Original Article Genomic and morphoproteomic correlates implicate the IGF-1/mTOR/Akt pathway in the pathogenesis of diffuse congenital hyperinsulinism

Senthil Senniappan^{1*}, Robert E Brown^{2*}, Khalid Hussain³

¹Department of Paediatric Endocrinology, Alder Hey Children's Hospital, Liverpool, L12 2AP, UK; ²Department of Pathology and Laboratory Medicine, UT Health-Medical School at Houston, Houston, Texas, USA; ³Genetics and Epigenetics in Health and Disease, Genetics and Genomic Medicine Programme, UCL Institute of Child Health, Great Ormond Street Hospital for Children, London, WC1N 3JH, UK. ^{*}Equal contributors.

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Abstract: Purpose: Patients with diffuse congenital hyperinsulinism (CHI), unresponsive to medical therapy require near total pancreatectomy. Morphoproteomic analysis of the pancreases in diffuse CHI has revealed constitutive activation of the mammalian target of rapamycin (mTOR) pathway. Treatment of infants with diffuse CHI with sirolimus (mTOR inhibitor) has recently been shown to be effective in avoiding pancreatectomy. The aim of this study was to gain insight into the biology of insulin production in diffuse CHI patients using genomic and morphoproteomic techniques. Methods: Gene expression microarray and immunohistochemical probes focusing on the insulin-like growth factor (IGF)/mTOR/Akt pathway were applied to pancreases of diffuse CHI and compared with non-CHI controls. Results: Genomic overexpression of MTOR, IGF1, IGF2 and IGF2BP3 and downregulation of TSC2 was noted in diffuse CHI. Correspondingly, morphoproteomics revealed: (a) strong plasmalemmal expression of phosphorylated (p)-mTOR (Ser 2448) in the exocrine pancreas; (b) p-Akt (Ser473) in the nuclear compartment and cytoplasm of the insulin-producing islet cells with variable expression of p-mTOR (Ser 2448); and (c) insulin and IGF-1R expression in the expanded islet cells in the context of minimal progression into the mitotic phase. Conclusion: Genomic and morphoproteomic findings indicate that the IGF-1R/mTORC2/Akt pathway is overexpressed in the expanded beta cell population in the absence of significant progression into the mitotic phase and a mass effect. These suggest that the observed efficacy of sirolimus in diffuse CHI could be the result of mTORC1 inhibition thereby reducing exocrine transdifferentiation to beta cells, and contributing to the disassembly of the mTORC2/Akt pathway, resulting in reduced insulin secretion and a propensity for increased apoptosis of insulin-producing beta cells.

Keywords: Congenital hyperinsulinism, hypoglycaemia, pancreatic beta cell, mTOR pathway, growth factors, gene expression

Introduction

Congenital hyperinsulinism (CHI) is a major cause of severe and persistent hypoglycaemia in the neonatal and infancy periods. CHI is characterised by inappropriate and unregulated insulin secretion from an expanded population of pancreatic beta cells in the presence of a low blood glucose concentration [1]. A rapid diagnosis and appropriate management of patients with CHI becomes essential to avoid the potentially devastating complications like epilepsy, cerebral palsy and developmental delay [2]. CHI is a heterogeneous condition in terms of clinical presentation, histology and molecular genetics [3]. CHI is caused by mutations in the key genes that are involved in the regulation of insulin secretion from the pancreatic beta cells. So far, mutations in *ABCC8, KCNJ11, GLUD1, GCK, HADH, SLC16A1, HNF4A, UCP2* and *HN-F1A* have been identified to be involved in the pathogenesis of CHI [4].

There are two histological subtypes of CHI: diffuse and focal [5]. The focal CHI is characterized by the presence of a small endocrine lesion in the pancreas, histologically corresponding to the adenomatous hyperplasia of apparently normally structured islets [6]. Diffuse CHI is histologically characterized by the presence of hyper-functioning pancreatic β-cells throughout the pancreas, some with abnormally large nuclei and replacing the exocrine pancreas without an obvious mass effect [6]. Diffuse CHI that is unresponsive to medical therapy requires a near total pancreatectomy, whereas the focal forms are cured by focal lesionectomy [3]. However, even with surgical removal of 95 to 98% of pancreas, some patients with diffuse CHI continue to have hypoglycaemic episodes and invariably all patients develop diabetes mellitus in the long-term [7]. Hence, there is a need for alternative therapies for the management of diffuse CHI.

Apart from the functional defect in K_{ATP} channels, pancreatic beta cell proliferation has been suggested to play a role in the pathogenesis of diffuse CHI. There are two major schools of thought regarding the histogenesis of such proliferation. One proposal is based on the increased Ki-67 labelling index and holds that the proliferation in diffuse CHI is associated with hyperplasia of pancreatic beta cells [8, 9]. A second proposal offers transdifferentiation of exocrine elements into insulin-producing islet cells as a histogenetic mechanism to account for the proliferation in diffuse CHI [10]. Evidence in favour of transdifferentiation in diffuse CHI includes: 1) intermediate forms in terms of acinar-islet and ductal-islet cells in diffuse CHI [10, 11]; 2) the over expression of the cyclindependent kinase inhibitor, p27 in diffuse CHI versus control pancreases in the absence of the S-phase kinase-associated protein (Skp)2 both consistent with a relative block at the G1 to S phase of the cell cycle [10, 12]; and 3) a relative paucity to absence of mitotic figures in the insulin-expressing beta cells and the absence of a "mass effect" in the diffuse form of CHI [10, 12]. Moreover, a relative block in cell cycle progression at the G1 to S phase could also explain the increase in the Ki-67 labelling index in diffuse CHI versus controls reported by some but not all investigators [8, 9, 13], given the fact that the Ki-67 detects cells in the G1 phase of the cell cycle, including those that have been blocked in that phase by cyclindependent kinase inhibitors [14].

The study by Alexandrescu S et al. supported the notion of transdifferentiation of acinar and ductal cells being a component of the beta cell proliferation [10]. Furthermore, in their study, comparing pancreatic tissues from two diffuse CHI patients with two controls, it was shown that mTOR (mammalian target of rapamycin) was overexpressed on the plasmalemmal aspects of acinar and ductal cells, thereby suggesting the possibility of constitutive activation of the mTOR pathway in diffuse CHI [10]. In a recent study, the use of mTOR inhibitor (sirolimus) has been shown to be effective in the management of severe diffuse CHI, thereby avoiding the need for near total pancreatectomy [15].

Gene expression microarray (GEM) technology is a high-throughput and versatile technology used for parallel gene expression analysis for thousands of genes of known and unknown function [16]. The application of GEM in cancer research has provided insights into the mechanisms and targets involved in the oncogenesis which has been used in the prediction of diseases, drug responses and tumour classification [17, 18]. Although several pancreatic GEM studies have been undertaken so far, majority evaluated gene expression in the cancer pancreatic tissues [19]. In the only gene expression study involving pancreatic tissues from patients with CHI, the authors noted up regulation of genes involved in the regulation of insulin secretion and synthesis (PFKL, SUCLG2, and PFKP), pathogenesis of cancer (PLCXD1, ERB-B3, ARPC5L, CBLL1, and PLXDC2), amino acid metabolism (GOT1), and calcium ion homeostasis (CALB2 and NUCB2) [19]. The study is limited by its small sample size and poor quality of RNA extracted from FFPE (Formalin Fixed Paraffin Embedded) tissues.

In this study, we aimed to combine the genomic and morphoproteomic techniques to further elucidate the role of various pathways in the pathogenesis of diffuse CHI. We have attempted to integrate the influence of mTOR pathway into the analysis of the pancreatic beta cells using a systems biology approach.

Materials and methods

The diffuse CHI patients who underwent near total pancreatectomy as a result of unresponsiveness to medical therapy were recruited into the study in a prospective manner. CHI was confirmed based on standard clinical (glucose requirement > 8 mg/kg/min) and biochemical (detectable insulin/c-peptide during hypo-

glycaemia [blood glucose < 3 mmol/L] with suppressed ketones and fatty acids) criteria. Diffuse CHI was suspected following genetic evaluation and or 18F-DOPA-PET CT scan and later confirmed by histology.

Genetics&18F-DOPA-PET CT scan

All CHI patients recruited into the study underwent genetic testing for known mutations. Genomic DNA was extracted using standard methods. The coding regions and conserved splice sites of the *ABCC8 and KCNJ11* genes were amplified by PCR (polymerase chain reaction) and the products were sequenced on an ABI 3730 capillary sequencer (Applied Biosystems, Warrington, UK). Fluorine-18-L dihydroxyphenylalanine positron emission tomography (18F-DOPA PET/CT) was undertaken to differentiate the focal from the diffuse CHI. A diffuse lesion was confirmed if SUV (standardized uptake value) was less than 1.3.

Ethics

The ethical approval was obtained for the project from NRES (National Research Ethics) Committee London-Hampstead (REC Reference Number 11/L0/2015). The institutional approval was obtained from Joint Research and Development Office, Great Ormond Street Hospital for Children NHS Foundation Trust (Reference 11CM44). Parent information sheets were provided and detailed discussion held with the families prior to obtaining written consent.

Tissue samples

Fresh frozen (FF) pancreatic tissue samples from the diffuse CHI patients undergoing surgery were obtained for RNA extraction and a corresponding frozen section is performed to confirm the specific histology. The samples were collected and stored directly in 5-10 volumes of RNA later solution (Ambion, Product number AM2020, Lot number 1111008, Applied biosystems, CA, USA) to avoid potential degradation of the RNA due to the high amount of ribonucleases in the pancreas. Two normal total RNA samples obtained from human tissue biobank (Cambridge Bioscience Limited) were used as controls for the study. The normal (control) RNA samples were extracted from fresh frozen pancreatic tissue samples (confirmed as normal by histology) which were obtained as part of treatment from adults aged 29 years and 33 years respectively.

RNA extraction and quality analysis

FF pancreatic tissue is homogenized and total RNA extracted using TRIzol reagent (Ambion, Invitrogen, CA, USA: catalogue number 1559-6026 and lot number 11602902) as per standard protocol. The Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to quantify as well as to determine the purity of RNA by estimating the optical density ratio (OD 260/280 ratio). Agilent 2100 Bioanalyser was used to stringently assess the quality (integrity) of RNA prior to microarray by evaluating RIN factor (RNA Integrity factor) and Intact 18 S and 28 S bands on the bioanalyser trace. The RNA Integrity Number (RIN) software algorithm allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact [20]. A combination of the following quality assessment criteria was used for the inclusion of RNA samples in the microarray experiment: (a) An optical density ratio (OD 260/280) between 1.8 and 2.1 (b) RNA Integrity factor (RIN) of 7 and higher (c) Intact 18S and 28S ribosomal bands.

Gene expression microarray (GEM)

GEM was performed using Affymetrix Human GeneChip 1.0 ST Array (Santa Clara, CA, USA) using standard protocol. The Affymetrix Human GeneChip 1.0 ST Array interrogates 28,869 well-annotated genes with 764,885 distinct probes [21]. It has been designed using a subset of the probes from the Human Exon 1.0 ST array (Affymetrix) and includes well-annotated exons based on RefSeq (www.ncbi.nlm.nih.gov/ refseq), Ensembl (www.ensembl.org), and putative complete coding sequences from GenBank (www.ncbi.nlm.nih.gov/genbank) [21].

Microarray data analysis

Prior to analysing the microarray data for differential expression, various quality assessment techniques including background correction, normalization, summarization, and batch correction was undertaken. The normalization procedure is aimed at making the distributions identical across arrays by eliminating the effects of obscuring variations that are intro-

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Patient Number	Gender M=Male F=Female	Birth Weight (Kg)	Gestation (weeks)	Postnatal age at presentation	18F DOPA PET scan	Age at surgery (months)	Type of surgery	Genetics
1	F	4.2	40	Day 1	Diffuse	1	Near total pan- createctomy	Homozygous ABCC8
2	М	5.05	40	Day 1	-	3	Near total pan- createctomy	Homozygous ABCC8
3	М	1.90	34	Day 22	Diffuse	8	Near total pan- createctomy	Homozygous ABCC8
4	F	5.36	38	Day 1	-	2	Near total pan- createctomy	Compound heterozy- gous ABCC8 mutation
5	F	3.76	37	Day 14	Diffuse	7	Near total pan- createctomy	None
6	F	5.39	39	Day 1	Diffuse	16	Near total pan- createctomy	None

 Table 1. Clinical features of the patients included in gene expression microarray study

duced during the sample preparation, manufacture of the arrays, and the processing of the arrays including labelling, hybridization, and scanning [22]. For batch correction, the ComBat (an Empirical Bayes method) approach [23] was used.

The data analysis was carried out using R and Bioconductor packages. The differentially expressed genes are identified by first fitting a linear model to the log transformed expression level of each gene [24]. This is followed by the application of an Empirical Bayes shrinkage method to reduce the standard error of the fit and hence stabilize the analysis even for experiments with small number of samples [25]. The R packages was used for this analysis [26]. Using the R library mutoss, Hochberg and Benjamini method was used for estimating the p-values [27]. The reported adjusted p-values (or q-values) are controlled at a 5% false discovery rate (for the Benjamini-Hochberg method). The q-value provides a measure of each feature's significance, automatically taking into account the fact that thousands of genes are simultaneously being tested [28].

Morphoproteomics

Morphoproteomics utilizes morphology by bright field microscopy and proteomics to help define the biology of disease processes. It incorporates the following with respect to the protein analytes in lesional and companionate cells: their immunohistochemical detection; quantification of their DAB chromogenic (brown) signal intensity on a 0 to 3+ scale by visual analysis and/or visual estimation of the percentage of immunopositive tumoral nuclei, in the case of cell cycle-related protein analytes; their subcellular compartmentalization; and an assessment of their state of molecular activation to include phosphorylation (p), compartmental translocation and functional grouping [29, 30]. Morphoproteomic analysis was performed on two of the pancreases in this series of CHI patients in an attempt to provide correlates to the genomic findings. Unstained slides from formalin-fixed paraffin-embedded (FFPE) blocks of tissue from their near total pancreatectomy specimens were stained with hematoxylin-eosin (H&E). Additionally, immunohistochemical probes for the following protein analytes were applied to individual sections to include: phosphorylated (p)-mammalian target of rapamycin (mTOR) at serine (Ser) 2448 (Cell Signaling Technology, 49F9); p-Akt (Ser 473; Cell Signaling Technology, 736E11); insulin (DA-KO, 10564); and total insulin-like growth factor-1receptor (IGF-1R; GenWay Biotech). Positive and negative controls were run concurrently. The details of the staining procedure have been previously described [10, 29, 30]. Examination was performed using bright-field microscopy.

Results

The patient characteristics are detailed in **Table 1**. Four out of the six patients with diffuse CHI had mutations in *ABCC8* while no mutation was identified in the two other patients. All six patients were unresponsive to medical therapy (diazoxide and octreotide) and underwent near total pancreatectomy. All the medications were stopped at least 48 hours before the surgery and the patients were only on high concentrations of glucose infusion at the time of surgery to maintain normoglycaemia. There was no difference in the biochemical or clinical character-



Figure 1. Boxplot shows the distribution of the log2 transformed probe intensities before (A) and after (B) batch correction. The plot shows the median, the upper (75th) and lower quartiles (25^{th}), the range, and individual extreme values. The central box in the plot represents the inter-quartile range (IQR) and the line in the middle of the box represents the median. The whiskers indicate the largest and smallest values not considered outliers. The distributions across the patient and the control samples are similar after batch correction.

istics between the patients with and without known mutation. The mean purity (OD260/280) of the total RNA from diffuse CHI patients was 2.01 and the mean RIN factor of the patient samples was 7.7 and that of the controls was

7.55. All the samples had intact 18S/28S bands and were assessed to be of good quality for microarray studies.

Quality assessment of array results

The microarray data was assessed for quality by stringent measures. The density histogram of the patients and the controls revealed the same density distribution indicating comparable data (figure not shown). The distribution of the log2 transformed probe intensities before and after batch correction is shown as boxplot in Figure 1. The distributions across the patient and the control samples are similar after batch correction. The standard versus rank of the mean is shown in Figure 2.

Microarray results

The expression levels of the genes were compared between diffuse CHI patients and normal controls. The results are detailed in the Tables 2 and 3. The absolute fold change and q-values are used to assess the significance of the results. The study has revealed a significant overexpression of genes coding for growth factors and their regulatory proteins like IGF1 (insulin like growth factor 1), IGF2 (insulin like growth factor 2) and IGF2BP3 (insulin like growth factor 2 binding protein 3) as well as mTOR in diffuse CHI (Table 2) and down-regulation of TSC2 (tuberous sclerosis protein 2) (Table 3). Although several ge-

nes were overexpressed in patients with diffuse CHI with *ABBC8* mutation compared to the diffuse CHI patients without any mutation, none of them reached a statistically significant level (data not shown).



Figure 2. Standard deviation versus rank of the mean. The figure shows a density plot of the standard deviation of the intensities across arrays on the y-axis versus the rank of their mean on the x-axis. The red dots show the running median of the standard deviation and this should be a horizontal line (as shown).

Morphoproteomics

Pancreatic tissue samples from 2 diffuse CHI patients who had undergone near total pancreatectomy (patients 2 and 4 in Table 1) were included for morphoproteomic analysis. Morphoproteomic correlates for the genomic findings include: 1. High expression of p-mTOR (Ser 2448) on the plasmalemmal aspect of the acinar and centroacinar and intercalated duct cells accompanied by plasmalemmal expression of IGF-1R (Insulin like Growth Factor 1 Receptor) on the acinar cells; and 2. IGF-1R and p-Akt (Ser 473) overexpression in the predominantly insulin-producing islet cells per se accompanied by variable, albeit weaker expression of p-mTOR (Ser 2448) in the nuclear and cytoplasmic compartments of the islet cells (Figures 3 and 4). Such findings coincide with a predominant expression of the mammalian target of rapamycin complex (mTORC) 1 pathway in the exocrine elements and a predominant IGF/mTORC2/Akt pathway in the formed islet cells [10, 31-33]. Moreover, the diffusely expanded and insulin-expressing, beta cell population appears to be replacing the exocrine elements, both without a mass effect and with only rare mitotic figures in the islet cells (i.e., no mitotic figures seen in the islet cells from patient 2 on H&E in 16 sets of 10 high power fields, 0 mitotic index, and only two mitotic figures in the islet cells from patient 4 on H&E in 3.5 sets of 10 high power fields, mitotic index of 0.6 mitotic figures per 10 high power fields; no mitotic figures were appreciated in the insulin-expressing beta cells in either case). The latter findings support the concept of transdifferentiation of exocrine elements into functional, insulin-producing beta cells, as a primary source of the expanded beta cell population [10].

Discussion

The data from this study comparing the gene expression profile of the diffuse CHI patients with that of controls (Tables 2 and 3) has shown significant overexpression of genes coding for growth factors and their corresponding proteins to include IGF1, IGF2, IGF2BP3 and MTOR and conversely, with down regulation of TSC2, which codes for tuberin that is inhibitory to the IGF/Akt/mTOR signalling pathway [34]. Correspondingly, morphoproteomic analysis on representative specimens from diffuse CHI patients from this study (Table 1, patients 2 and 4; and Figures 3 and 4) has confirmed the overexpression of constitutively activated mTOR phosphorylated on serine 2448 in the acinar cells, and correlative expressions of insulin-like growth factor-1receptor (IGF-1R) and p-Akt (Ser 473) on the plasmalemmal aspect of the acinar cells and in the expanded insulin-producing, islet-cell population along with nuclear p-mTOR (Ser 2448). The latter are consistent with IGF/ mTORC2/Akt signalling in the islet cells [31-33]. In addition, the microanatomical distribution of the insulin-producing islet cells shows admixture and contiguity with the exocrine pancreas in a diffuse pattern without a mass effect and with minimal progression into the mitotic phase (vide supra). This coincides with overexpression of the p-mTOR pathway and predominant cell cycle arrest at the GO-G1 phase in CHI patients from a previous study [10]. Together, these support a role for transdifferentiation of the exocrine pancreas, associated with overexpression and upregulation of the mTOR pathway, resulting in both the proliferation of insulin-producing beta cells and in contributing to the state of CHI.

The findings from the study suggest that the growth factors and their regulatory proteins potentially contribute to the development of beta cell proliferation in diffuse CHI. IGF2BP3 enhances the translation of IGF2, which potentially promotes the proliferation of islets as well as transdifferentiation of the exocrine cells into insulin producing cells. IGF1 is possibly contrib-

Table 2. Gene probesets with expression upregulated (absolute fold change > 1.4 and q-value < 0.05) in diffuse CHI (n=6) compared to controls (n=2). The genes are listed in the order of the relative upregulation in the diffuse patients (absolute fold change)

Gene Symbol	Gene title	Mean expres- sion (Diffuse)	Mean expres- sion (Control)	Absolute fold change (Diffuse/Control)	q-value
SFTA2	Surfactant associated 2	4.48248152	1.85199032	2.42035905	0.0135868
MTOR	Mammalian target of rapamycin	6.50424066	3.38668987	1.92053034	0.0205303
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3	5.48028777	3.32333398	1.64903311	0.0102585
FOXD4L6	Forkhead box D4-like 6	3.95733313	2.53680637	1.55996657	0.04107925
TOP2A	Topoisomerase	5.9689321	4.1347211	1.44361160	0.01358679
HIST1H3B	Histone cluster 1, H3b	6.9189861	4.8030661	1.44053511	0.02724006
C1orf54	Chromosome 1 open reading frame 54	7.2171931	5.0504121	1.42903121	0.02665911
HIST1H2AE	Histone cluster 1, H2ae	5.9400541	4.1630981	1.42683512	0.02315511
IGF2	Insulin-like growth factor 2 (somatomedin A)	9.7501978	6.8874492	1.41564713	0.0385005
HPGDS	Hematopoietic prostaglandin D synthase	5.6316341	4.0062682	1.40570621	0.01348923
UCP2	Uncoupling protein 2	7.2042258	5.1266053	1.40526243	0.0363704
IGF1	Insulin-like growth factor 1 (somatomedin C)	6.2893597	4.4796791	1.40397551	0.0134920
GLRX	Glutaredoxin (thioltransferase)	6.0604792	4.3211611	1.40251202	0.0313948

Table 3. Gene probesets with expression downregulated (absolute fold change < 0.72 [-1.4 fold] and q-value < 0.05) in diffuse CHI (n=6) compared to normal controls (n=2). The genes are listed in the order of the relative downregulation in the diffuse patients (absolute fold change)

Gene Symbol	Gene title	Mean expression (Diffuse)	Mean expres- sion (Control)	Absolute fold change (Diffuse/Control)	q-value
MIR217	MicroRNA 217	3.864124	7.246749	0.533222	0.011171
CA12	Carbonic anhydrase XII	5.186189	8.150467	0.636306	0.017879
TDH	L-threonine dehydrogenase	4.781031	7.445191	0.642164	0.003037
AZGP1	Alpha-2-glycoprotein 1, zinc-binding	6.448594	10.02059	0.643534	0.007181
ANKRD62	Ankyrin repeat domain 62	4.44924	6.812509	0.653099	0.007755
MIR29C	MicroRNA 29c	5.525513	8.312126	0.664753	0.009176
LOC148696	Hypothetical LOC148696	4.780071	7.164648	0.667175	0.001332
C15orf54	Chromosome 15 open reading frame 54	4.302897	6.333161	0.679423	0.014691
TSC2	Tuberous sclerosis 2	4.358815	6.322723	0.689389	0.028607
C8orf12	Chromosome 8 open reading frame 12	4.150246	5.963321	0.695962	0.015533
ANKRD62	Ankyrin repeat domain 62	4.181387	5.954232	0.702255	0.013479
CHAD	Chondroadherin	5.712001	7.967523	0.716912	0.003037

uting to this effect of IGF2. IGF1 receptors have been shown to promote beta cell development and survival through the IRS2 (insulin receptor substrate) signalling pathway [35, 36]. The net effect is an increase in the number of insulin producing cells leading to beta cell proliferation (**Figure 5**).

The mTOR protein integrates the input from several upstream pathways, including insulin, growth factors (IGF1 and IGF2), and amino acids and also senses cellular nutrient, oxygen, and energy levels. Growth factors activate the mTOR pathway through receptor tyrosine kinases [37]. Following the binding of growth factors, Akt (alpha serine/threonine protein kinase) is recruited to the plasma membrane and activated through phosphorylation by phosphoinositide 3 kinase (PI3K) and PDK1 (Phosphoinositide dependent kinase 1). Activated Akt in turn phosphorylates TSC2 (tuberous sclerosis complex 2), which prevents formation of the inhibitory TSC1/TSC2 heterodimer [37]. TSC2 serves as a GTPase-activating protein (GAP) and inactivates the small GTPase Rheb [38]. Hence the net effect of TSC2 inhibition allows Rheb to directly activate mTORC1 [39] (Figure 6).

The mTOR pathway is essential for growth and survival of beta cells [40]. In an experimental study involving rat islets, rapamycin (mTOR



Figure 3. Patients 2 and 4 from diffuse form of CHI illustrating contiguity and variable replacement of the exocrine pancreas by islet cells, some with karyomegaly (arrows) but without a mass effect (note acinar-like arrangement of islet cells in patient 4, arrow; H&E, original magnification ×400). Middle frames with insulin-producing islet cells in these patients with the diffuse form of CHI showing variable replacement of exocrine pancreas. (3,3' diaminobenzidine DAB [brown] chromogenic signal, original magnification ×200). Corresponding negative controls without primary antibody (lower frames) depicting absence of DAB chromogen stain and with replacement of portion of exocrine pancreas by islet cells without a mass effect (arrows, patient 2, original magnification ×200) and with karyomegaly in an occasional islet cell (arrow, patient 4, original magnification ×400).

inhibitor) was shown to inhibit 3H-thymidine incorporation (indicator of DNA synthesis) in a dose-dependent manner [40]. The inhibition of glucose-induced DNA synthesis by rapamycin was noted to be consistent with the unique role of mTOR to regulate growth and proliferation by

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mTOR pathway in congenital hyperinsulinism



Figure 4. Total insulin-like growth factor-1 receptor (IGF-1R [Tyr1165/1166]), upper row, is illustrated in the islet cell cytoplasm of the non-CHI control pancreas (original magnification ×400). It appears to be a constant in normal islet cells (arrow I) and shows uniform expression in the islet cells of the diffuse form (arrow I), but also with plasmalemmal expression on the acinar cells of both patients with CHI (contrast with non-CHI control (arrow A); DAB chromogen, original magnifications ×400). Strong expression of phosphorylated (p)-mTOR (Ser 2448) is evident in the intercalated duct/centroacinar cells of the exocrine pancreas, mild in the acinar cells and virtually absent in the islet cells of the non-CHI control pancreas (middle row, right hand frame ×400). This contrasts with the relatively strong expression in the contiguous islet cells but notably with some being in the nuclear compartment (arrow I), the latter consistent with mTORC2 (original magnifications, ×400). Akt, phosphorylated on serine 473 is expressed in the islet cells of the non-CHI control pancreas (arrow I, original magnification ×400). Similarly, the islets cells in diffuse CHI show chromogenic signal for this protein analyte in the cytoplasmic and nuclear compartments (arrows). In addition, there is variable chromogenic signal in the contiguous exocrine pancreas of both patients with CHI, for p-Akt (Ser 473) in the cytoplasm and/or plasmalemmal aspect (arrow P, original magnifications ×400).



Figure 5. Illustration of the possible mechanism of diffuse CHI due to the transdifferentiation of the exocrine pancreatic cells into insulin producing cells mediated by mTORC1 pathway following activation by the elevated growth factors and insulin. The reduced levels of TSC2 further activates mTORC1 pathway by the release of inhibition on mTORC1. Siro-limus acts by blocking the activated mTORC1 pathway.

sensing nutrient availability [40, 41]. S6K1 (S6 kinase 1), a target of mTOR, is proposed to control beta cell size by increasing mRNA translation as diminished beta cell size has been observed in S6K1 knockout mice [42].

An opportunity, afforded by the findings in this study, is to integrate the inactivating mutation (ABCC8) of the K_{ATP} channels, with: a) activation of the mTOR pathway; b) the factors contributing to the transdifferentiation of the exocrine and particularly acinar population into insulinproducing beta cells; and c) the upregulation of the IGF/mTORC2/Akt pathway into the functioning and survival of the expanded beta cell population resulting in hyperinsulinism. Moreover, the process of such integration should further define the genomic and proteomic factors that are contributing to the biology of CHI and enable us to produce correlates to the successful therapeutic application of sirolimus in such patients [15] and to raise other targeted therapies that could be used in a combinatorial fashion with sirolimus.

To that end, data mining of the clinical scientific literature has provided the following links: 1. Glucagon-like peptide (GLP)-1receptor antagonist exendin-(9-39) has been shown to suppress insulin secretion and to correct fasting hypoglycemia in SUR-1(-/-) mice and to elevate blood glucose levels in CHI associated with inactivating mutations in the K_{ATP} channel [43]; 2. GLP-1 receptor has been predominantly localized in beta cells of the human pancreas but with some expression in the acinar cells [44]; 3. GLP-1 receptor has been shown to protect beta cells against apoptosis by enhancing the activity of an IGF-2/IGF1-receptor autocrine loop and by enhancing IGF-1R expression and by stimulating IGF-2 secretion [45]; 4. GLP-1 has implications with regard to acinar to islet cell transdifferentiation to include: a) the ability of exendin-4, a long acting GLP analogue, given in the newborn period to increase Pdx1 expression and prevent the development of diabetes [46] [parenthetically, Pdx1 is one of the factors that contributes to the in vivo

reprogramming of pancreatic acinar cells to beta cells [47]]; b) pancreatic acinar cells can be induced to convert to insulin-producing cells and this effect is augmented by GLP-1 [48, 49]; and c) GLP-1 supplementation induced expression of insulin and PDX1 in a transdifferentiated pancreatic acinar cell line [50]. Notably, GLP-1, in inducing pancreatic ductal transformation into beta cells, effects an increase in p27Kip1 and an increase in the number of cells in the GO-G1 phase [51] as previously reported in CHI [10]. In addition, the activation of the mTOR pathway in CHI is consequent, at least in part, to the influx of calcium that collaborates with amino acids in the activation of Rag leading to mTORC1 [10]. A proof of concept regarding the role of GLP-1 in the pathogenesis and histogenesis of CHI is the association of hyperinsulinemic hypoglycaemia with persistent and exaggerated secretion of GLP-1 in adult patients following Roux-en-Y gastric bypass. The pancreases of such patients showed features of diffuse CHI to include "beta cell clusters scattered throughout the acinar parenchyma" [52].

The mTORC1 pathway may also contribute to the transformation of acinar cells into islet cells via influencing the Reg1 pathway. That is to say,



Figure 6. Illustration of mTORC1 activation by growth factors and amino acids. Growth factors activate Akt through phosphoinositide 3 kinase (PI3K). Activated Akt phosphorylates (inhibits) TSC2 (tuberous sclerosis complex 2). This releases the inhibition on Rheb (Ras homolog enriched in the brain), thereby activating mTORC1 (37). The phosphorylation (inhibition) of the mTORC1 component PRAS40 results in further mTORC1 activation (Green arrows indicate activation, red lines indicate inhibition, and dotted line indicates possible effect).

Reg1 expression in acinar cells is important in maintaining their lineage [53], and mTORC1 signalling apparently leads to cytoplasmic localization of Msn2 [54] a transcriptional activator of STRE (Stress Response Element. Furthermore, Msn2 potentially collaborates with Reg1 to effect nuclear signalling; and when Msn2 is confined to the cytoplasmic compartment by mTORC1, it theoretically, could passively facilitate such transdifferentiation of acinar cells by binding to Reg1 in the cytoplasm and limiting the nuclear influence of Reg1 in maintaining acinar lineage. The expression of the mTORC2 pathway in the formed islet cells in CHI coincides with the necessity of having Rictor/ mTORC2 to maintain beta cell mass, pancreatic insulin content and insulin secretion through Akt [55, 56]. Correspondingly, receptors for insulin, IGF-1 and insulin receptor substrate-1, mediate pathways that contribute to the regulation of islet hormone secretion and synthesis [57].

Integration of these pathways and pathogenetic mechanisms in transdifferentiation of acinar cells into insulin-producing beta cells and the synthesis and secretion of insulin are depicted in Figure 7. The efficacy of sirolimus in reducing the hyperinsulinaemic hypoglycemia and avoiding near total pancreatectomy in CHI [15] can be attributed, at least in part to the: 1) inhibition of mTORC1 generation in the acinar cells from Ca++/amino acid facilitated Rag pathway activation [10]: 2) inhibition of mTORC1 generation in the acinar cells thereby facilitating Msn2 nuclear translocation [54] a transcriptional activator of STRE (Stress Response Element, possibly complexed to Reg1, to favour acinar differentiation and potentially avoid acinar-islet transdifferentiation [53]; and 3) with prolonged application, the disassembly of the mT-ORC2 complex [58] interfering in the Akt signalling in the islets resulting in the reduction of insulin secretion and beta cell viabili-

ty via accelerated apoptosis [8, 12, 41, 59]. Additionally, other agents that target the pathogenetic sequence in CHI and might be considered in a combinatorial fashion with sirolimus include metformin to inhibit the Rag GTPases pathway, mTORC1 and IGF/mTORC2/Akt pathways [34, 58], nifedipine as a calcium channel blocker [10], and the GLP-1 receptor antagonist, exendin-(9-39) [43] (**Figure 7**).

The study limitations include a small sample size and the lack of age matched pancreatic control samples. As the study involved prospective collection of the fresh frozen pancreatic tissue samples, the sample size is limited due to the few number of patients undergoing surgery for this rare disorder. The control group did not include samples from paediatric pancreatic tis-



Potential Genomic and Molecular Correlates in CHI and Therapeutic Options

Figure 7. Schematic depicting the potential genomic and molecular correlates in CHI incorporating the inactivating mutation (*ABCC8*) of the ATP-sensitive K+ channels leading to Ca++ influx and activation of the mTORC1 pathway via Rag and glucagon-like peptide (GLP)-1 receptor(R) promoting acinar-islet transdifferentiation to insulin-producing beta-cell proliferation. Integration of gene amplifications of the IGF signaling pathway based on the genomic analyses in this study (\uparrow *IGF1/IGF2/IGF2PB3*/and *MTOR* and \downarrow *TSC2*) support both mTORC1 signaling in the acinar/ductal cells of the exocrine pancreas and mTORC2 in the beta-cell population promoting insulin synthesis and secretion and their survival. The efficacy of sirolimus in treating CHI is illustrated in the interference of the targeted pathways in the pathogenetic sequence with the possible application of additional combinatorial, targeted therapies such as metformin, nifedipine and exendin (9-39). [(+) promotes; (-) inhibits].

sues due to the extreme challenges involved in obtaining such samples. Prior treatment of the

patients could have had potential influences on the gene expression although all medications except glucose infusion were discontinued at least 48 hours before surgery.

In conclusion, the etiopathogenesis of diffuse CHI appears to be multifactorial with mutational alterations leading to an K_{ATP} channel defect, transdifferentiation of the acinar cells into insulin-producing beta cells and enhanced mTORC1 signalling in the acinar cells and genomic amplification with morphoproteomic evidence of constitutive activation of the IGFpathway/mTORC2/ Akt signalling in the islet cells, all leading to hyperinsulinism. This study has provided genomic and morphoproteomic correlates of the biology of CHI and targeted therapeutic options.

Disclosure of conflict of interest

None.

Authors' contribution

Senthil Senniappan (SS) and Robert Brown (RB) wrote the initial manuscript and Khalid Hussain (KH) contributed to the design and further development of the manuscript to the final stage. SS conducted RNA extraction and microarray data analysis and RB carried out the morphoproteomic experiments. KH supervised the project and contributed to the design and conduct of the overall study. All the authors made substantial contribution to conception and design, acquisition of data or analysis and interpretation of the version to be published.

Address correspondence to: Khalid Hussain, Genetics and Epigenetics in Health and Disease, Genetics and Genomic Medicine Programme, UCL Institute of Child Health, Great Ormond Street Hospital for Children, 30 Guilford Street, London WC1N 1EH, UK. Tel: 44 (0)2079052128; E-mail: Khalid.hussain@ucl.ac.uk

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