Title: Sex Specific Associations in Genome Wide Association Analysis of Renal Cell Carcinoma

Authors: Ruhina S Laskar[1], David C Muller[2], Peng Li[3], Mitchell J Machiela4, Yuanqing Ye[5], Valerie Gaborieau[1], Matthieu Foll[1], Jonathan N Hofmann[4], Leandro Colli[4], Joshua N Sampson[4], Zhaoming Wang[6], Delphine Bacq-Daian[7], Anne Boland[7], Behnoush Abedi-Ardekani[1], Geoffroy Durand[1], Florence Le Calvez-Kelm[1], Nivonirina Robinot[1], Helene Blanche[8], Egor Prokhortchouk[9,10], Konstantin G Skryabin[9,10], Laurie Burdette[4], Meredith Yeager[4], Sanja Radojevic-Skodric[47], Slavisa Savic[48], Lenka Foretova[11], Ivana Holcatova[12], Vladimir Janout[13,46], Dana Mates[14], Stefan Rascu[15], Anush Mukeria[16], David Zaridze[16], Vladimir Bencko[17], Cezary Cybulski[18], Eleonora Fabianova[19], Viorel Jinga[15], Jolanta Lissowska[20], Jan Lubinski[18], Marie Navratilova[11], Peter Rudnai[21], Neonila Szeszenia-Dabrowska[22], Simone Benhamou[23,24], Geraldine Cancel-Tassin[25,45], Olivier Cussenot[25,45], Antonia Trichopoulou[52], Elio Riboli[2], Kim Overvad[53], Borje Ljungberg[26], Raviprakash T Sitaram[26], Graham G Giles[49,50], Roger L Milne[49,50], Gianluca Severi[49,51], Fiona Bruinsma[49], Tony Fletcher[27], Kvetoslava Koppova[19], Susanna C Larsson[28], Alicja Wolk[28], Rosamonde E Banks[29], Peter J Selby[29], Douglas F Easton[30,31], Paul Pharoah[30,31], Gabriella Andreotti[4], Laura E Beane Freeman[4], Stella Koutros[4], Demetrius Albanes[4], Satu Männistö[32], Stephanie Weinstein[4], Peter E Clark[33], Todd L Edwards[33], Loren Lipworth[33], Hallie Carol[34], Matthew L Freedman[34], Mark M Pomerantz[34], Eunyoung Cho[35], Peter Kraft[36], Mark A Preston[37], Kathryn M. Wilson[36], J Michael Gaziano[37], Howard D Sesso[36], Amanda Black[4], Neal D Freedman[4], Wen-Yi Huang[4], John G Anema[38], Richard J Kahnoski[38], Brian R Lane[38,39], Sabrina L Noyes[40], David Petillo[40], Bin Tean Teh[40], Ulrike Peters[41], Emily White[41], Garnet L Anderson[42], Lisa Johnson[42], Juhua Luo[43], Wong-Ho Chow[5], Lee E Moore[4], Toni K Choueiri[34], Christopher Wood[5], Mattias Johansson[1], James D McKay[1], Kevin M Brown[4], Nathaniel Rothman[4], Mark G Lathrop[44], Jean-Francois Deleuze[7,8], Xifeng Wu[5], Paul Brennan[1], Stephen J Chanock[4], Mark P Purdue[4], Ghislaine Scelo[1]

Affiliations:

1. International Agency for Research on Cancer (IARC), Lyon, France
2. Faculty of Medicine, School of Public Health, Imperial College London, London, UK
3. Max Planck Institute for Demographic Research, Rostock, Germany
4. Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department Health and Human Services, Bethesda, Maryland, USA
5. Department of Epidemiology, Division of Cancer Prevention and Population Sciences, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA
6. Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA
7. Centre National de Recherche en Génomique Humaine, Institut de biologie François Jacob, CEA, Evry, France
8. Fondation Jean Dausset-Centre d'Etude du Polymorphisme Humain, Paris, France
9. Center 'Bioengineering' of the Russian Academy of Sciences, Moscow, Russian Federation
10. Kurchatov Scientific Center, Moscow, Russian Federation
11. Department of Cancer Epidemiology and Genetics, Masaryk Memorial Cancer Institute, Brno, Czech Republic
12. 2nd Faculty of Medicine, Institute of Public Health and Preventive Medicine, Charles University, Prague, Czech Republic
13. Department of Preventive Medicine, Faculty of Medicine, Palacky University, Olomouc, Czech Republic
14. National Institute of Public Health, Bucharest, Romania
15. Carol Davila University of Medicine and Pharmacy, Th. Burghele Hospital, Bucharest, Romania
16. Russian N.N. Blokhin Cancer Research Centre, Moscow, Russian Federation

17. First Faculty of Medicine, Institute of Hygiene and Epidemiology, Charles University, Prague, Czech Republic
18. International Hereditary Cancer Center, Department of Genetics and Pathology, Pomeranian Medical University, Szczecin, Poland
19. Regional Authority of Public Health in BanskaBystrica, BanskaBystrica, Slovakia
20. The M Sklodowska-Curie Cancer Center and Institute of Oncology, Warsaw, Poland
21. National Public Health Institute, Budapest, Hungary
22. Department of Epidemiology, Institute of Occupational Medicine, Lodz, Poland
23. INSERM U946, Paris, France
24. CNRS UMR8200, Institute Gustave Roussy, Villejuif, France
25. Sorbonne Université, GRC n°5, ONCOTYPE-URO, AP-HP, Tenon Hospital, Paris, France
26. Department of Surgical and Perioperative Sciences, Urology and Andrology, Umeå University, Umeå, Sweden
27. London School of Hygiene and Tropical Medicine, University of London, London, UK
28. Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden
29. Leeds Institute of Cancer and Pathology, University of Leeds, Cancer Research Building, St James's University Hospital, Leeds, UK
30. Department of Oncology, University of Cambridge, Cambridge, UK
31. Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK
32. National Institute for Health and Welfare, Helsinki, Finland
33. Vanderbilt-Ingram Cancer Center, Nasville, Tennesee, USA
34. Dana-Farber Cancer Institute, Boston, Massachusetts, USA
35. Brown University, Providence, Rhode Island, USA
36. Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA
37. Brigham and Women's Hospital and VA Boston, Boston, Massachusetts, USA
38. Division of Urology, Spectrum Health, Grand Rapids, Michigan, USA
39. College of Human Medicine, Michigan State University, Grand Rapids, Michigan, USA
40. Van Andel Research Institute, Center for Cancer Genomics and Quantitative Biology, Grand Rapids, Michigan, USA
41. Cancer Prevention Program, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA
42. Fred Hutchinson Cancer Research Center, Seattle, Washington, USA
43. Department of Epidemiology and Biostatistics, School of Public Health Indiana University Bloomington, Bloomington, Indiana, USA
44. McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada
45. CeRePP, Paris, France
46. Faculty of Health Sciences, Palacky University, Olomouc, Czech Republic
47. Institute of Pathology, Medical school of Belgrade, Belgrade, Serbia
48. Department of Urology, University Hospital “Dr D. Misovic” Clinical Center, Belgrade, Serbia
49. Cancer Epidemiology & Intelligence Division, Cancer Council of Victoria, 615 St Kilda Road, Melbourne, Victoria, 3004, Australia.
50. Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Parkville Victoria, 3010, Australia.
51. Inserm U1018, Center for Research in Epidemiology and Population Health (CESP), Facultés de Medicine, Université Paris-Saclay, Université Paris-Sud, UVSQ, Gustave Roussy, 114 rue Edouard Vaillant, 94805, Villejuif Cedex, France.
52. Hellenic Health Foundation, Alexandroupoleos 23, Athens 11527, Greece
53. Department of Public Health, Section for Epidemiology, Aarhus University, Aarhus C, Denmark

Key words: Sexual dimorphism, genetic susceptibility, cancer, renal cell carcinoma, GWAS

Corresponding author:
Ghislaine Scelo, PhD
International Agency for Research on Cancer (IARC-WHO)
150 cours Albert Thomas
69372 Lyon cedex 08
France
Tel.: +33-472738173.
Email: scelog@iarc.fr

Word count:
Abstract: 186; Text: 2,845
Number of Figures: 3; Number of Tables: 1

Abstract:

Renal cell carcinoma (RCC) has an undisputed genetic component and a stable 2:1 male to female sex ratio in its incidence across populations, suggesting possible sexual dimorphism in its genetic susceptibility. We conducted the first sex-specific genome-wide association analysis of RCC for men (3,227 cases, 4,916 controls) and women (1,992 cases, 3,095 controls) of European ancestry from two RCC genome-wide scans and replicated the top findings using an additional series of men (2,261 cases, 5,852 controls) and women (1,399 cases, 1,575 controls) from two independent cohorts of European origin. Our study confirmed sex-specific associations for two known RCC risk loci at 14q24.2 (*DPF3*) and 2p21(*EPAS1*) and identified two additional suggestive male-specific loci at 6q24.3 (*SAMD5*, male odds ratio (OR_{male})= 0.83[95% CI=0.78-0.89], $P_{male}=1.71x10^{-8}$ compared with female odds ratio (OR_{female}) = 0.98 [95% CI=0.90-1.07], $P_{female}=0.68$) and 12q23.3 (intergenic, OR_{male}= 0.75[95% CI=0.68-0.83], $P_{male}=1.59\ x10^{-8}$ compared with OR_{female} =0.93[95% CI=0.82-1.06], $P_{female}=0.21$) that attained genome-wide significance in the joint meta-analysis, but did not clearly replicate. Herein, we provide evidence of sex-specific associations in RCC genetic susceptibility and advocate the necessity of studies with greater statistical power to confirm the findings.

Introduction

Kidney cancer is the 12th most common malignancy in the world with estimated 337,860 new cases and 143,406 deaths in 2012 [1]. Renal cell carcinoma (RCC) accounts for approximately 90% of all kidney cancers [2]. The incidence differs significantly by sex, with two-fold higher rates for men than women. The 2:1 sex ratio has been consistent over time, across different age groups and geographical locations; and, hence, cannot be explained by differences in environmental or lifestyle exposures and hormonal factors alone [3]. Although there is recent evidence of sexual dimorphism at the genomic level, sex chromosome differences have gained most attention [4]. The first comprehensive sex-specific somatic alteration analysis of 13 cancer types from The Cancer Genome Atlas (TCGA) revealed extensive sex differences in autosomal gene expression and methylation signatures of kidney cancer, although it did not consider germline variation between sexes [5]. A genetic contribution to RCC susceptibility is well documented. Besides the rare inherited germline mutations implicated in some familial RCCs, e.g., *VHL* (von Hippel-Lindau disease), *MET* (hereditary papillary renal cancer), *FLCN* (Birt-Hogg-Dubé syndrome) and *FH* (hereditary leiomyomatosis and renal cell cancer) genes [6], large genome-wide association studies (GWAS) have identified 13 autosomal RCC susceptibility loci implicating several candidate genes [7-11]. A role for sex in modifying genetic susceptibility to RCC is possible, but, unlike many other sexually dimorphic diseases and traits [12-14], no genome-wide, systematic effort to study possible sex specific genetic contributions to kidney cancer risk has been undertaken.

We conducted a sex-specific genome wide association analysis of kidney GWAS datasets consisting of 13,230 individuals (8193 men, 5087 women) using approximately 6 million genotyped and imputed SNPs in sex-stratified and sex interaction models and replicated the top findings using another 8,113 men and 2,974 women. To explore the possibility of sex-specific gene regulation of the top genotypic variants, we performed an expression quantitative trait loci (eQTL) analysis using paired genotyping and gene expression data from normal and kidney tumour tissues.

Methods

Genetic association analysis

Discovery

The International Agency for Research on Cancer (IARC) kidney cancer GWAS have been previously described [11]. The dataset consisted of two IARC-Centre National de Genotypage (CNG) scans using 11 studies recruited from 18 countries and included a total of 5,219 RCC cases (1,992 women, 3,227 men) and 8,011 controls (3,095 women, 4,916 men) of European descent, the first being genotyped using HumanHap 317k, 550 or 610Q, and the second using Omni5 and OmniExpress arrays. Quality control assessments applied to the data have been previously described [7, 11]. Briefly, we used the following quality control measures at individual levels as exclusion criteria, genotype success rate of <95%, discordant sex, duplication or relatedness based on IBD score >0.185 and samples with < 80% European ancestry. SNP exclusion criteria included call rate <90%, departure from Hardy Weinberg equilibrium in controls at $P<10^{-7}$, and MAF<0.05. Imputation of genotypes was done by minimac version 3 using 1,094 subjects from the 1000 Genomes Project (phase 1 release 3) as

the reference panel and approximately 6 million SNPs were retained for the final analysis after post imputational QC steps (r^2>0.3). Population stratification analysis (implemented in EIGENSTRAT software) on the pooled dataset identified 19 significant (P<0.05) eigenvectors, showing significant association with the country of recruitment. Informed consent from the study participants and approval from the IARC Institutional Review Board (IARC Ethics Committee) was obtained.

SNP selection

Sexually dimorphic SNPs could have (i) a concordant effect direction (CED), if the association is present (i.e., significant after multiple testing correction) for one sex and nominally significant and directionally concordant for the other, (ii) single sex effect (SSE), if the association is present for one sex only, or (iii) opposite effect direction (OED), if the association is present for one sex, at least nominally significant and in opposite direction for the other sex [15]. Previous studies on sex-specific genetic associations indicated that sex-specific scans had a higher probability to select SNPs with CED or SSE signal, while sex-interaction scans had a higher probability to select SNPs with OED [15]. Therefore, in the discovery phase, we conducted both sex stratified and sex interaction scans. For the sex-stratified analysis, a log-additive model using unconditional logistic regression adjusted for age, study and the significant eigenvectors were used to identify associations. For the sex interaction analysis, a regression model including the main effects of the genotypes, sex, covariates and an interaction term for genotypes and sex was used to detect association. We applied a false-discovery-rate (FDR) approach separately for male and female datasets to account for multiple testing and the difference in sample size. FDR q-value cut offs of 5% and 30% were used to detect significant and suggestive SNPs respectively in each of the datasets. Accordingly, p-value threshold of 1x10-6 and 4x10-6 was considered to be significant (5% FDR) and p-value threshold of 1.1x10-5 and 5x10-5 was considered suggestive (30% FDR) for female and male datasets respectively. In addition to the significant and suggestive sex-specific p-values, a nominally significant (P<0.05) sex interaction p-value was taken into account in order to identify SNPs showing sex difference. The same FDR cut-offs were used to detect significant and suggestive signals in interaction tests (Supplementary figure S1). In addition, a clear LD cluster for the SNP was also considered as a criterion to avoid false positives. Among multiple SNPs in LD (r^2 > 0.8, with LD-window of 1Mb) showing an association, we choose the one with the lowest missing rate and p-value.

Replication and joint meta-analysis

Replication of the top hits from the discovery phase was conducted using 3,660 cases (1,399 women, 2,261 men) and 7427 controls (1,575 women, 5,852 men) from two previously published National Cancer Institute (NCI, Bethesda, Maryland, USA) and one MD Anderson Cancer Center (MDA, Texas, USA) kidney GWAS scans genotyped using HumanHap 550, 610 and 660W beadchip arrays. Quality control and genotype imputation was done as described previously [7, 8, 11]. For each study, sex-stratified and sex-interaction models for all significant and suggestive SNPs were tested assuming a log-additive model of genetic effects using unconditional logistic regression with adjustment for age, study centre, and significant

eigenvectors. The odds ratios and 95% confidence intervals per SNP from each study were meta-analysed using fixed-effect models implemented in GWAMA [16], to get the combined estimates from the replication series. We also performed a joined meta-analysis of results from the discovery and replication series to get the combined effect estimates of the tested SNPs. Heterogeneity in genetic effects across datasets was assessed using the I2 and Cochran's Q statistics.

Expression QTL analysis of the selected SNPs

To identify gene regulatory effects of the identified SNPs, we examined transcript expression near each of the SNPs in 279 normal and 574 tumour kidney tissues separately for men and women. Expression analysis was conducted using Illumina HumanHT-12 v4 expression BeadChips (Illumina, Inc., San Diego), normalised using variance stabilizing transformation (VST) and quantile normalization. Additive linear models were used to test the association between each transcript and SNP with age, country, tumour stage and grade as covariates. All transcripts within a 1 Mb upstream and downstream range of the SNPs were evaluated, and FDR adjusted p-value <0.05 using Benjamini-Hochberg procedure was used as statistical significance threshold. All probes with SNPs were filtered out.

Results

In the discovery phase, logistic regression testing separately for male and female datasets showed an excess of p-values less than 0.05 (Figure 1). The association Q-Q plots indicated little inflation for both the datasets (λ_{female}=1.02, λ_{male}=1.04; supplementary figure S2a, b). A total of 17 SNPs (6 significant and 11 suggestive) were selected for follow-up; of which 15 SNPs (5 significant and 10 suggestive) gave single sex-specific signals (SSE) and 2 SNPs namely, rs4903064 and rs6554676 (showing CED) were strongly associated in women and nominally in men (Supplementary table1). Among the 15 single sex-specific signals, 4 out of the 5 significant SNPs were male-specific, whereas, 7 of the 10 suggestive SNPs were female-specific. Also, 7 were associated with an increased RCC risk in women and 3 in men, whereas, 4 SNPs were associated with a decreased risk in men and 1 in women (Supplementary table 2). The strongest association was observed for rs4903064 in females (OR_{female}= 1.47 [95% CI=1.33-1.62], $P_{female}=9x10^{-14}$ compared with OR_{male}= 1.09 [95% CI= 1.01-1.19], P_{male}= 0.02; $P_{interaction}=1.7x10^{-5}$) at 14q24.2 mapping to an intronic region of *DPF3* (Figure 2). Other significant SNPs in discovery series, rs2121266 at 2p21, rs12930199 at 16p13.3 and rs1548141 at 3q11.2 mapped to the intronic regions of *EPAS1*, *RBFOX1* and *OR5H6*, respectively. Significant SNPs rs10484683 and rs78971134 mapped to intergenic regions at 7p22.3 and 6q24.3, with the nearest genes being *BTBD11* and *SAMD5*, respectively. For rs78971134 (*SAMD5*) the minor allele frequencies were similar for male and female cases. Regional LD plots for each of the loci are detailed in Supplementary Figure S3 (a) and (b). In contrast, the sex-interaction scan did not identify any SNP even at 30% FDR and no SNP could be carried forward (Supplementary figure 4a,b).

Sex-stratified analysis of the 17 SNPs in the replication phase exhibited stronger effect for *DPF3* in women compared with men (OR_{female}=1.24 [95%CI= 1.07-1.42], $P_{female}=3x10^{-3}$ compared with OR_{male}= 1.09 [0.98-1.21], P_{male}=0.09). In addition rs147304092 (*BBS9*), rs13027293 (*STEAP3*) rs6554676 (*SLC6A18*) showed nominally significant association with

RCC risk for either men or women in the follow-up series (Table 1). No other SNP exhibited any significant sex difference in the replication series.

We performed a joint meta-analysis of the discovery and replication series (8,061 women and 16,256 men) for the selected 17 SNPs. In addition to the consistent findings for *DPF3* ($metaOR_{female}$=1.38, $metaP_{female}$=1.54 x 10^{-14} compared with $metaOR_{male}$=1.09, $metaP_{meta}$=0.005; $metaP_{interaction}$ = 0.002), we found a stronger association for males for *EPAS1* ($metaOR_{male}$=1.18, $metaP_{male}$=1.84 x 10^{-09} compared with $metaOR_{female}$=1.09, $metaP_{female}$= 0.02; $metaP_{interaction}$ =0.03) but with significant study heterogeneity in the female dataset. Two additional SNPs reached genome-wide significance in the joint meta-analysis.Both rs10484683 at *SAMD5* ($metaOR_{female}$=0.98, $metaP_{female}$=0.68 compared with $metaOR_{male}$=0.83, $metaP_{meta}$=1.71x10^{-08},$metaP_{interaction}$=0.10) and rs78971134 near *BTBD11*($metaOR_{female}$=0.93, $metaP_{female}$=0.26, compared with $metaOR_{male}$=0.75, $metaP_{meta}$=1.59x10^{-08}; $metaP_{interaction}$= 0.14) showed an inverse association with risk for men but not women (Table 1). The results of replication and final meta-analysis of all the 17 SNPs are listed in supplementary table 2.

We also examined sex-specific expression of genes corresponding to the selected SNPs using Illumina expression data in normal and tumour kidney tissues. Although no gene with significant sex-difference in expression was detected in normal tissues, we observed a higher expression of *SAMD5* in tumour tissues of women (Supplementary table 3). We further tested the effect of the identified SNPs on expression of nearby genes by detecting *cis* expression quantitative trait loci (eQTL) in kidney tissues. No significant eQTL was identified for any of the 17 SNPs in normal tissues, but we identified rs4903064 as a significant eQTL for *DPF3* expression in tumours (P=1.88 x 10^{-8}). We further examined sex-specific *cis*-eQTLs and found a stronger association of rs4903064 on *DPF3* for women compared with men (β_{women}=0.06, P_{women}=2.69 x 10^{-6} vs β_{men}=0.03, P_{men}=0.004, $P_{sex\_interaction}$=0.03 Figure 3). A borderline association was also observed for rs6554676 and SLC6A18 expression in male tumour tissues only (β_{male}=-0.21, P_{male}=0.05 vs β_{female}=-0.01, P_{female}=0.94).

Discussion

We conducted the first systematic sex-specific genome-wide association analysis of RCC and observed sexually dimorphic associations for two previously known risk SNPs on *DPF3* and *EPAS1* at 14q24 and 2p21, respectively. In a joint meta-analysis of top hits using 8,061 women and 16,256 men, we also identified two additional suggestive SNPs (rs10484683 at *SAMD5* and rs78971134 near *BTBD11*) with possible sex-specific associations – both being associated with a lower risk for men, and with no strong evidence of association for women.

We report a higher risk of RCC for women for rs4903064 at *DPF3* gene and also provide evidence that the association might be mediated through expression of the gene, with the magnitude of the association between the SNP and expression being greater for women than men. rs4903064 was previously reported to be associated with increased RCC risk in a large GWAS [11], and our analysis confirms the previous reports of its sex-specific association. Polymorphisms at intron 1 of *DPF3* are also associated with increased risk of breast cancer for women of European origin, but the SNPs were not in linkage disequilibrium with rs4903064 [17]. *DPF3* is a histone acetylation and methylation reader of the BAF and PBAF chromatin remodeling complexes. Other components of the complexes like *BAP1* and *PBRM1* are

frequently mutated in RCC and show sex differences in their mutation frequency and association with survival [18]. Chromatin-remodeling complexes regulate gene expression and loss of these chromatin modifiers has been associated with characteristic gene expression signatures in RCC [19, 20]. Sexually dimorphic gene expression is frequent in both murine [21] and human [5, 22] kidney normal and tumour tissues, and is hypothesized to contribute to the mechanism underlying sex-difference in kidney diseases including cancer [4, 23]. Therefore, polymorphisms and mutations of chromatin remodeling complex associated genes might modify RCC risk differently for men and women through sex-specific gene expression but the exact mechanism remains speculative and requires detailed functional studies in vitro.

The SNP rs2121266 mapping to intron 1 of the *EPAS1* gene is in strong linkage disequilibrium (r^2=0.97, D'=1.00) to the previously described risk SNP rs11894252 at 2p21[7]. We report a sex-specific association of this variant showing stronger association for men in the discovery set. This is in agreement with previous findings of stronger associations for the proxy SNP rs11894252 for men (OR_{male}=1.18 compared with OR_{female}=1.06, $P_{interaction}$=0.03) in RCC. Additionally, sexually dimorphic associations for *EPAS1* variants were also observed for rs13419896 in lung squamous cell carcinoma [24] and rs4953354 in lung adenocarcinoma [25] in two independent reports from a Japanese population. *EPAS1* (*HIF2α*) is a key gene in RCC and functions as a transcription factor in the *VHL–HIF* signalling axis [26, 27]. The intron 1 of *EPAS1* contains estrogen response elements (EREs) and estrogen-dependent downregulation of *EPAS1* occurs in invasive breast cancer cells [28]. RCC related polymorphisms near other important genes like *CCND1, MYC/PVT1* have been found on enhancers at tissue-specific *HIF*-binding loci in renal tubular cells [29, 30], implying a role for *HIF* in transactivation of key oncogenic pathways in RCC. Although rs2121266 and rs11894252 were not eQTLs for *EPAS1*, it is possible that the role of these polymorphisms in sex hormone mediated regulation of *EPAS1* and transactivation of downstream genes may result in sex-specific susceptibility to RCC.

Two other SNPs that reached genome-wide significance in the joint analysis of discovery and replication series, namely rs10484683 at *SAMD5* and rs78971134 near *BTBD11* have not been previously reported to be associated with risk of RCC. For rs10484683 (*SAMD5*), the sex-specific finding from the discovery stage was driven by MAF differences in the controls only. Hence, the result remains unclear and might be the reason that the apparent association did not replicate. The SNP rs10484683 was not a significant cis eQTL in normal or tumour kidney tissues in our series, but expression of SAMD5 varied significantly between tumour samples from men and women. Although not implicated in RCC, *SAMD5* overexpression has been found to be associated with bile duct and cholangiocarcinoma [31]. *BTBD11* gene codes for an ankyrin repeat and BTB/POZ domain-containing protein involved in regulation of proteolysis and protein ubiquitination. Functional implications of this gene is not well known in RCC, but SNPs near the *BTBD11* gene were previously reported to be associated with kidney function traits[32] and diabetic kidney diseases[33] by large genome-wide studies.

In the discovery series, we observed that rs147304092 at 7p14.3, mapping to an intronic region of the *BBS9* gene, was associated with substantially lower risk for women but not men. *BBS9* is implicated in Bardet-Biedl Syndrome (BBS), a rare autosomal recessive ciliopathy with a wide spectrum of clinical features including obesity, renal abnormalities, mental retardation, hypogonadism etc. [34]. This gene is also a candidate tumour suppressor gene in Wilms'

tumour, the most common paediatric malignancy of the kidney [35]. Nevertheless, this observation was not replicated and did not reach genome-wide significance in the meta-analysis; fine mapping of this locus in larger studies might identify new global or sex-specific candidates of RCC.

We confirmed sex specific genetic associations of known RCC risk SNPs and identified new suggestive associations for one or the other sex, but without clear replication. Also, no clear pattern of an increased risk for men or decreased risk for women could be observed in the top sexually dimorphic SNPs, as would be otherwise anticipated for explaining the 2:1 sex ratios. Therefore, these SNPs are not conclusive for untangling the sex-specific genetic susceptibility that might contribute to the sex ratio in incidence. Due to technical constraints we could not examine sex chromosomal associations in the current study. Even given its large sample size, a drawback of the study is its limited statistical power to detect subtle sex-specific associations, particularly when analysing men and women separately. A male-specific association may simply reflect the lack of power to detect association in women, owing to the smaller sample size for women compared with men. To increase the power to detect sex-specific associations, the combination of results from different GWAS in sex-stratified meta-analyses is warranted. Therefore, considering sex-specific scans and sex chromosomes in performing genome-wide association studies will also provide an opportunity to examine sex associations in addition to overall associations in sexually dimorphic diseases and traits.

Acknowledgements

The authors thank all of the participants who took part in this research and the funders and support staff who made this study possible. Funding for the genome-wide genotyping was provided by the US National Institutes of Health (NIH), National Cancer Institute (U01CA155309) for those studies coordinated by IARC, and by the intramural research program of the National Cancer Institute, US NIH, for those studies coordinated by the NCI. Funding for the IARC gene expression and eQTL study was provided by the US National Institutes of Health (NIH), National Cancer Institute (U01CA155309). RSL received IARC Postdoctoral fellowship from the IARC, partially supported by EC FP7 Marie Curie Actions – People – Co- Funding of regional, national and international programmes (COFUND). DCM is supported by a Cancer Research UK Population Research Fellowship. MMCI- supported by MH CZ - DRO (MMCI, 00209805)

Competing financial interests

The authors declare that they have no competing financial interests.

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Legends to Figures:

Figure 1. **Sex stratified genome-wide association scan in renal cell carcinoma**: Manhattan plots of male and female specific association P-values from the discovery series.

Figure 2.**Regional plot of the most significant sex-specific loci**: P-values and LD among SNPs at 14q24.2 mapping to the DPF3 gene in women and men.

Figure 3. **cis-eQTL**: boxplot displaying expression levels of *DPF3* gene stratified by the risk SNP rs4903064 in women and male kidney tumour tissues.