Sequence Read Archive, accession number ERP002635. The detected variations in each sample were filtered out using the minimum Phred SNP quality score of 100, the minimum depth of 10 reads, and the minor allele with an allelic fraction >0.2. The variants were then annotated by the ANNOVAR software [http://www.openbioinformatics.org/annovar/annovar_db.html], for filtering common variants reported in dbSNP132 [http://www.ncbi.nlm.nih.gov], in 1000 Genomes Project [http://www.1000genomes.org/], and in-house database.

Selection of Gene of Interest and Validation

For a focused evaluation of the whole-exome data, a list of 272 genes arising from three functional criteria of inclusion was used: ten genes known to be causative for CHI (ABCC8, KCNJ11, GLUD1, GCK, HADH, HFN1A, SLCE1A1, UCP2, INSR and HNF14); 51 genes suggested by TDT association analysis (Table S1); 145 genes associated to regulation of insulin secretion reported in the Rat Genome Database [58] and 66 genes indicated by reviewing the literature concerning the β-cell function and its metabolic regulation (Table S3).

To predict the effect of non-synonymous mutations on the encoded proteins of the selected gene list, we used two different web tools: SIFT [59] and PolyPhen v2.0 [60]. We reported non-synonymous mutations that were predicted deleterious at least by one predictor tool with a high score. To investigate the presence of functional interactions among genes, including direct (physical) and indirect (functional) associations, we used STRING, a database of known and predicted protein interactions [61].

Computer-assisted analysis for splice-site prediction was accomplished using the Neural Network Promoter Prediction Tool (NNPPT) program [http://www.fruitfly.org/seq_tools/splice.html] and the NetGene2 (release 2.4) program [http://www.cbs. dtu.dk/services/NetGene2].

All variants were validated by PCR amplification of exons carrying the identified mutation (primers sequences and PCR conditions are available on request) using genomic DNA from CHI probands. The amplified products were purified using montague the Micro PCR96 plates (Merk Millipore, Billerica, MA, USA) and then directly sequenced with the forward and reverse primers previously used for the amplification. Sequenced products were purified using the Montage SEQ 96 Sequencing Reaction Cleanup kit (Merk Millipore). Sequencing analysis was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit v3.1 and an automated DNA sequencer (ABI-3100 XL Genetic Analyser; Applied Biosystems, Foster City, CA, USA). Factura and Sequence Navigator software Packages (Applied Biosystems) were used for mutation detection.

Supporting Information

Figure S1 Study design. The initial study sample consisted of a total of 32 subjects, belonging to 32 CHI families. Overall 33 probands of these families were pre-screened for mutations in the two main CHI genes (ABCC8, KCNJ11). The 17 probands non consanguineous (lacking causal mutations in ABCC8/KCNJ11) together with their relatives were taken forward to whole-genome scan by the Affymetrix GeneChip Human Mapping 250 K Array. We applied TDT and haplotype analyses to highlight possible susceptibility/modifier CHI genes. Finally, 5 patients from the TDT study and 5 newly-enrolled CHI patients (not pre-screened for ABCC8/ KCNJ11) were analyzed by whole-exome sequencing (WES).

Figure S2 Manhattan plot. Results of single-locus test of association between each of the 112,740 performing SNPs and CHI using the TDT association analysis. 112,740 single-marker permuted results (-log10 p-values) from the TDT test are plotted on each chromosome; red line represents P≤10⁻³ threshold, blue line represents P≤5×10⁻³ threshold.

Figure S3 Schematic representation of insulin secretion in β-cell and the hypothetical role of novel CHI gene. The entry of glucose in the β-cell trough the glucose transporter
GLUT2) stimulates insulin secretion by metabolic activating pathways producing ATP. The increase of ATP/ADP ratio leads to the closure of K_ATP channels, to the depolarization of the plasma membrane and to the subsequent activation of VDCC promoting influx of calcium into the cell. The overall modulation of the cytosolic free concentration [Ca^{2+}] is essential for the triggering pathways of the insulin secretion. The binding of secreted insulin to its receptors (INSR), might activates the PI3K/Akt pathway and some transcription factors controlling insulin gene expression. Insulin exocytosis can also be influenced by neurotransmitters and hormones. Indeed, GLP1 activates AC to the closure of KATP channels, to the depolarization of the membrane, to the activation of CaM and finally the Ca^{2+} mobilizes intracellular Ca^{2+} to activate CaMK and inducing the secretory process of insulin. Moreover, CDKAL1 is implicated in the control of the first phase of insulin exocytosis via K_ATP responsiveness. Other transmembrane ion channels might modulate electrical activity of the cellular membrane regulating the insulin secretion (KCN, TRP, SCN). Abbreviations: VDCC, voltage dependent calcium channel; TRP, transient receptor potential channels; KCN, potassium voltage-gated channel; SCN, sodium channel; SERCA, sarco/endoplasmic reticulum Ca^{2+}ATPase; GIP, glucose-dependent insulominotropic peptide; AC, adenyl cyclase; PLC, phospholipase C; IP3, Inositol trisphosphate; PKC, protein kinase C; DAG, diacylglycerol; Gs, Gi, Gq, G proteins; PKA, Protein kinase A; PI3K, phosphatidylinositol 3-kinase; CaM, calmodulin; Ach, acetylcholine; FA, fatty acid; FFA, free fatty acid. Yellow box indicate the CHI causative genes.

**(TIF)**

**Table S1** TDT data and associated alleles and genes. 144 SNP resulted from TDT analysis at P<0.005. OR, TDT odd ratio; CI L95 and CI U95, lower and upper 95% confidence interval for TDT odds ratio; Adjusted p-value, empirical p-value by adaptive procedure; A1/A2, A1: minor allele, A2: major allele; NP: number of permutations.

**Table S2** Haplotype analysis of TLL1 locus.

**Table S3** Refined gene list. The table is including 221 gene symbols and criteria of inclusion. PMID, pubmed identification number; RGD, rat genome database (http://rgd.mcw.edu/).

**Acknowledgments**

We thank all patients with CHI disease and their families for participating in this study. We also thank Maria Vurchio (CNR-ITB) for technical and administrative support.

**Author Contributions**

Conceived and designed the experiments: PSV SMC CB. Performed the experiments: AG MCP RB IZ CD. Analyzed the data: EM RS AG MCP RA GDB. Contributed reagents/materials/analysis tools: AS MCN SMC CB. Wrote the paper: CB MCP GDB. Clinical and ethical issues of the study: AS MCN PSV SDC SM.

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