

# The 57 kb deletion in cystinosis patients extends into *TRPV1* causing dysregulation of transcription in peripheral blood mononuclear cells

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## ABSTRACT

**Background** Cystinosis is an autosomal recessive disease characterised by the abnormal accumulation of lysosomal cystine. Mutations in the cystinosis gene (*CTNS*) represent known causes for the disease. The major cystinosis mutation is a 57 kb deletion on human chromosome 17p13 that removes the majority of *CTNS* and the entire adjacent gene, *CARKL/SHPK*.

**Objectives** In order to identify other genes that may influence the cystinosis pathobiological pathway, peripheral blood mononuclear cells (PBMC) were collected from cystinosis family members, and DNA and RNA extracted.

**Results** Using whole genome transcriptional profiling, transient receptor potential vanilloid 1 (*TRPV1*) was found to be differentially expressed in association with cystinosis. This was verified using TaqMan qRT-PCR. There was a 72% reduction in PBMC *TRPV1* mRNA levels in cystinosis individuals homozygous for the 57 kb deletion (n=6) compared to unaffected individuals without the deletion (n=6) (p=0.002). *TRPV1* is a sensory receptor located on chromosome 17p13, adjacent to *CARKL/SHPK*. It was ascertained that the 57 kb deletion extends from exon 10 of *CTNS*, upstream through *CARKL/SHPK*, to intron 2 of *TRPV1*, thus deleting the first two non-coding exons.

**Conclusion** This is the first study to report that the 57 kb deletion extends into the *TRPV1* gene causing dysregulation of transcription in PBMC isolated from cystinosis patients.

## INTRODUCTION

Cystinosis is an autosomal recessive disorder with an estimated incidence of one in 100 000–200 000 live births.<sup>1</sup> It has been classified as a lysosomal storage disorder based on cytologic and biochemical evidence pointing to the intra-lysosomal accumulation of the disulfide amino acid, cystine. While a spectrum of disease phenotypes is observed, affected individuals are generally assigned to one of three clinical forms, based on the age at diagnosis and magnitude of lysosomal cystine deposition. The most common and severe form, infantile nephropathic cystinosis (MIM 219800), generally appears between 6 and 12 months of age with a proximal renal tubulopathy (the renal Fanconi syndrome).<sup>2</sup> Endocrinological, hepatic, gastrointestinal, muscular, and neurological abnormalities have also been documented.<sup>1–3</sup>

The chromosomal region associated with cystinosis was mapped to the short arm of chromosome

17 in 1995.<sup>4</sup> In 1998, positional cloning was used to isolate the *CTNS* gene (HGNC: 2518).<sup>5</sup> *CTNS* encodes the lysosomal cystine transport protein, cystinosin, and mutations in the *CTNS* gene represent the known causes of cystinosis. *CTNS* is composed of 12 exons<sup>5</sup> and at least 90 *CTNS* mutations have been described,<sup>6–8</sup> the most common being a 57 257 bp deletion (57 kb deletion) located on human chromosome 17p13.<sup>9</sup> The deletion breakpoints for the 57 kb deletion were characterised<sup>9</sup> and were found to extend from within exon 10 of *CTNS* and upstream through the adjacent gene, *CARKL/SHPK*.

While the *CTNS* gene was identified by a classical genetic approach, there has been limited scientific investigation of other genes that may influence critical parts of the cystinosis pathobiological pathway. Thus, one of the goals in our cystinosis research programme was to identify variation in transcript specific mRNA associated with cystinosis. In this study we used peripheral blood mononuclear cells (PBMC) collected from cystinosis families to identify genes that are differentially expressed in association with cystinosis.

## METHODS

### Sample collection and processing

Blood samples were collected from individuals with cystinosis and their first degree relatives who were attending the 2007 Cystinosis Research Network Family Conference in San Antonio, Texas. All protocols were approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio. After obtaining informed consent, peripheral whole blood was collected by venepuncture into EDTA tubes. Within 4 h of collection, PBMC were isolated by Ficoll gradient centrifugation. DNA and RNA were subsequently extracted from these samples (RNeasy Mini kit, QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). The RNA was DNase treated during the course of the extraction.

### PCR

PCR was used to classify the samples according to the status of the 57 kb deletion.<sup>10</sup> To identify subjects that were either heterozygous or did not carry the deletion, PCR reactions for each subject were also performed containing primers that annealed within the 57 kb deletion region in intron three of *CTNS* (F 5'-AGCCCAATATCTCAGTT

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GCTG-3', R 5'-CAGGATGCTGAAAGTGATGC-3'). Amplicons (5  $\mu$ l) were visualised by agarose gel electrophoresis and ethidium bromide staining.

## Whole genome transcriptional profiling

PBMC RNA was used to generate amplified RNA (aRNA) (Ambion MessageAmp II Amplification Kit, Applied Biosystems, Foster City, California, USA). Total yield was determined spectrophotometrically using the NanoDrop ND-1000. aRNA was hybridised to Illumina Sentrix Human Whole Genome (WG-6) v3 BeadChips (Illumina, San Diego, California, USA). Samples were scanned on the Illumina BeadArray 500GX Reader. Illumina BeadStudio software (version 3.1.7) was used to view control summary reports, perform a standard background normalisation, and determine differential gene expression. A t test statistic was used to determine p values.

## Gene expression quantitation by real-time reverse transcription PCR

Reverse transcription and PCR amplification (RT-PCR) were performed using the TaqMan Gene Expression Assay system (Applied Biosystems). Total RNA (150 ng) extracted from PBMC was used to generate cDNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor. Each reaction was performed in duplicate and was accompanied by a negative control in which the reverse transcriptase enzyme was replaced by nuclease-free water.

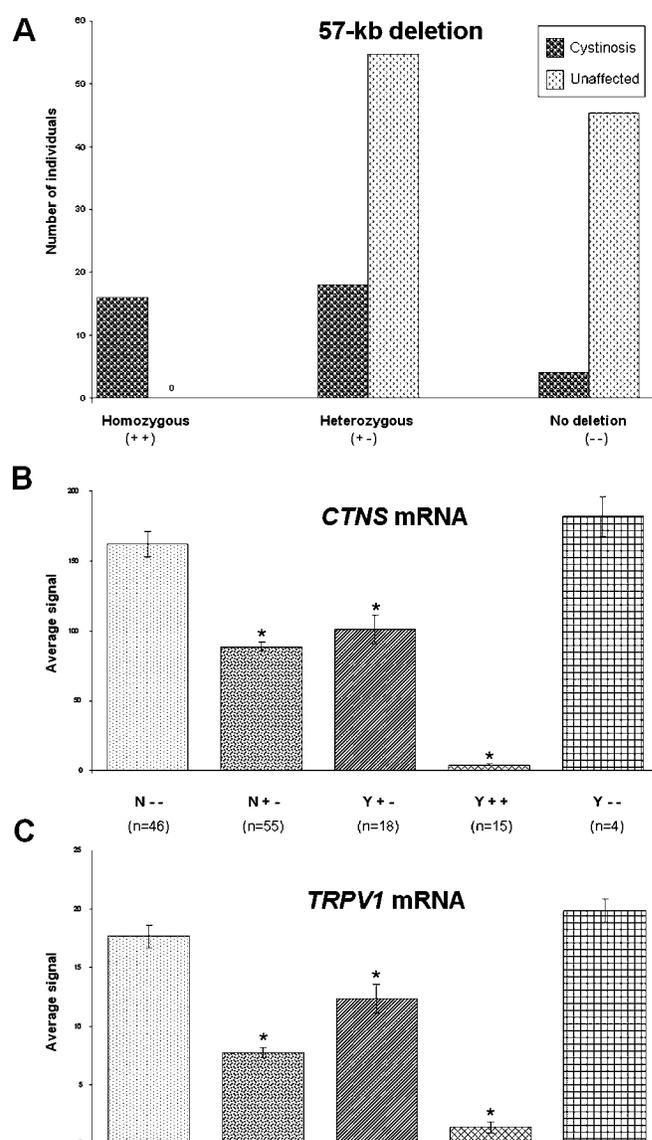
A pre-optimised TaqMan Gene Expression Assay for transient receptor potential vanilloid 1 (*TRPV1*) (Hs00218912\_m1, RefSeq NM\_080704.3, exon boundary 9–10, Assay Location 1661) was used to measure mRNA expression levels relative to the endogenous reference gene, *GAPDH* (4333764E, RefSeq NM\_002046.3, exon 3). The *TRPV1* TaqMan probe spans an exon junction and detects all known human *TRPV1* RefSeq transcripts. PCR was performed using a Prism 7900 Fast Real Time HT PCR instrument.

Comparative threshold cycle ( $C_T$ ) quantification was used to calculate differential mRNA.  $C_T$  values were initially standardised by reference to the housekeeping gene, *GAPDH*. The  $\Delta C_T$  values were computed as the difference between the mean value for *TRPV1* and the mean of the  $C_T$  values of *GAPDH*. Fold change values were calculated, based on the differences in  $\Delta C_T$  values between individuals with cystinosis that were homozygous for the 57 kb deletion ( $n=6$ ) and from unaffected individuals who do not carry the deletion ( $n=6$ ) ( $2^{-\Delta\Delta C_T}$ ). The non-parametric Wilcoxon rank sum test (Mann–Whitney U test) evaluated the difference between the  $2^{-\Delta\Delta C_T}$  values.

## RESULTS

Initially PCR was used to classify the samples according to the status of the 57 kb deletion on chromosome 17p13. The distribution of the 57 kb deletion in the cystinosis families is presented in figure 1A. As expected, all of the individuals who are homozygous for the 57 kb deletion have cystinosis. In accordance with previously published data of cystinosis patients in the USA,<sup>8</sup> approximately half of the individuals with cystinosis are homozygous for the 57 kb deletion.

We then performed whole genome transcriptional profiling to characterise the gene expression profiles of PBMC collected from the cystinosis family members. In the first instance it was important to validate our gene expression data. This was done by examining the expression profiles for *CTNS* and the adjacent genes on chromosome 17p13, *TAX1BP3* (HGNC: 30684), *SHPK*



**Figure 1** DNA and RNA profiles of cystinosis family members. (A) 57 kb deletion status. DNA was extracted from peripheral blood mononuclear cells (PBMC) collected from cystinosis families and the 57 kb deletion status (homozygous, heterozygous or no deletion) of each individual was determined by agarose gel electrophoresis. Represents patients who have cystinosis, while represents unaffected individuals. Expression levels of *CTNS* (B) and *TRPV1* (C) in PBMC RNA collected from cystinosis families was determined by whole genome transcriptional profiling. The subjects were classified according to clinical outcome (N=not affected, Y=cystinosis patient) and the status of the 57-kb deletion. (N-- ) Subjects who are unaffected without the 57 kb deletion, (N+- ) subjects who are unaffected who are heterozygous for the 57 kb deletion, (Y+- ) patients who have cystinosis who are heterozygous for the 57 kb deletion, (Y++) patients who have cystinosis who are homozygous for the 57 kb deletion, and (Y-- ) patients who have cystinosis without the 57 kb deletion. The number of individuals in each group is indicated in parentheses. Data represent mean  $\pm$  SEM. \*Represents significant difference from N-- ,  $p < 0.05$ .

(HGNC: 1492) (originally called *CARL*), and *TRPV1* (HGNC: 12716). There was no differential expression of the *TAX1BP3* transcript in association with cystinosis (data not shown). However, as expected, the level of the *CTNS* transcript in individuals homozygous for the 57 kb deletion was negligible

compared to individuals without the deletion (figure 1B). As predicted, those individuals who are heterozygous for the 57 kb deletion have approximately half of the level of *CTNS* mRNA compared to individuals without the deletion, irrespective of clinical outcome. This expression profile was duplicated for the *SHPK* transcript (data not shown). Given that the 57 kb deletion covers the entire *SHPK* gene and the majority of the *CTNS* gene,<sup>9</sup> these data are consistent with the 57 kb deletion knocking out both *CTNS* and *SHPK*. However, somewhat surprisingly, *TRPV1* showed a similar expression profile (figure 1C) to both *CTNS* and *SHPK*.

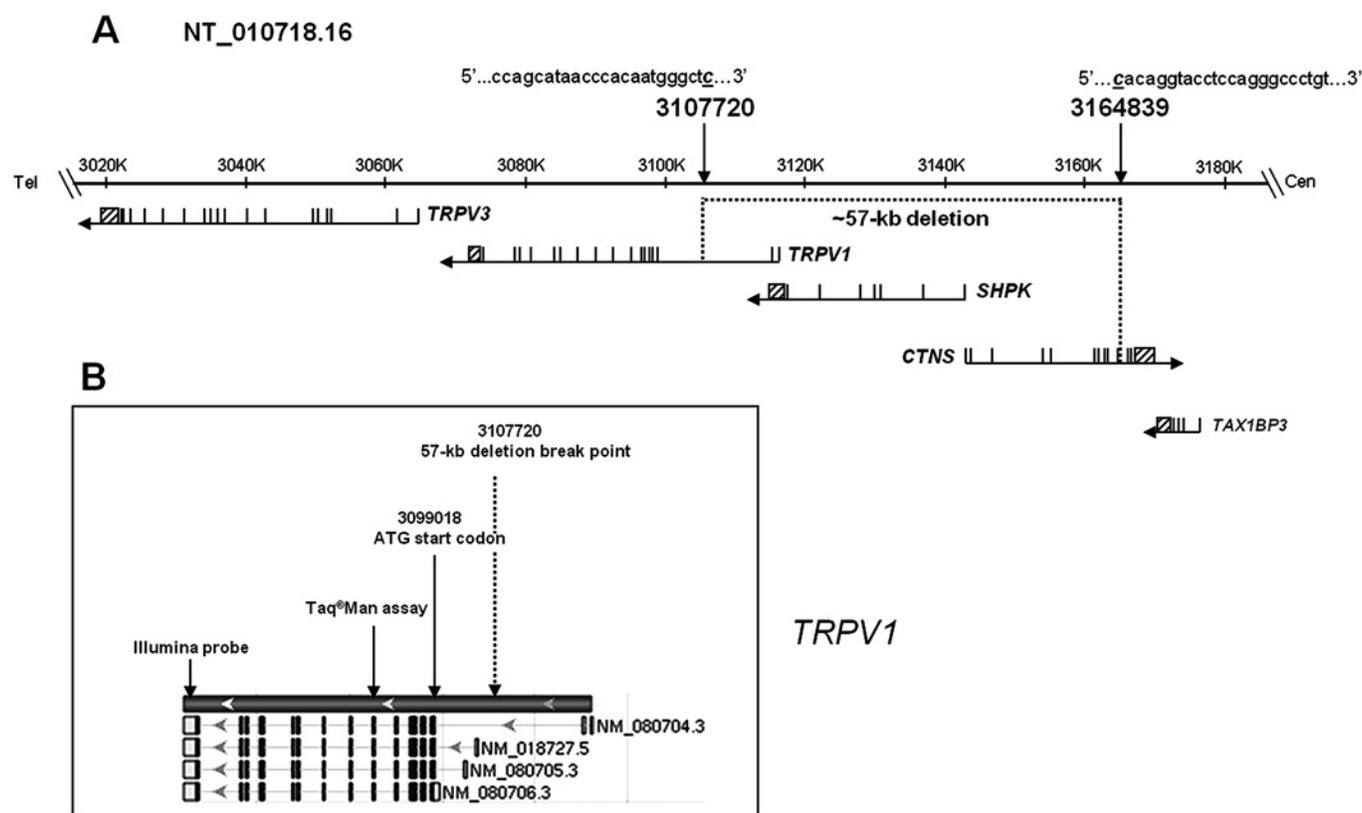
This result prompted a closer examination of the *Homo sapiens* chromosome 17p13 genomic region that harbours the 57 kb deletion (figure 2). The NCBI genomic contig (NT\_010718.16) was examined using NCBI Sequence Viewer of Entrez Gene (<http://www.ncbi.nlm.nih.gov/gene/>). It was ascertained that the deletion extends from within exon 10 of *CTNS*, upstream through *SHPK* to within 9 kb from the start of translation of the largest *TRPV1* transcript (NM\_080704.3), deleting the first two non-coding exons along with putative regulatory elements (figure 2).

Although Touchman *et al*<sup>9</sup> detected *TRPV1* (originally named human ortholog of rat vanilloid receptor subtype 1) in the 17p13 genomic region which harbours the 57 kb deletion in 2000, no further investigation of *TRPV1* in relation to cystinosis has been pursued. Therefore, we now report that the 57 kb deletion

extends into *TRPV1* and, for the first time, show that the expression of the *TRPV1* gene is knocked down in PBMC in those individuals who are homozygous for the 57 kb deletion.

This has potentially widespread implications as TRPV1 is involved in the transmission and modulation of pain and is activated by a wide variety of exogenous and endogenous physical and chemical stimuli.<sup>11</sup> The TRPV1 channel belongs to the TRP family of cation channels which are involved in transmitting a broad range of environmental information to the cell interior, generally via calcium entry. The receptor is activated by vanilloids, such as capsaicin, moderate heat ( $\geq 43^{\circ}\text{C}$ ) and low pH ( $\text{pH} \leq 5.9$ ).<sup>12</sup>

Given the diverse and extensive role of TRPV1 in normal and pathological states and its potential contribution to cystinosis, we went on to validate the *TRPV1* gene expression data generated from the microarray experiment with quantitative real time RT-PCR. In this first study, we focused on using patients with severe cystinosis—that is, those individuals homozygous for the 57 kb deletion—as a confirmation of the validity of the microarray data. From the microarray data, we observed a statistically significant decrease ( $p < 0.05$ ) in *TRPV1* transcript levels in the patients with severe cystinosis compared to unaffected subjects without the deletion. The TaqMan qRT-PCR data confirmed the differential expression. There is a 7.2-fold change (72% reduction,  $p = 0.002$ ) in *TRPV1* expression levels in PBMC derived from individuals who are homozygous



**Figure 2** Genomic features of the region on human chromosome 17p13 that harbours the 57 kb deletion. (A) Genomic organisation of *TRPV3*, *TRPV1*, *SHPK*, *CTNS* and *TAX1BP3* on NCBI contig NT\_010718.16 on chromosome 17p13. The positions of the introns and exons are depicted schematically while the arrows on the genes indicate the direction of transcription. The 57 kb deletion extends from contig position 3164839 in exon 10 of *CTNS* to contig position 3107720 in intron two of *TRPV1* (transcript NM\_080704.3). The sequences flanking the deletion breakpoints are shown. The 17p telomere (Tel) is leftward and the 17p centromere (Cen) rightward. (B) Schematic representation of the known RefSeq human *TRPV1* transcripts. The positions of the 57 kb breakpoint (NT\_010718.16 contig position 3107720), ATG start codon (NT\_010718.16 contig position 3099018), *TRPV1* TaqMan assay and *TRPV1* Illumina probes are indicated by arrows. The 57 kb deletion extends into intron 2 of the largest *TRPV1* transcript (RefSeq NM\_080704.3) and is located 8702 bp upstream from the start of translation. Both the TaqMan and Illumina systems designed to quantitate *TRPV1* mRNA levels will detect all known human RefSeq *TRPV1* transcripts.

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for the 57 kb deletion (n=6) compared to unaffected individuals without the deletion (n=6).

## DISCUSSION

We report for the first time that the 57 kb deletion on chromosome 17p13 extends into the *TRPV1* gene, causing dysregulation of transcription in PBMC isolated from cystinosis patients homozygous for the 57 kb deletion. The consequence of *CTNS* gene knockout has been extensively documented for cystinosis patients and there has been limited investigation into the knockout of *SHPK*,<sup>13</sup> the gene immediately upstream of *CTNS*.

However, there has been no consideration given to altered expression of TRPV1 in cystinosis patients. There are a range of potential implications, given that TRPV1 plays a regulatory role in a number of physiological processes<sup>11</sup> including thermoregulation. The physiological consequences of reduced TRPV1 have been studied extensively using the *TRPV1* knockout mouse model. Mice genetically modified to lack the *TRPV1* receptor were impaired in the detection of painful heat and demonstrated changes in behaviour and taste responses.<sup>12 14 15</sup> Disturbances in thermoregulation,<sup>3</sup> cognitive functions<sup>16</sup> and taste perception have also been reported in cystinosis patients.

In relation to food preference, cystinosis patients have reported craving for salty<sup>1</sup> and spicy food.<sup>17</sup> It is possible that cystinosis individuals who have reduced levels of TRPV1 may display reduced sensitivity to capsaicin (N-vanillyl-8-methyl-6-(E)-nonamide), the pungent compound of hot chilli peppers. There is one report<sup>18</sup> of a woman who showed total insensitivity to hot peppers. RNA and protein levels of TRPV1 in her buccal mucosa were less than half of those in a normal subject. Interestingly, sequence analysis identified no single nucleotide polymorphisms (SNPs) in the *TRPV1* cDNA but seven genomic SNPs were identified within intron two (NCBI contig NT 010718.16, NM\_080704.3). Closer examination of two of these SNPs reveals that rs161375 is within the 57 kb deletion while rs161380 is located 20 bp from the 57 kb deletion breakpoint.

There has been limited investigation into the 5' transcriptional regulatory region of *TRPV1* in humans. Xue *et al*<sup>19</sup> demonstrated that transcription of rat TRPV1 utilises a dual promoter system. However, given that the 57 kb deletion breakpoint is around 9 kb upstream of the start of translation of human *TRPV1*, it is more likely that there are enhancer and/or repressor elements within this region that are influencing the expression levels of *TRPV1* in PBMC. Enhancers can reside several hundred kilobase pairs upstream of a promoter and are well known for their role in activating the transcription of target genes in a time and tissue dependent manner.<sup>20</sup>

Despite the considerable amount of data generated from the *TRPV1* knockout mouse, there is a paucity of information in humans on the consequence of a genetic defect leading to the substantial reduction in the expression of *TRPV1*. In our efforts to utilise peripheral blood to identify disease biomarkers, we have shown that *TRPV1* is knocked down in cystinosis patients homozygous for the 57 kb deletion. Further research is required to ascertain the functional consequences for those individuals and whether this dysregulation extends to other cell types. Therefore, these data now present an important opportunity to extend our basic understanding of the pathophysiology of cystinosis.

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**Competing interests** None.

**Ethics approval** This study was conducted with the approval of the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

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