International Urology and Nephrology
https://doi.org/10.1007/s11255-018-1892-z

NEPHROLOGY - ORIGINAL PAPER

Transcriptome analysis supports viral infection and fluoride toxicity as contributors to chronic kidney disease of unknown etiology (CKDu) in Sri Lanka

Saravanabavan Sayanthooran[1] · Lishanthe Gunerathne[2] · Tilak D. J. Abeysekera[3] · Dhammika N. Magana-Arachchi[1]

Received: 8 February 2018 / Accepted: 10 May 2018

Abstract

Purpose Chronic kidney disease of unknown etiology (CKDu), having epidemic characteristics, is being diagnosed increasingly in certain tropical regions of the world, mainly Latin America and Sri Lanka. They have been observed primarily in farming communities and current hypotheses point toward many environmental and occupational triggers. CKDu does not have common etiologies of chronic kidney disease (CKD) such as hypertension, diabetes, or autoimmune disease. We aimed to understand the molecular processes underlying CKDu in Sri Lanka using transcriptome analysis.

Methods RNA extracted from whole blood was reverse transcribed and used for microarray analysis using the Human HT-12 v.4 array (Illumina). Pathway analysis was carried out using ingenuity pathway analysis (IPA—Qiagen). Microarray results were validated using real-time PCR of five selected genes.

Results Pathways related to innate immune response, including interferon signaling, inflammasome signaling and TREM1 signaling had the most significant positive activation *z* scores, where as EIF2 signaling and mTOR signaling had the most significant negative activation *z* scores. Pathways previously linked to fluoride toxicity; G-protein activation, Cdc42 signaling, Rac signaling and RhoA signaling were activated in CKDu patients. The most significantly activated biological functions were cell death, cell movement and antimicrobial response. Significant toxicological functions were mitochondrial dysfunction, oxidative stress and apoptosis.

Conclusions Based on the molecular pathway analysis in CKDu patients and review of literature, viral infections and fluoride toxicity appear to be contributing to the molecular mechanisms underlying CKDu.

Keywords EIF2 signaling · Innate immunity · Interferon signaling · Microarray · mTOR signaling · RT-qPCR

Introduction

Chronic kidney disease (CKD) is a global public health problem and the etiology for the disease is said to be affected by racial, geographic and economic disparities [1, 2]. Systemic diseases such as hypertension and diabetes contribute to majority of the cases in all the developed countries and

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11255-018-1892-z) contains supplementary material, which is available to authorized users.

✉ Dhammika N. Magana-Arachchi
cellbio@ifs.ac.lk

Saravanabavan Sayanthooran
sayan_28@yahoo.com

Lishanthe Gunerathne
lishanthe@yahoo.co.uk

Tilak D. J. Abeysekera
tilak_1@hotmail.com

[1] Molecular Microbiology and Human Diseases, National Institute of Fundamental Studies, Kandy 20000, Sri Lanka

[2] Renal Research Centre, District Hospital, Girandurukotte 90750, Sri Lanka

[3] Centre for Education, Research and Training on Kidney Diseases (CERTKID), Faculty of Medicine, University of Peradeniya, Peradeniya 20400, Sri Lanka

Published online: 28 May 2018

many developing countries, whereas glomerulonephritis and unknown causes are more common in countries of Asia and sub-Saharan Africa [3].

Many tropical regions of the world including Latin America [4], Egypt [5], India [6], and Sri Lanka [7] have cases of CKD that cannot be linked to any of the known etiologies and thus has been given the name chronic kidney disease of unknown etiology (CKDu). The affected individuals usually belong to the farming community, and hypotheses have thus mainly focused towards environmental sources of renal toxicity [8–11], and dehydration due to heat stress [10, 12]. Recent hypotheses have also focused on genetic susceptibility [13–15] and an infectious etiology [16].

One of the earliest hypotheses for the cause of CKDu in Sri Lanka, based on its geographical specificity, was the presence of high amounts of fluoride in the groundwater, which was the major source of drinking water for the people of the affected areas. Another hypothesis stated that not only the presence of high fluoride, but the use of aluminum vessels for cooking resulted in the leaching of aluminum and formation of various aluminum fluoride complexes which have been documented to cause disease [17].

A study testing for nine heavy metals in dietary and water sources [18] overall concluded that their findings strongly identify cadmium, present in sedimentation of the reservoirs and working their way up the food chain through fish, lotus roots, rice, milk, and tobacco, accumulating over the years, as the cause of the chronic renal failure. They mention that this may be further aggravated when combined with the fluoride rich water in some of the areas [18]. Recently synergistic effect of cadmium, fluoride and hard water was shown to have a high impact on kidney disease and was suggested as the cause for CKDu [19].

More recent studies have hypothesized viral etiologies for the disease with patients showing seropositivity to Hantavirus [16], although infectious etiology and autoimmunity have been previously ruled out [20].

As in CKD of other known etiologies, the CKDu patients do not present with overt symptoms early in the disease and therefore are diagnosed only during later stages. The histopathology of CKDu also has different patterns to that of CKD of diabetic or hypertensive etiologies, showing mainly tubulointerstitial nephritis, and small echogenic fibrotic kidneys [20]. The Sri Lankan CKDu was also associated with less arterial stiffening than in known causes of CKD [21]. In view of the differences in causative etiologies and histopathology, the underlying molecular mechanisms of CKDu are expected to be different from that of other forms of CKD.

Genome wide association studies and whole exome sequencing have identified single nucleotide polymorphisms (SNPs) in CKDu patients indicating genetic predisposition [13, 14], whereas pilot studies of gene expression using selected genes identified oxidative stress in a CKDu endemic population including healthy group, as opposed to a non-endemic healthy population, indicating environmental source of oxidative stress [15]. We hypothesized that analyzing whole transcriptome of CKDu patients and underlying pathways associated to the disease should provide a better understanding of the molecular mechanisms of CKDu, and could narrow down on causative factors. Genomic expression profiling has been used as a tool in classifying disease subtypes for other diseases such as human dilated cardiomyopathy [22] and breast tumor [23]. As this is the first study profiling the transcriptome in blood of CKDu patients, we expected to identify key pathways controlling CKDu in relation to currently held hypotheses for the disease.

Materials and methods

Ethical procedures

Ethical approval for the study was obtained from the Ethics Review Committee of the Postgraduate Institute of Science, University of Peradeniya. All study subjects provided written informed consent for the collection of blood samples and subsequent analysis.

Study subjects

A discovery cohort with a total of 36 male subjects were recruited for microarray analysis; six patients from each of stage 2–5 of CKDu, six apparently healthy individuals from an endemic area of CKDu in Sri Lanka, Girandurukotte, and six apparently healthy volunteers from a non-endemic area of Sri Lanka, Kandy. The sample collection and microarrays were carried out during the period of July 2014 to March 2016.

A validation cohort was recruited for real-time PCR analysis of selected genes and consisted of a total of 50 male subjects; 30 CKDu patients, 10 apparently healthy volunteers from Girandurukotte, and 10 apparently healthy volunteers from Kandy. The characteristics of the population in the validation group are presented in Table 1.

Inclusion criteria for the CKDu participants were having unknown etiology based on criteria set by the Ministry of Health, Sri Lanka; no past history of diabetes mellitus, chronic or severe hypertension, snake bite, glomerulonephritis or urological diseases; normal HBA1C (< 6.5%), blood pressure < 160/100 mmHg untreated or < 140/90 mmHg on up to two antihypertensive medications.

Inclusion criteria for the healthy individuals from both the endemic as well as non-endemic areas were absence of family history of CKD in first degree relatives and no prior diagnosis of any chronic disease. Exclusion criteria for healthy individuals were smoking and presence of known

Table 1 Characteristics of the population in the validation cohort

	Chronic kidney disease of unknown etiology ($n=30$)				Girandurukotte healthy ($n=10$)	Kandy healthy ($n=10$)
	Stage 2 ($n=4$)	Stage 3 ($n=13$)	Stage 4 ($n=7$)	Stage 5 ($n=6$)		
Age	46.25 ± 6.85	48.69 ± 7.78	42.43 ± 8.32	49.50 ± 8.76	41.11 ± 8.32	35.4 ± 9.09
Serum creatinine	1.2 ± 0.08	1.84 ± 0.33	3.47 ± 0.57	6.54 ± 1.42	–	–
eGFR	69.61 ± 5.79	43.41 ± 8.82	21.40 ± 4.27	10.08 ± 2.08	–	–

history of any chronic disease. The healthy individuals from the endemic area were identified as negative for CKD/CKDu in village screening programs.

RNA processing and quantification

Total RNA was extracted from peripheral blood using Qiazol Lysis Reagent (Qiagen) and consecutively purified using the RNeasy MinElute cleanup kit (Qiagen) according to manufacturers' protocols. For the microarray studies, we constructed pools of the six individuals of each group, to finally have six pools for analysis. Technical triplicates were used for analysis. The purified RNA was shipped to Macrogen Inc., South Korea, where quality control of the RNA and microarray was performed (Supplementary information 1).

Data analysis

Data were processed and analyzed using *R* packages; beadarray [24], Biobase [25], dplyr [26], and Complex Heatmap [27], and *Microsoft Excel*. Raw signal intensities with detection p values < 0.05 were filtered out, quantile normalized and log transformed. Batch correction was carried out using the ComBat script [28] prior to fold change and false detection rate (FDR) calculations.

Pathway analysis

Fold change and FDR were entered to the Ingenuity Pathway Analysis (IPA) software (Qiagen) and analysis was initially performed using expression cut-off of 1.0 fold and FDR of 0.001. This relaxed fold change cut-off (1.0) was used to capture the slightest variations in disease pathways with high significance (FDR < 0.001).

Quantitative real-time RT-PCR validation

Genes from the most activated pathways with fold change greater than 1.5 fold with FDR of 0.05 were then used for real-time PCR validation. These genes included the Interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), Interferon-induced transmembrane protein 3 (IFITM3), Myxovirus resistance 1 (MX1), and Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) from the interferon signaling pathway and the Interleukin 1B (IL1B) from the inflammasome pathway. Primer details of the genes are presented in Table 2.

Briefly, cDNA synthesized using the GoScript Reverse Transcription System (Promega) were subjected to qPCR and amplifications performed with SYBR Green I (Invitrogen) in the Rotor-Gene Q PCR machine (Qiagen). Specificity of all individual amplification reactions was confirmed

Table 2 Primer details of genes selected for RT-qPCR validation

Gene	Direction	Sequence (5′–3′)	Primer design
IFIT3	Forward	GAAGGAACTGGGCCGCCTGCTAAG	Niess et al. [56]
	Reverse	GCCCTGGCCCATTTCCTCACTACC	
IFITM3	Forward	TGTCCAAACCTTCTTCTCTCC	Zhao et al. [57]
	Reverse	CGTCGCCAACCATCTTCC	
MX1	Forward	CAGCACCTGATGGCCTATCA	Martinez et al. [58]
	Reverse	ACGTCTGGAGCATGAAGAACTG	
IFIT1	Forward	TCATCAGGTCAAGGATAGTCTG	Tang et al. [59]
	Reverse	GGTGTTTCACATAGGCTAGTAG	
IL1B	Forward	ACAGATGAAGTGCTCCTTCCA	Hedl and Abraham [60]
	Reverse	GTCGGAGATTCGTAGCTGGAT	
B2M	Forward	TGCCGTGTGAACCATGTGA	Koop et al. [61]
	Reverse	CCAAATGCGGCATCTTCAA	
GAPDH	Forward	TGACCTCAACTACATGGTTTA	Sayanthooran et al. [15]
	Reverse	GCCCCACTTGATTTTGGA	

by melt curve analysis. Real-time expression values were calculated by using the delta–delta CT method [29] with Kandy healthy as calibrators, and B2M and GAPDH as the housekeeping genes.

Results

Data deposition

The data discussed in this manuscript have been deposited in National Center for Biotechnology Information's (NCBI) Gene expression Omnibus (GEO) and are accessible through GEO series accession number GSE62792, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62792.

Microarray raw data analysis

A total of 25,171 out of the 47,314 probes in the Human HT-12 array showed detection p values < 0.05. Following batch correction, fold change calculations, and p value corrections, 1500 genes showed FDR < 0.001 for at least any one of the three study groups. This was further narrowed down to 1166 genes where the fold change was either up- or down-regulated more than 1.0 fold. A heatmap of these genes clustered the CKDu patients together and the Girandurukotte and Kandy healthy individuals together (Fig. 1).

Pathway analysis

The fold change and FDR values obtained following the raw data analysis was then used for further analysis with the IPA software (Qiagen). Pathway analysis was therefore carried out with 1166 genes that fulfilled the threshold cut-off of 1.0 fold change and FDR < 0.001 in the CKDu

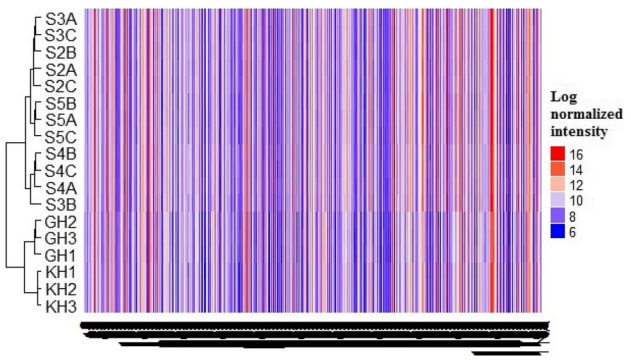

Fig. 1 Heatmap for differentially expressed genes used in pathway analysis, with expression greater than or less than 1.0 fold and having FDR adjusted p value < 0.001. CKDu patients are labeled with their stages (S2-stage 2, S3-stage 3, S4-stage 4, S5-stage 5), Girandurukotte healthy (GH), and Kandy healthy (KH), each having three replicates A–C or 1–3

population and the Girandurukotte healthy compared to the Kandy healthy. Canonical pathways that had activation z score values were taken for further analysis (Supplementary Table 1) while disregarding pathways with no activity pattern available.

EIF2 signaling ($-\log p = 12.3$) and interferon signaling ($-\log p = 6.08$) were the most significant canonical pathways. EIF2 signaling pathway had a negative activation z score of -1.877 indicating overall down regulation of the pathway, whereas the interferon signaling pathway showed a positive activation z score of 3.464 indicating overall up regulation of the pathway (Fig. 2a, b). mTOR signaling was the second most significant pathway with a negative z score, whereas inflammasome pathway, type I diabetes mellitus signaling pathway, TREM1 signaling, IL-8 signaling and Th1 pathways were also among the top significant pathways with positive activation z scores. The Girandurukotte healthy population showed significant difference (FDR < 0.001; fold change > 1.5 fold) in only RNVU1-18, although when applying a relaxed threshold of fold change 1.5 and FDR < 0.05, showed DEFA1, MCMDC2, RN7SK, RNU1-3 and RNVU1-18 to be differentially regulated.

Diseases and bio functions analysis

Only those diseases and bio functions having predicted activation states (Supplementary Table 2) were taken for analysis. Diseases and bio functions related to cancer, cell death and survival, organismal injury and abnormalities, tumor morphology, cellular movement, free radical scavenging, infectious diseases, antimicrobial response, and inflammatory response had predicted increased activation scores. Biological function categories including lymphoid tissue structure and development, tissue morphology, organism survival and protein synthesis had decreased states with negative activation z scores. The category of hematological system development and function had both increased and decreased activation z scores with annotations of accumulation of myeloid cells, accumulation of phagocytes and toxemia having activated scores and quantity of blood cells, quantity of leukocytes, and quantity of lymphoid cells having negative scores.

Toxicological functions analysis

Increased mitochondrial dysfunction, liver, renal and cardiac necrosis, cell death, oxidative stress, and pro-apoptosis were some of the main and repeatedly seen toxicological functions in our CKDu population. Stacked bar chart of the toxicological functions is presented (Fig. 3).

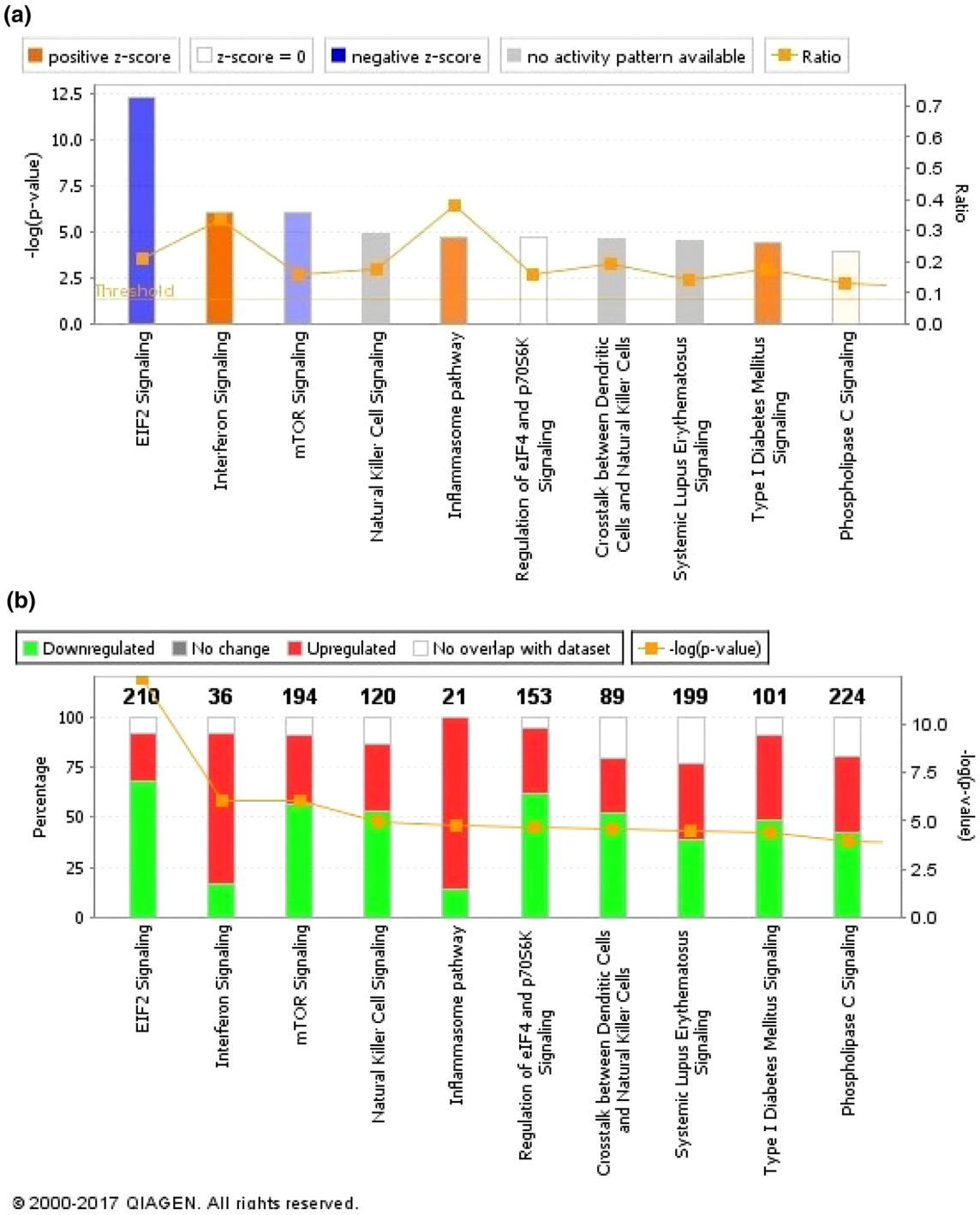

Fig. 2 Top ten significant canonical pathways ($p < 0.01$) when comparing CKDu patients with Kandy healthy individuals; **a** bar chart colored according to activation z score and **b** stacked bar chart showing proportion of up-regulated and down-regulated genes in each pathway. The ratio defines the number of genes responsive from that particular pathway in our CKDu population divided by the total number of genes involved in that canonical pathway. The total number of genes involved in the pathways is mentioned above the bars

RT-qPCR validation

Five genes downstream in two of the most activated pathways; interferon signaling pathway and inflammasome pathway that showed greater than 1.5 fold changes were selected for real-time PCR validation using the validation cohort. Activation states of the genes in the respective pathways are shown (Fig. 4). Real-time PCR and microarray

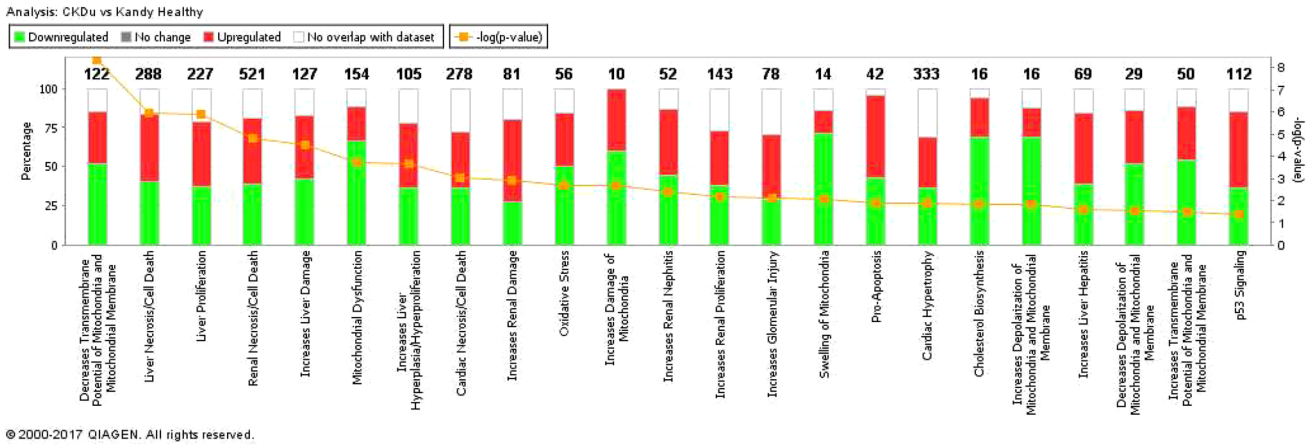

Fig. 3 Toxicological functions inferred from differentially expressed genes in CKDu patients compared to Kandy healthy individuals using IPA software. The total number of genes involved in the pathways is mentioned above the bars

showed consistency direction-wise for the mean log-normalized expression values of the five genes (Fig. 5). There was however a larger standard deviation seen in the validation cohort with samples even showing down regulation of the genes (Fig. 5).

Discussion

In this study, we took an approach of analyzing the transcriptomic profiles in CKDu patients to better understand molecular mechanisms of the disease and pathways that may be involved, in order to infer possible causes for the disease. The discovery groups in microarray clustered with clear separation between the healthy individuals and the CKDu patients. The results were analyzed in relation to current hypotheses, clinical observations and their possible influence on the affected pathways in order to narrow down on the most probable causes of the disease. It should be noted however that a single type of signal can lead to different responses in different cells and also that different signaling pathways can lead to a single response. It is therefore the interaction of multiple signaling pathways which modifies a response and gives it the fine tuning and specificity needed [30].

The histopathology of CKDu is mainly tubulointerstitial disease, with interstitial fibrosis and tubular atrophy [31]. This pathogenic process is brought about by the release and actions of the cytokines PDGF, TGF-β, and complement C5 fibroblast chemoattraction and of interaction of fibroblasts with metalloproteinases and interleukin-1 (IL-1), tumor necrosis factor (TNF-α), epidermal growth factor [32, 33]. The activation of PDGF signaling, TNF receptor signaling and IL1B was seen in our CKDu patients in accordance with this. Molecular mediators of the bone morphogenic protein (BMP) and hepatocyte growth factor (HGF) signaling pathways have also been associated with renal interstitial fibrosis [33], and these pathways were also activated in our CKDu patients.

The interferon signaling pathway was seen as the most significantly enriched pathway with a positive activation *z* score. Interferons are a family of cytokines that are involved in the regulation of different functions including antiviral, antiproliferative, antitumor, and immunomodulatory activities [34]. Different classes of interferons stimulate different interferon receptors complexes, which when activated, results in the transcription of the interferon stimulated genes (ISGs) [35]. Although type I (IFNα/β) are most commonly induced by viruses, type II (IFNγ) also has antiviral properties and is involved in bridging the gap between the innate and adaptive immune responses [36]. Upregulation of the MX1 gene which was seen in our CKDu patients was shown to be specific to type I interferon response [37], however activation of the interferon gamma receptor 1 (IFNGR1) was also seen in our study, which is indicative of a type II response [38]. The activation of JAK/STAT, NF-κB, PKCθ, Rac, p38, and ERK signaling pathways are seen with the type I IFN signaling [38] and this was also observed in our study group.

The other canonical pathways in relation to the innate immune system, including the inflammasome pathway, TREM1 signaling pathway, acute phase response signaling pathway, toll-like receptor (TLR) signaling pathways, and lipopolysaccharide (LPS) stimulated MAPK signaling pathway further point towards infectious response. The innate immune system gets activated by recognizing pathogens using certain pattern recognition receptors like the TLRs and NOD-like receptors and are not dependent on the antigen. Activation of these pattern recognition receptors results in the production of cytokines, chemokines and interferons

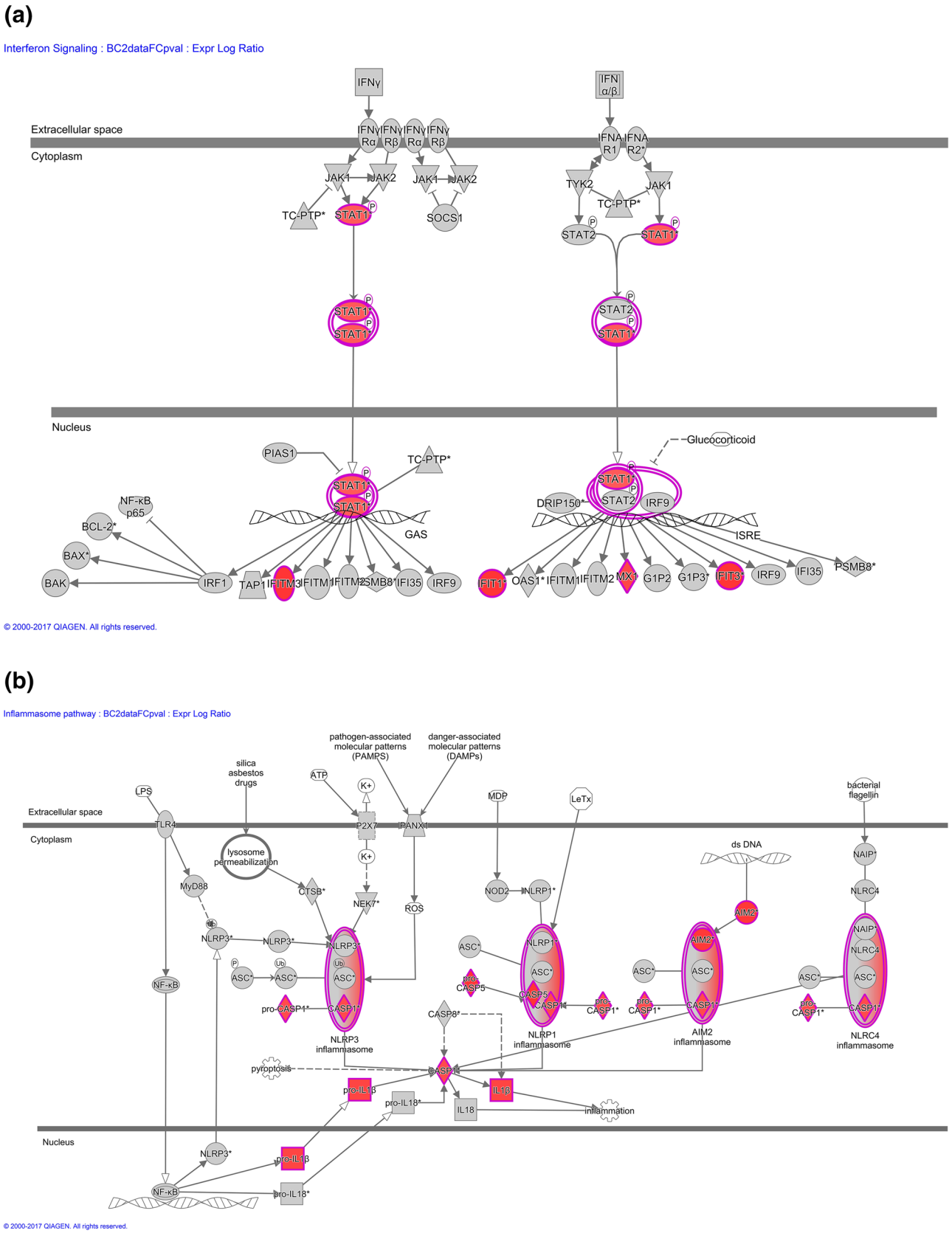

Fig. 4 Activated genes in the CKDu group of the **a** interferon signaling pathway and **b** inflammasome pathway as reproduced from the IPA software. Up-regulated genes are highlighted in red

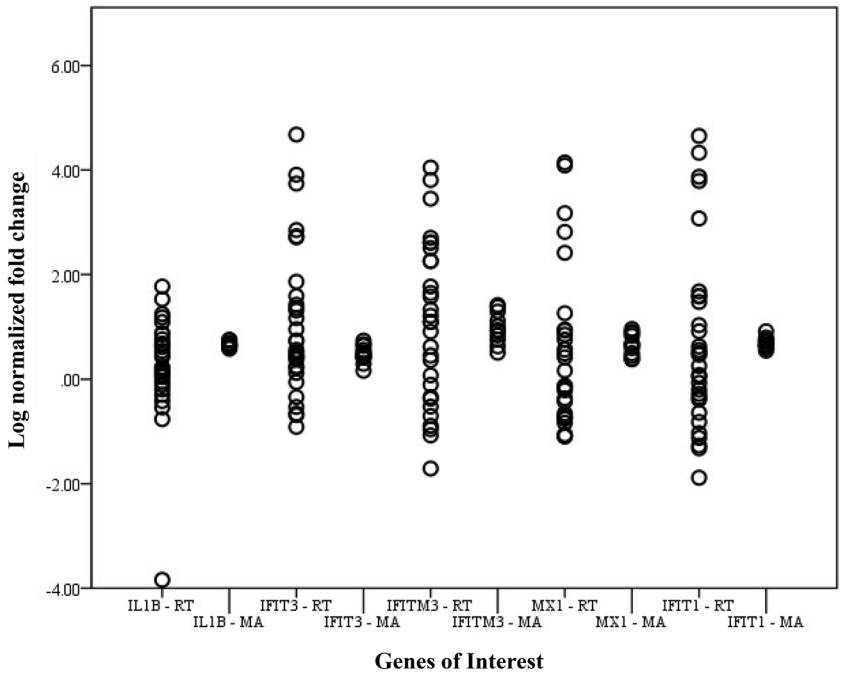

Fig. 5 Log-normalized fold changes in expression of selected genes using real-time PCR (RT) in a validation cohort compared to microarray (MA) in the discovery cohort

which further activate the JAK/STAT pathway inducing the transcription of ISGs [35].

Infections are known to contribute to the development and progression of CKD and complicate the course of patients with pre-existing CKD [2]. Histopathological findings of kidney biopsies of CKDu patients in Sri Lanka also showed tubulointerstitial disease [20, 31] which is characteristic of pathogenic infections [39]. A recent study showed higher than expected seropositivity to Hantavirus with 54.5% of CKDu and 13.5% of healthy individuals from Girandurukotte being seropositive [16] supporting the possibility of viral infections in these patients.

The EIF2 signaling pathway showed the most significant negative activation score of all the pathways. Both bacterial and viral infections generally increase the host response to the infection by up regulating of the EIF2 mediated translational control, reducing general protein synthesis and consequently viral replication, increasing cytokine expression and opposing bacterial infection [40]. Certain bacteria and viruses, however, have evolved to inhibit the EIF2 signaling pathway. The virulence factor YopJ of *Yersinia* inhibits EIF2 mediated functions [40], and several viruses have independently evolved to affect the initiation step of protein synthesis by hijacking and modifying the EIFs to ensure efficient translation of viral mRNAs [41].

The other notable pathways include the phosphatidylinositol-3′-kinase-Akt (PI3K-Akt) and mammalian target of rapamycin (mTOR) pathways which showed negative activation *z* scores in the CKDu patients. Fluoride toxicity was seen to inhibit the phosphorylation of mTOR, thereby decreasing PI3 and Akt mRNA expression in mice Leydig cells [42], whereas arsenic and cadmium were seen to activate the PI3K-Akt-mTOR pathway [43]. The PI3K-Akt-mTOR pathway has also been implicated in viral infections [44, 45], where this pathway is inhibited by the activation of cellular stress responses during viral infection [45]. Mammalian DNA viruses have evolved mechanisms to activate this pathway [45] whereas it was seen inhibited by RNA virus [44]. As this pathway showed down regulation in our study, it could be postulated that the possible viral infection is more likely a RNA virus than a DNA virus, and the influencing environmental factor is more likely to be fluoride than cadmium.

The up regulation of both the type I and type II interferon responses in our study indicates that there is possibly more than one trigger to the immune response. Although all types of interferons are important in fighting infections, the type I interferons; IFN α and IFN β are the main subtypes of interest with an immunological perspective and are produced in response to viral infection [38]. The suppressors of cytokine signaling (SOCS) are induced by prolonged type I interferon and act in a negative feedback loop [38] and this was not observed in our study thereby suggesting a possible acute response. It is also notable that of the genes differentially expressed in the Girandurukotte healthy, DEFA1 belongs to the class of defensins, which are produced by neutrophils, the cells of the immune system that are first to travel to the sites of bacterial, fungal, and viral infections [46].

Interferons are also activated by environmental toxins. Cadmium was seen to enhance interferon gamma in newborn cord blood leukocytes [47], and fluoride ions were seen to have the ability to influence interferon

gamma release thereby influencing the immune system [48]. Fluoride has the ability to augment the human lymphocyte reactivity to a specific antigen as shown by the increased levels of interferon gamma (IFNγ) and sIL-2R [48]. Sodium fluoride was also seen to induce the IFNγ by itself at levels ten times higher than normal plasma fluoride level [38].

While studying the other pathways affected by fluoride, the activation of G-protein alpha subunit signaling, Cdc42 (cell-division cycle 42) signaling, Rac signaling and RhoA signaling in our study further strengthens the possible role of fluoride in CKDu. Epithelial cells treated with sodium fluoride were shown to have these pathways activated as a response [49]. Fluoride toxicity in the form of aluminum fluoride mainly induces its toxic effects by affecting the G-protein functions by replacing the gamma phosphate of GTP [49, 50]. The G-protein signaling showed positive activation z scores in our study with G-protein alpha-s, beta-gamma, alpha-i and alpha-q subunits showing activated z scores of 2.53, 1.63, 0.816, and 0.577, respectively.

The toxic effects of fluoride lead to inflammatory reactions, cell contractile responses, inhibition of protein synthesis and cell cycle progression, oxidative stress and DNA damage which ultimately leads to apoptosis and cell death [51]. Apoptosis is a normal process that takes place in cell proliferation and differentiation, tissue homeostasis, and aging [51]; however, the apoptosis induced by fluoride, includes stimulation of G-protein-dependent signaling systems, oxidative stress, ATP depletion, activation of the cell surface death receptors, disruption of the outer mitochondria membrane, activation of caspases, alteration in the ratio of anti-apoptotic to apoptotic Bcl-2 proteins, upregulation of p53 expression, expression of apoptosis-related genes, ER stress and disturbances in the protein synthesis [51]. The activation of biological processes including G-protein upregulation and p53 signaling, oxidative stress, mitochondrial dysfunction, disturbances in protein synthesis, and apoptosis were seen in the biological and toxicological functions in our CKDu group, which further supports fluoride toxicity.

It is notable that groundwater fluoride is also high in the other regions of the world where CKDu is seen increasingly. Fluoride levels were shown to be high in Latin America [52, 53], and Andhra Pradesh in India [54] and these regions also belong to the tropical zones of the world. It is possible that they share similar molecular pathways underlying the disease.

Gene expression patterns associated to oxidative stress caused by cadmium toxicity were also noticed. The oxidative stress induced by cadmium was seen to increase NF-κB, nuclear factor E2-related factor 2 (Nrf2) and mitogen-activated protein kinases (MAPK) pathways [55]. These were all activated in our study group. However, while comparing cadmium toxicity versus fluoride toxicity, there were a larger number of activated pathways and biological functions supporting fluoride toxicity than cadmium toxicity.

Conclusion

The differentially expressed genes in the CKDu patients and the pathways that they represent provide an insight into the underlying mechanisms of the disease. Activation of the innate immune response and the interferon signaling pathway, and decreased activity of EIF2 and Akt/PI3K/mTOR pathway indicates the presence of a viral infection. The type II IFN response could be due to the same infectious process or due to environmental stimuli such as cadmium or fluoride. Activation of G-protein signaling, Cdc42 signaling and RhoA signaling, and down regulation of the Akt/PI3K/mTOR pathway further support the possibility of fluoride toxicity in the CKDu patients.

Limitations of the study

Biological and technical replication was carried out after pooling only in the CKDu group. In the healthy groups only pooling of six samples per group and technical replication was carried out. While pooling cancels out small differences between individuals of the group and highlights common differences of a group, the individual differences in gene expression are not observable from the data. Validation with real-time PCR, which was carried out with individual samples showed similar mean fold changes, but larger variation between individuals of the validation cohort. Another limitation was that a specific blood cell type was not isolated for extracting RNA and rather whole blood was used. This blood sample would include RNA from leukocytes, premature red blood cells, exosomes, and free RNA in serum. Complete blood counts were not considered for normalizing this variation and instead it was assumed that a difference in a particular cell population in the disease group will be reflected in the overall gene expression and resulting pathways. The results are discussed with respect to literature from particular cell types and conclusions arrived at from an overall perspective.

Acknowledgements The authors thank the staff of the Renal Research Centre, District Hospital, Girandurukotte for their support in sample collection.

Funding The real-time PCR machine used in this study was funded by the National Research Council, Sri Lanka (NRC Grant 11-059).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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