Mutation screening of *ACKR3* and *COPS8* in kidney cancer cases from the CONFIRM study

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Abstract

An apparently balanced t(2;3)(q37.3;q13.2) translocation that appears to segregate with renal cell carcinoma (RCC) has indicated potential areas to search for the elusive genetic basis of clear cell RCC. We applied Hi-Plex targeted sequencing to analyse germline DNA from 479 individuals affected with clear cell RCC for this breakpoint translocation and genetic variants in neighbouring genes on chromosome 2, *ACKR3* and *COPS8*. While only synonymous variants were found in *COPS8*, one of the missense variants in *ACKR3*:c.892C>T, observed in 4/479 individuals screened (0.8%), was predicted likely to damage *ACKR3* function.

Identification of causal genes for RCC has potential clinical utility, where risk assessment and risk management can offer better outcomes, with surveillance for at-risk relatives and nephron sparing surgery through earlier intervention.

Keywords (4-6)

Kidney cancer; *ACKR3*; *COPS8*; mutation screening; massively parallel sequencing; Hi-Plex

Introduction

An apparently balanced t(2;3)(q37.3;q13.2) translocation appears to segregate with renal cell carcinoma (RCC) in a previously reported family [1]. The breakpoint was identified incidentally by virtue of being in the regions of highest genetic linkage during a genome scan for vesicoureteric reflux [2]. The breakpoint on chromosome 2 is between Atypical Chemokine Receptor 3 (*ACKR3*), previously known as CXC Chemokine Receptor 7 (*CXCR7*) and COP9 Signalosome Subunit 8 (*COPS8*). *ACKR3/CXCR7* is known to be involved in kidney development and is overexpressed in ~50% of RCC [3,4]. Expression measured by immunohistochemistry in the tumours of the family members carrying the translocation has been found not to be consistent (unpublished data). The breakpoint on chromosome 3 bisects *CD96*, which has been implicated in C (Opitz trigonocephaly)

syndrome [5] but not in RCC. On the basis of these findings, it is plausible that genes neighbouring the t(2;3) translocation may have a role in the genesis of RCC. We, therefore, examined *ACKR3* and *COPS8* and tested for t(2;3)(q37.1;q13.2) in the germline DNA obtained from participants from the Consortium for the Investigation of Renal Malignancies (CONFIRM).

Materials and Methods

Specimens

CONFIRM utilizes a case-control design to investigate the epidemiological and genetic risk factors for renal cell carcinoma. Cases were recruited via the Victorian and Queensland Cancer Registries and familial cancer centers across Australia. Eligible participants are those diagnosed at <75 years of age. Participation involves completing epidemiological questionnaires about lifestyle, medical history, diet and family history of cancer and providing a blood sample.

DNA from 479 participants with a renal malignancy were extracted using the Roche MagNA Pure 96 Blood protocol system and quantitated using the Qubit dsDNA Assay system (ThermoScientific, Waltham, MA, USA), according to the manufacturer's instructions.

Ethics approval was granted by the Human Research Ethics Committee of the Cancer Council of Victoria. Informed consent was obtained from all participants included in the study.

Hi-Plex mutation screening

28 gene-specific primer pairs (GSP) were designed to target protein-coding region co-ordinates for *ACKR3* (NM_020311) and *COPS8* (NM_006710). One additional primer pair (5' to 3' DNA sequences CCTCTGAATGAGAGATCCTC and GACTAATATTCAGTAAGACCAGA, respectively) spans the chr2:237,594,844-237,594,846 and chr3:111,305,167-111,305,169 (hg19) breakpoint genomic rearrangement. These were designed so that the breakpoint was significantly offset from the centre of the amplicon to facilitate subsequent mapping to the hg19 human

reference genome-build. The sequences of other GSPs are available on request. Their general structures were similar to those described in earlier reports [6]. Since the present work, we have developed an improved primer design algorithm that minimises primer-dimer off-target effects, yields more uniform coverage across amplicons and allows more stringent reaction conditions for improved assay performance [7]. The non-GSP library insert regions were designed in a narrow size range around 100 bp to facilitate complete overlap of read-pairs-based analyses [8,9]. All primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA) and purified to standard desalting grade. 'F8_bridge' and 'R5_bridge' abridged adapter universal primers have 5' to 3'-oriented DNA sequences of CTCTCTATGGGCAGTC and CTGCGTGTCTCCGAC, respectively [10]. Full-length, dual-indexing adapter primer sequences have been reported previously in [11]. We used additional Illumina Nextera indices for dual indexing to enable 192 pooled specimen libraries to be analysed on a single sequencing run.

Individual Hi-Plex PCR reactions were conducted in wells of a skirted PCR plate, each in a final volume of 25µl with 1xPhusion® HF PCR buffer (ThermoScientific), 1 unit of Phusion Hot Start II High-Fidelity DNA Polymerase (ThermoScientific), 400µM dNTPs (Bioline, London, UK), 0.125M gene-specific primer pool aggregate (individual gene-specific primer concentrations vary and are described in [11]), 1µM 'Bridge' primers [12], 2.5mM MgCl₂ (ThermoScientific) and 25 ng input genomic DNA. The following steps were then applied: 98°C for 1 min, 25 cycles of [98°C for 30 sec, 50°C for 1 min, 55°C for 1 min, 60°C for 1 min, 65°C for 1 min, 70°C for 1 min], addition of 1 µM dual-indexed hybrid adapter primers, then a further three cycles of [98°C for 30 sec, 68°C for 1 min, 70°C for 1 min], followed by incubation at 68°C for 20 min. Pooled library size-selection, quantification and sequencing were performed as detailed in [12]. Five µl of each reaction were pooled before subjecting the resulting barcoded library (including the 96 sub-libraries) to electrophoresis on a 1.5% HR-agarose gel (ThermoScientific). Size selection, gel extraction and purification were performed as described previously [12].

The library was then sequenced on a MiSeq instrument, using the 300bp MiSeq Reagent kit v2 (Illumina). Prior to performing the run, 3.4 µL of 100 µM sequencing primers were added to the respective read1, read2 and i7 primer reservoirs in the reagent cartridge as described in [11]. Sequencing data were mapped to the entire human genome (hg19) using bowtie2-2.1.0 [13] applying default parameters except for --trim5 20 --trim3 20. Bedtools v2.16.1 [14] was used to compute on-target coverage. ROVER variant caller [8] was applied using a variant proportion threshold of 0.25 and minimum required variant depth of 10 read-pairs. We also filtered mapping artefacts from the data according to methods described in [15]. Genetic variants were annotated using the web version of ANNOVAR (accessed on 29th November 2016) [16]. Predictions were obtained from the following programs, using their default settings: SIFT [17], PolyPhen-2 [18], Mutation Taster [19] and CADD [20]. For breakpoint analysis, the parameters --trim5 0 --trim3 60 were applied with bowtie2-2.1.0. An in-house script was used to count reads mapping to the chromosome 2 region of the breakpoint rearrangement.

The threshold for successful sequencing was set to a minimum 26/28 amplicons covered by ≥ 10 read pairs. Variant confirmation was performed by Sanger Sequencing using BigDye Terminator v3.1 (Life Technologies), according to the manufacturer's instructions.

Results

We screened the coding region and proximal intron-exon junctions of *ACKR3* and *COPS8* in 479 subjects by targeted-MPS, using Hi-Plex. The target regions consisted of a total of 28 Hi-Plex amplicons. Successful sequencing was achieved for 472/479 specimens (98.5%). The average on target rate was 98.98% across the 28 amplicons. The median coverage was 1258 reads.

The mutation screening identified 12 genetic variants in *ACKR3* consisting of five missense variants (*ACKR3*:c.1007C>T, p.(A336V), rs143408017; *ACKR3*:c.124G>A; p.(V42I),

rs146407095; *ACKR3*:c.236C>T, p.(T79M), rs200095631; *ACKR3*:c.752G>A, p.(R251Q) and *ACKR3*:c.892C>T, p.(H298Y), rs150632398) and seven synonymous variants (*ACKR3*:c.138G>A, p.(T46T); *ACKR3*:c.189C>T, p.(S63S), rs35767135; *ACKR3*:c.603C>T, p.(P201P), rs144225712; *ACKR3*:c.630C>T, p.(I210I), rs146961395; *ACKR3*:c.72C>T, p.(S24S), rs1153382174; *ACKR3*:c.796C>T, p.(L266L), rs1045879 and *ACKR3*:c.93G>A, p.(T31T), rs147894231) (Table 1).

Of the five missense variants, *ACKR3*:c.892C>T, was predicted to be probably damaging to protein function by PolyPhen-2, Mutation Taster and CADD. SIFT did not provide a prediction because of low sequence diversity in the protein multiple sequence alignment at that position. This variant was observed in 4/479 individuals screened (0.83%) and is present in the Exome Variant Server with a carrier frequency of 0.47% in the non-Finnish European population [21]. In these four affected participants, age at diagnosis ranged from 40 to 57 years and two came from families with multiple cancers. More information on these participants is presented in Table 2. There was no other missense variant for which the prediction tools were in agreement. *ACKR3*:c.1007C>T was not annotated by SIFT for the same reasons as described above and Mutation Taster predicted this variant as “probably deleterious”. However, it was not ranked as high as *ACKR3*:c.892C>T by PolyPhen-2 (“possibly damaging”, whereas *ACKR3*:c.892C>T was classified as “probably damaging”- the most deleterious class for PolyPhen-2). CADD phred-scaled score for *ACKR3*:c.1007C>T is below 20, which means this variant is not predicted to be amongst the top 1% of deleterious variants in the human genome (the CADD score for *ACKR3*:c.892C>T is 23.3). Screening of *COPS8* identified three genetic variants, all of which are synonymous variants: *COPS8*:c.378T>C, p.(T126T), rs147957647; *COPS8*:c.387C>T, p.(I129I) and *COPS8*:c.514C>T, p.(L172L), rs34344319 (Table 3).

The Hi-Plex assay also included primers designed to detect a breakpoint genomic rearrangement. The assay detected the breakpoint mutation in two known carriers used as positive controls in the assay. However no new carriers of this rearrangement were identified in this screening.

Discussion

Approximately 3- 5% of renal cancer is familial [22]. RCC with specific histologies can be attributed to genetic syndrome, such as the chromophobe RCC often observed with pneumothorax and skin lesions and associated with mutations in *FLCN* and Birt-Hogg-Dube Syndrome [23] and the papillary type 2 RCC associated with cutaneous leiomyoma in Hereditary Leiomyomatosis Renal Cell Carcinoma Syndrome caused by mutations in *FH* [24]. While the genetic basis of syndromic renal cancer is well known, susceptibility genes for clear cell RCC are yet to be described. In von Hippel-Lindau Syndrome, clear cell RCC unusually occurs alone; heterozygous mutations in the *VHL* gene cause a highly penetrant clinical constellation, including haemangioblastoma, pheochromocytoma and clear cell RCC [25].

It is plausible that the breakpoints of the previously reported apparently balanced t(2;3)(q37.3;q13.2) translocation incorporate a potential clear cell RCC susceptibility gene, especially since there were three individuals affected with RCC in the family who also carried this translocation.

ACKR3 acts as a receptor for the inflammatory chemokine CXCL11 and for the constitutive chemokine CXCL12, which is involved in tumor cell survival, migration, and tumour development. Activation of ACKR3 leads to arrestin recruitment as a signalling response. Studies of mouse models have shown that ACKR3 facilitates angiogenesis and that tumour growth is inhibited when ACKR3 is blocked [26]. Thus, it has been suggested to participate in tumour angiogenesis and Maishi *et al.* have reported that tumour blood vessels highly express ACKR3 in RCC [4]. Five missense variants were identified in *ACKR3* of which one, *ACKR3*:c.892C>T, was predicted probably damaging to protein function by three of four *in-silico* prediction tools. This missense

substitution falls into the highly conserved 7 transmembrane receptor domain (7tm_1). Missense variants are challenging to interpret and further studies will be required to investigate the functional impact of *ACKR3*:c.892C>T on protein function if future large scale screening studies confirm the association of *ACKR3* in predisposition to RCC. A possible mechanism by which missense variants in *ACKR3* might affect protein function is by introducing an amino acid substitution that would increase the stability of AKCR3 and thus the relative cellular abundance. As *ACKR3* is a receptor, a missense substitution such as *ACKR3*:c.892C>T might render the protein constitutively active, *i.e.* active without the requirement of binding from a ligand. The possible consequences of such a gain-of-function mutation would be consistent with previously reported overexpression of *ACKR3* in RCC cases [3,4].

Screening of *COPS8* identified three genetic variants, all of which were synonymous providing no evidence *COPS8* playing a role in RCC predisposition.

The Hi-Plex assay also included primers designed to detect a breakpoint genomic rearrangement. We were able to detect the breakpoint mutation in two known carriers used as positive controls in the assay, showing this to be an effective methodology.

Identification of susceptibility genes for RCC has potential clinical utility, as improved risk assessment and risk management can offer better outcomes, particularly with earlier nephron sparing surgery. Studies to elucidate such genes are continuing.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 Genetic variants identified during mutation screening of *ACKR3* in 479 subjects participating in the Consortium for the Investigation of Renal Malignancies (CONFIRM) study.

	Nucleotide change ^a	Protein change	rs number ^b	PolyPhen-2 ^c	SIFT ^d	Mutation Taster ^e	CADD ^f
Missense variants							
	<i>ACKR3</i> :c.124G>A	p.(V42I)	rs146407095	B	T	N	6.737
	<i>ACKR3</i> :c.236C>T	p.(T79M)	rs200095631	B	D	D	14.4
	<i>ACKR3</i> :c.752G>A	p.(R251Q)	-	B	NA	D	13.52
	<i>ACKR3</i> :c.892C>T	p.(H298Y)	rs150632398	D	NA	D	23.3
	<i>ACKR3</i> :c.1007C>T	p.(A336V)	rs143408017	P	NA	D	19.23
Synonymous variants							
	<i>ACKR3</i> :c.72C>T	p.(S24S)	rs1153382174	-	-	-	
	<i>ACKR3</i> :c.93G>A	p.(T31T)	rs147894231	-	-	-	
	<i>ACKR3</i> :c.138G>A	p.(T46T)	-	-	-	-	
	<i>ACKR3</i> :c.189C>T	p.(S63S)	rs35767135	-	-	-	
	<i>ACKR3</i> :c.603C>T	p.(P201P)	rs144225712	-	-	-	
	<i>ACKR3</i> :c.630C>T	p.(I210I)	rs146961395	-	-	-	
	<i>ACKR3</i> :c.796C>T	p.(L266L)	rs1045879	-	-	-	

^a Number based on transcript sequence NM_020311, +1 as A of ATG start codon. ^b rs number from dbSNP v.137

^c PolyPhen2 prediction (D: probably damaging, P: possibly damaging, B: benign)

^d SIFT prediction (D: deleterious, T: tolerated, NA: not available due to low sequence diversity in the protein multiple sequence alignment)

^e Mutation Taster prediction (D: probably deleterious, N: probably harmless)

^f CADD phred-scaled score

^g COSMIC database version 70

Table 2 Details of gender, age at diagnosis, type of renal cell carcinoma (RCC) and family history from the four carriers of *ACKR3*:c.892C>T identified in the Consortium for the Investigation of Renal Malignancies (CONFIRM) study.

	Gender	Age at diagnosis	Type of RCC	Family history
Carrier 1	Female	51	Clear Cell, 120mm, 3A, secondaries in lung and liver	Brother: mantle cell lymphoma Paternal grandmother, aunt, cousin: breast

Carrier 2	Male	40	Clear cell, 20mm, IA	Sister: melanoma (aged 17) Paternal grandfather: lung cancer Maternal grandfather: bowel cancer Paternal aunt – pancreatic cancer
Carrier 3	Male	57	Clear cell, 42mm, IB	no family history listed
Carrier 4	Male	43	Clear cell, 20mm, 2	Maternal uncle: unknown cancer type

Table 3 Genetic variants identified during mutation screening *COPS8* in 479 subjects participating in the Consortium for the Investigation of Renal Malignancies (CONFIRM) study.

	Nucleotide change ^a	Protein change	rs number ^b	COSMIC ^c	# Carriers (frequency)
Synonymous variants	<i>COPS8</i> :c.378T>C	p.(T126T)	rs147957647	-	1 (0.2%)
	<i>COPS8</i> :c.387C>T	p.(I129I)	rs367631675	-	1 (0.2%)
	<i>COPS8</i> :c.514C>T	p.(L172L)	rs34344319	-	3 (0.6%)

^a Number based on transcript sequence NM_006710, +1 as A of ATG start codon

^b rs number from dbSNP v.137

^c COSMIC database version 70