Trp53 inactivation leads to earlier phaeochromocytoma formation in pten knockout mice

Esther Korpershoek¹, Nanne K Kloosterhof²,³, Angelique Ziel-van der Made¹, Hanneke Korsten¹, Lindsey Oudijk¹, Jan Trapman¹, Winand N M Dinjens¹ and Ronald R de Krijger¹

Departments of ¹Pathology, and ²Neurology, Josephine Nefkens Institute, Erasmus Medical Centre, University Medical Centre Rotterdam, PO Box 2040, 3000 CA Rotterdam, The Netherlands
³Department of Paediatric Oncology and Haematology, Erasmus Medical Centre, Sophia Children’s Hospital, Rotterdam, The Netherlands

(Correspondence should be addressed to E Korpershoek; Email: e.korpershoek.1@erasmusmc.nl)

Abstract

Phaeochromocytomas (PCCs) are benign neuroendocrine tumours of the adrenal medulla. Approximately 10% of PCC patients develop metastases, but this frequency is much higher in specific subtypes of patients. The reliable diagnosis of malignant PCC can only be made after identification of a metastasis. To study the effect of Trp53 inactivation on PCC pathogenesis in Pten KO mice, we investigated the adrenals of a large cohort of mice with conditional monoallelic and biallelic inactivation of Trp53 and Pten. The adrenal weights were determined for all mice, and in a proportion of these mice, immunohistochemistry for tyrosine hydroxylase and dopamine β-hydroxylase was performed on the adrenals and corresponding lungs. Finally, comparative genomic hybridization (CGH) was performed. The histological and immunohistochemical results confirmed that the adrenal tumours were PCCs. Inactivation of one or both alleles of Trp53 resulted in earlier tumour occurrence in the PtenloxP/loxP mice as well as in the PtenloxP/+ mice. In addition, lung metastases were found in up to 67% of mice. The CGH results showed that the most frequent genomic alterations were loss of chromosome 19 (86%) and gain of chromosome 15 (71%). In this study, we have shown that Pten/Trp53 KO mice showed metastatic PCC at high frequency and primary tumours occurred at younger ages in mice with Trp53 inactivation. Therefore, the present model appears to be a suitable model that might allow the preclinical study of new therapeutics for these tumours.

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Introduction

In humans, phaeochromocytomas (PCCs) are relatively rare tumours that occur in the adrenal medulla and usually overproduce catecholamines, such as adrenaline or noradrenaline (Lenders et al. 2005). This overproduction causes high blood pressure, which in some cases may be fatal, through myocardial infarction or stroke (Maher & Eng 2002). PCCs occur sporadically as well as in the context of hereditary syndromes, which include the multiple endocrine neoplasia syndrome type 2, von Hippel–Lindau (VHL) disease, neurofibromatosis type 1 and the PCC–paraganglioma (PGL) syndrome (Neumann et al. 2002). The latter syndrome is characterized by both PCC and PGLs. PGLs are histologically similar to PCCs but are not always biochemically active. PGLs can be subdivided into parasympathetic PGLs (pPGLs) and sympathetic PGLs (sPGLs). pPGLs occur in the head and neck regions and usually do not produce catecholamines, whereas sPGLs are biochemically active tumours that arise from chromaffin tissue outside the adrenal glands, occurring in the thorax and abdomen along the neural crest (Neumann et al. 2002).

In addition to the genes described earlier, in the last decade, other tumour suppressor genes have been identified that were associated with PCC and/or PGL,
such as SDHA, SDHAF2, TMEM127 and MAX (Hao et al. 2009, Burnichon et al. 2010, Qin et al. 2010, Comino-Mendez et al. 2011). Most PCCs are benign, but ~10% of sporadic cases present with metastases at diagnosis (Gimenez-Roqueplo et al. 2006). This frequency can be much higher, varying from 34 to 98%, in patients with a germline mutation in the succinate dehydrogenase subunit B gene, although these numbers involve both PCC and pGGL (Neumann et al. 2004, Benn et al. 2006, Brouwers et al. 2006, Amar et al. 2007, Timmers et al. 2007). Currently, only one study has come up with a potentially interesting gene that could differentiate metastatic and recurring PCCs from benign tumours. However, the study was performed on a limited amount of PCCs, and it has not been validated in other series (Lee et al. 2011). Besides this promising study, there are no validated markers that can predict the malignant clinical behaviour of PCC in humans.

Currently, patients with metastatic PCC or PGL have few therapeutic options, which are on a palliative basis. Surgery is usually not curative for patients with metastases, but surgical removal of the primary tumour and metastasis can prolong the patient’s life and improve life quality. Radiotherapy with 131I-MIBG is an option if patients have a good uptake of 123I-MIBG in active metastases. In addition, systemic chemotherapy has also been used to treat metastatic disease; however, the number of patients in these studies was too less to draw evidence-based conclusions (Adjalle et al. 2009, Plouin et al. 2012). A recent study on two mouse PCC cell lines showed that a combination of drugs that specifically block mTORC1/2, PI3K, ERK1/2, AKT, VEGF and EGF receptor functions can have a significant effect on the growth of these tumour cells. These results could indicate a new therapeutic approach for human malignant PCC (Nolting et al. 2012).

There are many distinct knockout mouse models that have been shown to develop almost exclusively benign PCC (Korpershoek et al. 2012). Genes that were silenced in these models included Nf, Rbl1, p130 (Rbl2), p18(Ink4c) (Cdkn2c), p27(Kip1) (Cdkn1b) and Pten (Nikitin et al. 1999, Franklin et al. 2000, Powers et al. 2000, Di Cristofano et al. 2001, Dannenberg et al. 2004, Bai et al. 2006). Only two studies have reported mice with spontaneous development of PCC lung metastases. These included the Pten KO mice of You et al. (2002), which showed metastases in a small percentage (15%), and our previous study in which we reported a conditional Pten KO mouse model that also presented with lung metastasis, which occurred in 35% of the mice of 10 months or older (Korpershoek et al. 2009).

The Pten gene is a tumour suppressor gene that is involved in the pathogenesis of many tumour types, such as prostate and breast cancer, and belongs to the most frequently mutated genes in human cancer. Although mouse models that involve the inactivation of the Pten gene develop PCC, human PCC has never been associated with Pten mutations (van Nederveen et al. 2006). Nevertheless, the genomic alterations found in the PCC of Pten KO mice show similarities with the genomic alterations found in human PCC, and loss of the Pten locus has also been reported in human PCC (You et al. 2002, Korpershoek et al. 2009).

Another gene that belongs to the most frequently mutated genes in human cancer is the TP53 gene. TP53 has been associated with aggressiveness of tumour behaviour, including local invasion and metastasis (Muller et al. 2011). Although TP53 mutations have never been found in human PCC, frequent loss of the TP53 genomic locus has been demonstrated in PCC by loss of heterozygosity analysis and fluorescence in situ hybridization (Petri et al. 2008). The aggressive behaviour of TP53-mutated tumours is also illustrated in different mouse models, in which metastatic lymphomas and osteosarcomas occur (Donehower et al. 1992, Jacks et al. 1994). In addition, another study combined Rb with Trp53 to obtain a mouse model with highly aggressive medulloblastomas (Marino et al. 2000).

In this study, we have investigated the effect of inactivation of the Trp53 gene (on a Cre-loxP basis, under the control of the PSA promoter) on the behaviour of PCC in single or double Pten KO mice. Parameters investigated included the frequency of metastases, the age of tumour presentation, size (weight) of the tumours and chromosomal alterations.

Materials and methods

Generation of the Pten/Trp53 mice

Generation of the Pten\textsubscript{loxP/loxP} and Trp53\textsubscript{loxP/loxP} mice was described previously (Marino et al. 2000, Ma et al. 2005). The Pten\textsubscript{loxP/loxP} animals were cross-bred with the Trp53\textsubscript{loxP/loxP} mice to obtain Pten\textsubscript{loxP/+}, Trp53\textsubscript{loxP/+} offspring. This F1 offspring was inbred to obtain Pten\textsubscript{loxP/loxP}, Trp53\textsubscript{loxP/loxP} mice, which were cross-bred with the previously reported PSA-Cre\textsubscript{+-} mice (Ma et al. 2005). The PSA-Cre: Pten\textsubscript{loxP/loxP}, Trp53\textsubscript{loxP/loxP} offspring was interbred with Trp53\textsubscript{loxP/loxP} to obtain the six genotypes investigated.
All mice used in this study were male mice that carried the PSA-Cre construct, with the exception of the mice used as healthy controls for the immunohistochemistry. The current Pten/Trp53 KO mice were generated to obtain a mouse model with prostate carcinomas that behave more aggressively, compared with the prostate tumours of the Pten KO mouse model. These tumours will be described in a separate report (Korsten H, Ziel-van der Made, Hermans K, van Leenders A, van der Kwast T, Ma X, de Ridder C, Kraaij R, Nigg A, Trapman J, unpublished observations, 2005–2007). Mice were housed according to institutional guidelines, and procedures were carried out in compliance with the standards for use of laboratory animals. In addition, animal experiments performed in this study have been approved by the animal experimental committee of the Erasmus MC Medical Centre (DEC-consult). A total of 236 male mice of all genotypes were killed at various preset ages, and all organs were systematically investigated macroscopically and microscopically for abnormalities. Numbers of mice per age group and genotype are displayed in Table 1. Alterations not related to PCC or PCC metastases will be described in a separate report (Korsten H, Ziel-van der Made A, Hermans K, van Leenders A, van der Kwast T, Ma X, de Ridder C, Kraaij R, Nigg A, Trapman J, unpublished observations, 2005–2007). In addition, the weight of the adrenals was determined for all mice. Adrenal glands that weighed 8 mg (twice the weight of a healthy adrenal) or more were considered as tumour containing. Mice were genotyped using tail DNA and PCR was performed with forward and reverse primers for Pten and Trp53 (primer sequences available on request). Forty-eight adrenal tumours and 28 corresponding lungs of mice of different genotypes were available for investigation.

**Immunohistochemistry**

Forty-eight formalin-fixed paraffin-embedded adrenals of 25 PSA-Cre;PtenloxP/loxP, eight PSA-Cre;PtenloxP/loxP;Trp53loxP/+ and 15 PSA-Cre; PtenloxP/loxP; Trp53loxP/loxP were investigated with immunohistochemical markers according to the previously described method with antibodies directed against tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) (Koropershoek et al. 2009). In addition, immunohistochemistry with an antibody for succinate dehydrogenase subunit B (Rabbit polyclonal HPA002686; Sigma–Aldrich; 1/250) was performed according to procedures reported previously (van Nederveen et al. 2009). Furthermore, the 28 corresponding lungs were histologically and immunohistochemically (TH and DBH) investigated for the presence of PCC metastases. For each tumour and lung, negative controls were performed by omission of the primary antibody. Lungs were systematically investigated for metastases by three-step sections of 5 μm at intervals of at least 10 μm. The adrenals of Cre-negative littermates were used as positive controls.

**Comparative genomic hybridization**

Of the 14 mice of which snap-frozen PCC tissue was available, DNA was isolated using the Gentra Puregene Tissue Kit (Qiagen) according to the manufacturer’s procedures. Labelling, hybridization conditions and methods were described previously (Koropershoek et al. 2009). For hybridization, high-density whole-genome mouse bacterial artificial chromosome microarray slides produced by the Central Microarray Facility of The Netherlands Cancer Institute in Amsterdam were used (Chung et al. 2004). Slide scanning was performed using the ScanArray Express HT (Perkin Elmer Life Science, Boston, MA, USA). Spot intensities were measured using GenePix Pro 5.1 Software (Axon Instruments, Leusden, The Netherlands). Excel 2000 and the aCGH-smooth application (http://www.few.vu.nl/~vumarray/acs-mooth.htm) were used to analyse the normalized data. The default settings of aCGH-smooth were used as healthy controls for the immunohistochemistry.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>2 Months</th>
<th>4–5 Months</th>
<th>7–8 Months</th>
<th>11–12 Months</th>
<th>15 Months</th>
<th>18 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtenloxP/loxP</td>
<td>0/7</td>
<td>0/10</td>
<td>0/9</td>
<td>9/10 (90%)</td>
<td>7/7 (100%)</td>
<td>–</td>
</tr>
<tr>
<td>PtenloxP/loxP;Trp53loxP/+</td>
<td>0/2</td>
<td>1/10 (10%)</td>
<td>11/12 (92%)</td>
<td>12/12 (100%)</td>
<td>8/8 (100%)</td>
<td>–</td>
</tr>
<tr>
<td>PtenloxP/loxP;Trp53loxP/loxP</td>
<td>0/7</td>
<td>2/10 (20%)</td>
<td>27/29 (93%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PtenloxP/+</td>
<td>0/2</td>
<td>0/11</td>
<td>0/10</td>
<td>2/8 (25%)</td>
<td>1/4 (25%)</td>
<td>3/5 (60%)</td>
</tr>
<tr>
<td>PtenloxP/+;Trp53loxP/+</td>
<td>0/2</td>
<td>0/7</td>
<td>0/5</td>
<td>2/6 (33%)</td>
<td>3/3 (100%)</td>
<td>3/3 (100%)</td>
</tr>
<tr>
<td>PtenloxP/+;Trp53loxP/loxP</td>
<td>0/3</td>
<td>0/10</td>
<td>0/9</td>
<td>7/9 (78%)</td>
<td>5/5 (100%)</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>0/23</td>
<td>2/58 (3.4%)</td>
<td>38/75 (51%)</td>
<td>32/45 (71%)</td>
<td>24/27 (89%)</td>
<td>6/8 (75%)</td>
</tr>
</tbody>
</table>

The columns contain the number of mice presenting with PCC per total numbers of mice and the corresponding percentages are inside the brackets. Above the columns are the different age groups at the time of killing.
used, except for $\lambda = 8.5$. The Student’s $t$-test was used to determine the statistical differences between the genomic alterations found in the tumours with and without $Trp53$ knock-down.

**Results**

**Trp53 inactivation leads to an earlier PCC presentation**

All average tumour percentages and adrenal weights per age group and genotype are listed in Tables 1 and 2 respectively. PCC occurred from the age of 11 months onwards in the $Pten^{loxP/loxP}$ and the $Pten^{loxP/loxP}; Trp53^{loxP/+}$ mice. In contrast, if one or two $Trp53$ alleles were inactivated in the $Pten^{loxP/loxP}$ mice, PCC occurred already at the age of 4–5 months. Both the $Pten^{loxP/loxP}; Trp53^{loxP/+}$ and the $Pten^{loxP/loxP}; Trp53^{loxP/loxP}$ mice showed a nearly full penetrance at the age of 7–8 months (92 and 93% respectively). The main difference between the two genotypes was that all $Pten^{loxP/loxP}; Trp53^{loxP/loxP}$ mice had to be killed at the age of 8 months because of severe health problems that could be related to the PCC or prostate cancer, whereas the $Pten^{loxP/loxP}; Trp53^{loxP/+}$ stayed healthy until the age of 15 months. $Pten^{loxP/+}$ mice showed the first PCC at 11–12 months, similar to the $Pten^{loxP/loxP}$, but at a much lower frequency (33 vs 90%). In addition, monoallelic and biallelic inactivation of $Trp53$ in the $Pten^{loxP/+}$ did not result in an earlier tumour presentation. However, used, except for $\lambda = 8.5$. The Student’s $t$-test was used to determine the statistical differences between the genomic alterations found in the tumours with and without $Trp53$ knock-down.

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the frequency of PCC occurrence was higher per age group compared with the PtenloxP/+ mice, indicating the acceleration effect of Trp53 inactivation.

**Immunohistochemistry**

Forty-eight primary tumours were immunohistochemically stained for TH and DBH to confirm the tumours were PCC. All tumour cells stained positive for TH and DBH, as well as the positive controls, which included the adrenals of Cre-negative mice. SDHB immunohistochemistry was positive for all PCCs investigated (Fig. 1).

**Presence of lung metastases**

In total, 28 lungs of mice with three different genotypes were available for investigation of the presence of metastases (Table 3). Lungs were examined by histology and immunohistochemistry with markers for TH and DBH. Metastases typically encompassed few cells, usually approximately five to ten cells with a maximum of 25 cells (illustrated in Fig. 1). Generally, up to five metastases were seen scattered throughout the lungs. PtenloxP/loxP and PtenloxP/loxP;Trp53loxP/+ mice investigated showed PCC metastases in 67% of mice (eight in 12 mice and two in three mice respectively). The PtenloxP/loxP;Trp53loxP/loxP showed lung metastases at a similar frequency of 61.5% of mice (in eight of 13 mice).

**Comparative genomic hybridization**

CGH was performed on 14 PCCs of mice with four genotypes. An overview of the CGH results is shown in Table 4 and a typical example is shown in Fig. 2.

Overall, the most frequent chromosomal alterations were complete loss of chromosome 19 (in 85.7% of mice) and gain of (parts of) chromosome 15 (in 71.4% of mice). Furthermore, loss of (parts of) chromosome 14 and chromosome 6 (in 42.9 and 35.7% of mice respectively) was demonstrated.

To investigate whether Trp53 inactivation influenced the occurrence of genomic alterations, we split the mice in two groups. Group 1 included the recent PtenloxP/loxP KO mice (n=4) and PtenloxP/loxP KO mice (n=8) of our previous study (Korpershoek et al. 2009), as they are genotypically identical. Group 2 included the PtenloxP/loxP;Trp53loxP/+ (n=1), PtenloxP/loxP;Trp53loxP/loxP (n=7) and PtenloxP/loxP;Trp53loxP/loxP (n=2) mice. The tumours of group 1 showed loss of chromosome 6 and 19 in 67% (8/12) mice, whereas tumours of group 2 showed loss of chromosome 6 in 40% (4/10) and loss of chromosome 19 in 100% (10/10). Gain of chromosome 15 was seen in 42% (5/12) of tumours of group 1, while 80% (8/10) of the tumours in group 2 showed gain of chromosome 15. The difference in frequency of chromosomal alterations between the two groups was significant only for chromosome 15 (P=0.046).

**Discussion**

In this study, we have shown that the Pten/Trp53 KO mouse model presents metastatic PCC at a high frequency and that Trp53 inactivation leads to PCC, arising at similar frequencies, but at earlier ages. In addition, monoallelic or biallelic Trp53 inactivation in Pten KO mice does not lead to higher frequencies of metastases. The most frequently occurring genomic alterations in PCC of mice with or without Trp53 knock-down involved the same chromosomes. Although the frequencies of losses and gains were
Table 4 Overview of comparative genomic hybridization results

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Genotype</th>
<th>Chr. 2</th>
<th>Chr. 4</th>
<th>Chr. 6</th>
<th>Chr. 7</th>
<th>Chr. 8</th>
<th>Chr. 9</th>
<th>Chr. 13</th>
<th>Chr. 14</th>
<th>Chr. 15</th>
<th>Chr. 16</th>
<th>Chr. 19</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>(\text{Pten}^{\text{loxP/loxP}})</td>
<td>WC –</td>
<td></td>
<td></td>
<td>62–103 Mb +</td>
<td>WC –</td>
<td>62–103 Mb +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(\text{Pten}^{\text{loxP/loxP}})</td>
<td>WC +</td>
<td></td>
<td></td>
<td>WC –</td>
<td>WC –</td>
<td>WC –</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>(\text{Pten}^{\text{loxP/loxP}})</td>
<td>WC –</td>
<td>51–153 Mb –</td>
<td></td>
<td>WC –</td>
<td>WC +</td>
<td>WC –</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>WC –</td>
<td></td>
<td></td>
<td>WC –</td>
<td>WC +</td>
<td>WC –</td>
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<tr>
<td>14</td>
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<td>88–103 Mb +</td>
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<td>112–153 Mb –</td>
<td></td>
<td></td>
<td>WC +</td>
<td>WC –</td>
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<td>WC –</td>
<td></td>
<td></td>
<td>100–153 Mb –</td>
<td>WC –</td>
<td>WC –</td>
<td>0–104 Mb</td>
<td>WC –</td>
<td>WC –</td>
<td>WC –</td>
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</tbody>
</table>

This table shows an overview of the genomic alterations found in 14 mouse PCCs. WC, whole chromosome; –, loss; +, gain; Chr., chromosome. Some losses or gains are parts of chromosomes, then megabases are shown.
dissimilar between the two groups, this difference was only significant in case of chromosome 15. 

The \textit{PTEN} gene belongs to the most frequently mutated genes in human cancer. The main function of PTEN is to convert phosphatidylinositol (3,4,5)-triphosphate to phosphatidylinositol (4,5)-diphosphate, thereby inhibiting the activation of \textit{AKT} and its downstream targets (Di Cristofano & Pandolfi 2000). Although \textit{PTEN} mutations have not been found in human PCCs, overexpression of its downstream target \textit{AKT} has been associated with human PCCs (Fassnacht et al. 2005). To investigate the effect of \textit{PTEN} inactivation in tumourigenesis, many \textit{Pten} KO mouse models have been generated (Suzuki et al. 1998, Podsypanina et al. 1999, Di Cristofano et al. 2001, Lesche et al. 2002, You et al. 2002, Ma et al. 2005, Freeman et al. 2006, Korpershoek et al. 2009). In the conventional \textit{Pten} KO mouse models, \textit{Pten} nullizygosity leads to embryonic lethality, whereas \textit{Pten} heterozygotes present with many different tumours, mainly prostate cancer and often PCCs (Suzuki et al. 1998, You et al. 2002). To investigate the exact effect of \textit{PTEN} inactivation in the prostate, a prostate-specific conditional mouse model was generated using the Cre-lox system under the control of the PSA-promoter (Ma et al. 2005). The \textit{Pten}^{loxP/loxP} KO mice developed invasive prostate carcinomas and metastatic PCCs, of which the earliest PCCs occurred in mice aged 7–9 months. In this study, the \textit{Pten}^{loxP/loxP} mice again presented metastatic PCC at similar frequencies as the mice of the previous study (Korpershoek et al. 2009). In addition, PCC lung tumours were confirmed to be TH and DBH immunohistochemistry, as these markers are normally not present in cells of the lungs.

\textit{TP53} is the most frequently mutated gene in human cancer. It acts as a tumour suppressor gene and regulates different cellular functions, including cellular senescence and apoptosis. In a healthy cell, p53 is suppressed by E3 ubiquitin ligase mouse double minute 2 (MDM2), which will bind to p53 and ubiquinate p53 for degradation (Nigro et al. 1989, Muller et al. 2011). In a stressed cell, p53 will be activated, promoting cell cycle arrest or apoptosis. Recently, mutated \textit{TP53} has been associated with cell migration and invasion, suggesting that p53 inactivation can contribute to a tumour’s metastatic potential (Muller et al. 2011). In this study, the monoallelic and biallelic inactivation of \textit{Trp53} resulted in an earlier tumour presentation in the \textit{Pten} KO mice. In addition, PCC lung metastases also occurred at younger ages, but without an increased frequency compared with that in mice with solely \textit{Pten} inactivation. It must be noted that numbers were too small to perform statistical analysis. No \textit{TP53} mutations have been found in human PCCs, although chromosomal loss of the \textit{TP53} gene has been demonstrated. However, there appeared to be no difference in frequencies between benign or malignant tumours (Petri et al. 2008).

Genetic alterations have been investigated by CGH in two \textit{Pten} KO mouse models (You et al. 2002, Korpershoek et al. 2009). The first study investigated the PCC of (conventional) heterozygous KO mice and found loss of chromosome 4 as the most frequent genetic alteration (You et al. 2002). In contrast, in our previous study, we found almost no loss of chromosome 4 in our (conditional) \textit{Pten} KO mice but found loss of chromosome 6 and 19 as the main chromosomal alterations (Korpershoek et al. 2009). In this study,
we performed CGH on 14 PCC of four different genotypes. The most frequent aberration found was loss of chromosome 19 and gain of chromosome 15. The frequency of loss of chromosome 19 was similar to that of the previous study (86 and 75% respectively).

To determine the effect of Trp53 inactivation on the chromosomal alterations, the mice were split into two groups: group 1 included the Pten\textsuperscript{loxP/loxP} mice of the this and previous study (as they are genotypically identical; Korpershoek \textit{et al}. 2009), and group 2 included Pten\textsuperscript{loxP/+};Trp53\textsuperscript{loxP/loxP}, Pten\textsuperscript{loxP/loxP};Trp53\textsuperscript{loxP/+} and Pten\textsuperscript{loxP/loxP};Trp53\textsuperscript{loxP/+} mice. Inactivation of Trp53 did not result in changes other than minor frequency differences in chromosomal alterations. Loss of chromosome 6 occurred in 40% of the group 1 tumours and in 67% of the group 2 tumours. In addition, all group 2 tumours showed loss of chromosome 19, whereas group 1 tumours showed loss in 67%. Finally, the group 2 tumours showed gain of chromosome 15 in 80% of cases, whereas the group 1 tumours showed gain in 40%. Gain of chromosome 15 was the only significant difference between the two groups. Notably, no loss of the Trp53 wild-type allele was seen in tumours of Pten\textsuperscript{loxP/loxP};Trp53\textsuperscript{loxP/+} mice, indicating that loss of one Trp53 allele is sufficient to accelerate PCC pathogenesis in these mice.

Mouse chromosome 19 includes a region that is syntenic to human chromosome 11q13, which is associated with SDHD-related and VHL-related PCC, indicating involvement of this chromosomal area in the pathogenesis of these tumours. Mouse chromosome 15 is syntenic to human chromosome 5p13–15, of which 5p has been associated with the pathogenesis of human malignant PCC (personal observations of a large series of human malignant PCC). This shows that these mice PCC have genetic aberrations that could provide clues about chromosomal areas containing genes involved in human PCC. However, these areas include too many genes to predict which genes could be of importance.

In SDHB-related human PCC, the frequency of metastases is relatively high (34–98%) compared with sporadic PCC (Neumann \textit{et al}. 2004, Gimenez-Roqueplo \textit{et al}. 2006, Amar \textit{et al}. 2007, Timmers \textit{et al}. 2007). In this study, we performed SDHB immunohistochemistry on the PCC of the Pten and Trp53 KO mice and found only tumours with positive tumour cells indicating that there are no SDH mutations present (van Nederveen \textit{et al}. 2009). Therefore, it is highly unlikely that succinate dehydrogenase (mitochondrial complex II) is involved in the pathogenesis of these mouse tumours. This is in accordance with our previous findings, as Sdhb or Sdhd mutations were not found in the Pten\textsuperscript{loxP/loxP} mice (Korpershoek \textit{et al}. 2009).

In conclusion, we have investigated a large cohort mouse adrenal glands from six different genotypes showing that tumours occurred at much earlier ages in the Pten\textsuperscript{loxP/loxP};Trp53\textsuperscript{loxP/loxP} and the Pten\textsuperscript{loxP/loxP}, Trp53\textsuperscript{loxP/+} mice, compared with the Pten\textsuperscript{loxP/loxP} mice, indicating the accelerating effect of Trp53 inactivation on pathogenesis of these tumours. Although Trp53 inactivation leads to earlier PCC occurrence, it does not result in higher frequencies of metastases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

E Korpershoek, R R de Krijger, W N M Dinjens and J Trapan were involved in study design and data interpretation. E Korpershoek, N K Kloosterhof, A Ziel-van der Made, H Korsten and L Oudijk collected the samples and carried out the experiments. E Korpershoek and N K Kloosterhof analysed the data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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