Author's response to reviews

Title: Prevalence of Von Hippel-Lindau gene mutations in sporadic Renal Cell Carcinoma: results from the Netherlands Cohort Study

Authors:
- Kjeld P. van Houwelingen (K.vanHouwelingen@ncmls.kun.nl)
- Boukje A.C. van Dijk (Boukje.vanDijk@epid.unimaas.nl)
- Christina A. Hulsbergen-van de Kaa (C.Hulsbergen@pathol.azn.nl)
- Leo J. Schouten (LJ.Schouten@epid.unimaas.nl)
- Hanneke J.M. Gorissen (H.Gorissen@ncmls.kun.nl)
- Jack A. Schalken (J.Schalken@ncmls.kun.nl)
- Piet A. van den Brandt (PA.vandenBrandt@epid.unimaas.nl)
- Egbert Oosterwijk (E.Oosterwijk@ncmls.kun.nl)

Version: 3 Date: 5 April 2005

Author's response to reviews: see over
Dear editorial team,

Thank you for the opportunity to revise and resubmit the article entitled “Prevalence of Von Hippel-Lindau gene mutations in sporadic Renal Cell Carcinoma: results from the Netherlands Cohort Study” (MS ID: 1065222680582079). Suggestions and comments by the reviewers were very valuable for improving our manuscript. We have addressed all comments/suggestions by the reviewers. Please find our responses below. Additions and alterations to the document are indicated in the response including corresponding page numbers of the main document. We hope the manuscript is now suitable for publication in BMC Cancer.

On behalf of the authors, with best regards,

Boukje van Dijk

Reviewer 1 (Peter Schraml)

Major Compulsory Revisions

1. It is not clear how the authors verified missense mutations. For example, two separate PCRs would clearly demonstrate whether or not the mutation is true (same mutation in two PCR products or artefact in one sample and no or another mutations in the second one). Therefore, forward and/or reverse sequencing of a shifted PCR product, as described by the authors, is not sufficient especially if formalin-fixed paraffine-embedded material from 52 different labs(!) is used. This would also explain the uncommonly high rate of VHL mutations in clear cell RCC and the observation of different mutations in different paraffin blocks from one tumor.

Response: We agree with the reviewer that missense mutations may pose a particular problem in studies like ours. However, we would like to emphasize that we first identified mutations by PCR-SSCP, followed by direct sequencing of a new PCR product, i.e., we have used two independent methods to verify VHL mutations. We therefore feel that these mutations are true observations and not technical flaws.

2. The authors classified tumor stage according to the 1987 revision of the UICC-TNM classification. The last (5th) edition of the staging system of malignant tumors appeared in 1997 and is commonly used nowadays. Compared to the former edition there was a dramatic change in the pT classification of organ-confined renal cancer in which the break point between category pT1 and pT2 was increased from 2.5 cm to 7 cm. The introduction of the cut point brought considerable changes to the number of cases in each category, with more cases now being categorized as pT1. It is also important to be aware that the new pT stage is strongly correlated with patient survival. The use of the new system might therefore have great influence on the calculations regarding correlations between tumor stage, patient outcome and VHL mutation.

Response: The cases of this study were diagnosed in the period September 1987 through December 1997. In this period, the cancer registries used the following UICC TNM versions: the revised 3rd edition (1986-1987), the 4th edition (1988-1992) and 2nd revision of the 4th edition (1993-1997). Several editions were considered: the 5th edition could not be used, because not only the T-category but also the N-category changed and we could not derive all information regarding the N-status, hence we would not be able to validly recode the N-category in all cases. We decided to use the 4th edition (1987) of the TNM classification, because both the revised 3rd edition (1982) and the 2nd revision of the 4th edition (1992) could
be recoded into the 4th edition (1987). Furthermore, we decided to collect information on the exact tumor size using the information from the pathology report, because of the different cut point in the 5th TNM edition (1997) and because it offered the opportunity to investigate more accurately whether VHL status was associated with tumor size.

3. Obviously the authors have access to additional information including follow-up data, lifestyle and dietary habits of cancer patients. A possible correlation between VHL mutations, survival and risk factors would strengthen the impact of this paper.

Response: We fully agree with the reviewer that we will be able to link risk factors to VHL mutations in future studies. However, we feel that both the methods of the risk factor assessment and the laboratory methods should be described in detail. This adds significantly to the size and complexity of the article, which is why we decided to describe all relevant laboratory methods, relevant clinical/pathological parameters and the quality of the mutation analysis in this manuscript.

4. Samples having at least 10% tumor cells were considered for sequence analysis, which is very uncommon. Does this mean the authors were still able to detect VHL mutations in heterozygous tumors with only one allele being mutated? How many tumors consisted of only 10% malignant cells? How did the authors retrieve the material from these samples (microdissection, scraping)?

Response: 180 out of 190 samples contained at least 50% tumor cells. The other 10 samples contained the following percentages of vital tumor tissue: 10%, 20%, 30%, 30%, 33%, 33%, 40%, 40%, 45%, and 48%. The lowest percentage of vital tumor tissue with a mutation was 30%. Thus it may be true that we missed existing mutations in the two samples with less than 30% tumor tissue, which has been added to the discussion (page 12). The protocol to retrieve material from these samples was not different from the general protocol, i.e. 5 20 µm slides were cut from which DNA was extracted.

Minor Essential Revisions
1. Grammar and spelling should be re-checked (f.e. subtypes will be assessed…op page 11).
Response: We rechecked the document on grammar and spelling.

2. SSCP analysis is a well-described and accepted tool for mutational screening. SSCP analysis and findings should be written as concise as possible. Table 2 and 3 should be omitted.
Response: Table 3 and 4 were combined and supplemented with characteristics for all observed mutations (see next question). Table 2 was not omitted since we felt we needed to give additional information on the performance status of the SSCP preceding sequencing as compared to direct sequencing only (the other method frequently used). In fact, this table allows the reader insight in the validation of SSCP as a tool to detect VHL mutations versus direct sequencing.

3. The distribution and types of VHL mutations from all tumor subtypes should be listed in one table. Table 4 and 5 should be combined (without data about sex, age, and % tumor tissue). Tumor parameters (stage, grade, nodal status) should be briefly summarized in an additional table. Table 6 is too complex and should be omitted.
Response: Table 3 and 4 were combined into table 3 (pages 25-27) and supplemented with characteristics for all observed mutations. Table 5 can now be deduced from the new table 3,
so we have decided to leave this out. Table 6 shows the data on which tests on differences between \textit{VHL} mutated and wildtype \textit{VHL} tumors were calculated. We have tried to decrease its complexity by leaving out the information on tumor size (which is now in the text of the results section only) and by changing the lay-out (table 4, page 28).

4. There is no significant correlation between tumor size and \textit{VHL} mutation. Therefore, the result should be briefly mentioned in the results section.
Response: This has been changed accordingly.

\textit{Reviewer 2 (Christophe Beroud)}
\textbf{Major Compulsory Revisions}

The authors should provide a complete description of all mutations at the molecular level. They should use the international nomenclature described in the Human Genome Variation Society website (http://www.geneomic.unimelb.edu.au/mdi/mutmomen/) and complete the tables #3 and #4 accordingly. This will particularly be useful in order to evaluate the pathogenicity of each mutation.
Response: A new table #3 (pages 25-27) has been constructed containing all observed mutations, using the nomenclature mentioned by the reviewer.

We also recommend a discussion on silent mutations, splice mutations and missense mutations to evaluate if they could be considered as true mutations or as polymorphisms.
Response: The goals of our study were to describe mutations observed in sporadic RCC arising from a large population-based study and investigate the association with clinical/pathological parameters. By definition, the only difference between a mutation and a polymorphism is the frequency by which it is present. A common definition states that a polymorphism is a genetic variation (mutation) which is present in \textgreater 1\% of the general population. Because information on the presence of polymorphisms in the \textit{VHL} gene observed in the general population is not available, it is not possible to define nucleotide changes as mutations or polymorphisms. However, the incidence of individual mutations described in our manuscript makes it very unlikely that these should be considered polymorphisms.

Table #2 should be modified, as it is not easy to find the number of samples with a specific SSCP result.
Response: As requested by the reviewer, we have modified table 2, and trust that this has increased the readability (page 24).

In addition, it is necessary to discuss about mutations found only on one strand DNA.
Response: Based on our analyses, we cannot discriminate whether mutations are on the same strand of DNA or not.

\textbf{Minor Essential Revisions}

Page #9, paragraph #1: The authors report that they can use paraffin embedded tissues with at least 10\% malignant cells. They should give more information about these samples as one can expect that the mutated allele is then present at a very low fraction (\textgreater 5\%). Are the SSCP and sequencing techniques sensitive enough to detect such a low allele fraction?
Response: See response to major compulsory revision 4 from reviewer 1
The authors report various genotypes from various tumor blocks from the same patient. Did they reproduce their experience in order to prove that the same genotype was consistently found on each block?
Response: We agree with the reviewer that reproduction would be advisable, preferably beginning with the isolation of DNA. Obviously, the results surprised us, whereupon we re-examined these cases, i.e., confirmed particular genotypes. It appeared that the results were not the consequence of technical errors. However, unfortunately, we were not able to isolate new DNA from these tissue blocks, because we no longer have access to the paraffin material, since it was returned to the original laboratories.

The authors should modify values in all the manuscript. For example: 50.7% should be written 50.7%
Response: We would like to thank the reviewer for the attentiveness. Indeed, we noticed inconsistencies in the use of dots and commas. We rechecked the entire document for correct use.

Discretionary Revisions
Page #13 paragraph #2: The authors say that the percentage of point mutations was much higher in exon #1 (50.7%) than in exon #2 and #3 (29.6 and 23.1, respectively). This is expected as this exon contains 340 nt (53% of coding sequence) while exon #2 contains 122 nt (19%) and exon #3 175 nt (27%). Thus this should not be considered as a hot spot.
Response: The reviewer is correct. We may inadvertently have implied that we viewed this as a hot spot. To circumvent confusion the paragraph was changed (page 10).

Reviewer 3 (Eamonn Maher)
Major Compulsory Revisions
Details of all VHL mutations should be listed individually with the corresponding clinical and pathological characteristics of the specific tumour to allow future meta-analysis studies.
Response: A new table describing all mutations has been constructed (table 3, pages 25-27)

Some mutations were detected in the 5’ of codon 54 – these are unusual and would be predicted to affect pVHL30 but not pVHL 19 translation products – this should be discussed.
Response: A section discussing this has been added to the discussion (pages 15-16).

RCC without VHL mutations may have epigenetic silencing of VHL by promotor methylation – this should be mentioned in the Discussion as it will reduce the likelihood of detecting clinical differences between VHL mutation positive and mutation negative groups.
Response: We fully agree with the reviewer and have added this to the discussion section of the paper (page 15).