Trp53 inactivation leads to earlier phaeochromocytoma formation in *pten* knockout mice

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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 $Pten^{loxP/loxP}$ mice as well as in the $Pten^{loxP/+}$ 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,

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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 sPGL (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 ^{131}I-MIBG is an option if patients have a good uptake of ^{123}I-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*, *Rb1*, *p130* (*Rbl2*), *p18*(I*nk4c*) (*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 $Pten^{loxP/loxP}$ and $Trp53^{loxP/loxP}$ mice was described previously (Marino *et al*. 2000, Ma *et al*. 2005) The $Pten^{loxP/loxP}$ animals were cross-bred with the $Trp53^{loxP/loxP}$ mice to obtain $Pten^{loxP/+}$; $Trp53^{loxP/+}$ offspring. This F1 offspring was inbred to obtain $Pten^{loxP/loxP}$;$Trp53^{loxP/loxP}$ mice, which were cross-bred with the previously reported *PSA-Cre*$^{+/-}$ mice (Ma *et al*. 2005). The *PSA-Cre*;$Pten^{loxP/+}$; $Trp53^{loxP/+}$ offspring was interbred with $Pten^{loxP/loxP}$; $Trp53^{loxP/loxP}$ 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 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). 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 the additional 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*;*Pten*$^{loxP/loxP}$, eight *PSA-Cre*; *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/+}$ and 15 *PSA-Cre*; *Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/loxP}$ were investigated with immunohistochemical markers according to the previously described method with antibodies directed against tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) (Korpershoek *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 (Korpershoek *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 (Perklin 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/acgs-mooth.htm) were used to analyse the normalized data. The default settings of aCGH-smooth were

Table 1 Presentation of phaeochromocytoma (PCC) in mice of different ages and different genotypes

Genotype	2 Months	4–5 Months	7–8 Months	11–12 Months	15 Months	18 Months
Pten$^{loxP/loxP}$	0/7	0/10	0/9	9/10 (90%)	7/7 (100%)	–
Pten$^{loxP/loxP}$;*Trp53*$^{loxP/+}$	0/2	1/10 (10%)	11/12 (92%)	12/12 (100%)	8/8 (100%)	–
Pten$^{loxP/loxP}$;*Trp53*$^{loxP/loxP}$	0/7	2/10 (20%)	27/29 (93%)	–	–	–
Pten$^{loxP/+}$	0/2	0/11	0/10	2/8 (25%)	1/4 (25%)	3/5 (60%)
Pten$^{loxP/+}$;*Trp53*$^{loxP/+}$	0/2	0/7	0/5	2/6 (33%)	3/3 (100%)	3/3 (100%)
Pten$^{loxP/+}$;*Trp53*$^{loxP/loxP}$	0/3	0/10	0/9	7/9 (78%)	5/5 (100%)	–
Total	0/23	2/58 (3.4%)	38/75 (51%)	32/45 (71%)	24/27 (89%)	6/8 (75%)

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.

Table 2 Adrenal weights at different ages per different genotype

Genotype	2 Months (mg)	S.D.	4–5 Months (mg)	S.D.	7–8 Months (mg)	S.D.	11–12 Months (mg)	S.D.	15 Months (mg)	S.D.	18 Months (mg)	S.D.
Pten$^{loxP/loxP}$	3.8	1.1	5.0	1.1	5.0	1.3	44.0	50.0	122	93.5	–	
Pten$^{loxP/loxP}$; *Trp53*$^{loxP/+}$	6.3	0.9	5.9	3.6	17.5	11.1	225	183	326	209	–	
Pten$^{loxP/loxP}$; *Trp53*$^{loxP/loxP}$	3.7	1.4	8.6	9.2	38.0	99.3	–		–		–	
Pten$^{loxP/+}$	4.0	1.3	3.5	0.6	2.8	0.7	6.0	4	72.7	142	26.8	44.4
Pten$^{loxP/+}$; *Trp53*$^{loxP/+}$	3.8	1.2	3.5	1.4	3.3	1.7	158	377	210	310	338	286
Pten$^{loxP/+}$; *Trp53*$^{loxP/loxP}$	3.6	0.9	3.0	1.1	3.6	1.0	72.7	117	163	126	–	

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*$^{loxP/+}$ 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,

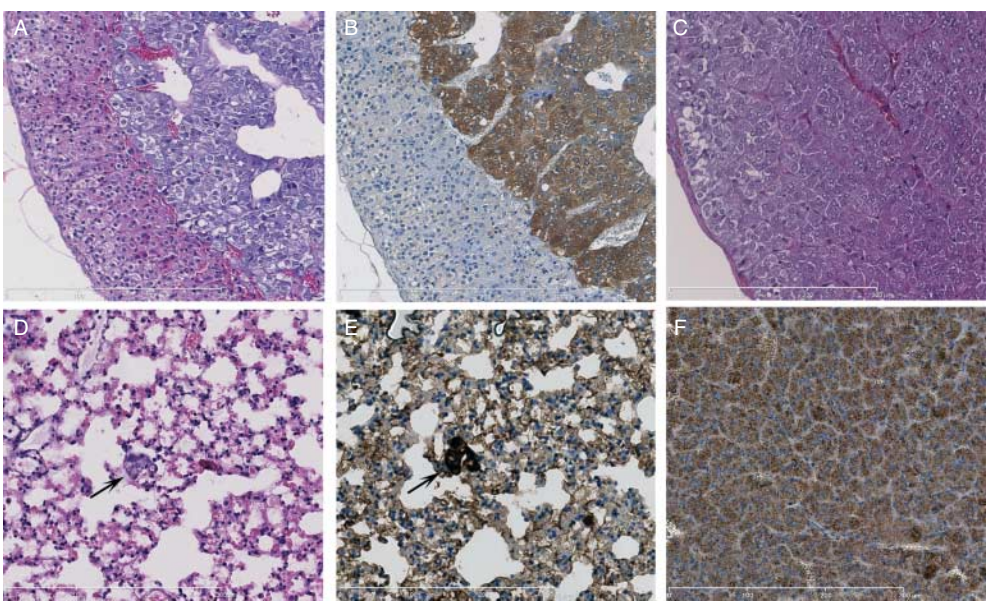

Figure 1 Haematoxylin–eosin staining of normal adrenal (A), phaeochromocytomas (C) and lung metastasis (D); TH immunohistochemistry of normal adrenal (B) and lung metastasis (E) and SDHB immunohistochemistry of phaeochromocytoma (F). Arrows indicate metastasis.

the frequency of PCC occurrence was higher per age group compared with the *Pten*$^{loxP/+}$ 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. *Pten*$^{loxP/loxP}$ and *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/+}$ mice investigated showed PCC metastases in 67% of mice (eight in 12 mice and two in three mice respectively). The *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/loxP}$ showed lung metastases at a similar frequency of 61.5% of mice (in eight of 13 mice).

Table 3 Presence of phaeochromocytoma (PCC) lung metastases

Mouse no.	Adrenal weight (mg)	Age (months)	Lung metastasis present
Pten$^{loxP/loxP}$			
1	32	10	Y
2	41.4	12	N
3	20.5	12	Y
4	133	12	Y
5	32.1	12	Y
6	140	12	Y
7	9.6	12	N
8	26.2	15	N
9	66.4	15	Y
10	103	15	Y
11	80.3	15	Y
12	69.7	15	N
Pten$^{loxP/loxP}$; *Trp53*$^{loxP/+}$			
13	286	11	Y
14	117	12	Y
15	71	15	N
Pten$^{loxP/loxP}$; *Trp53*$^{loxP/loxP}$			
16	40.5	7	Y
17	8.0	7	N
18	21.8	7	Y
19	27.5	8	Y
20	33.7	8	N
21	18.0	8	N
22	27.3	8	Y
23	18.1	8	N
24	46.8	8	Y
25	38.2	8	Y
26	15.1	8	Y
27	34.8	8	Y
28	55.6	11	N

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 *Pten*$^{loxP/loxP}$ KO mice ($n=4$) and *Pten*$^{loxP/loxP}$ KO mice ($n=8$) of our previous study (Korpershoek *et al.* 2009), as they are genotypically identical. Group 2 included the *Pten*$^{loxP/+}$;*Trp53*$^{loxP/loxP}$ ($n=1$), *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/+}$ ($n=7$) and *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/loxP}$ ($n=2$) 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

Mouse no.	Genotype	Chr. 2	Chr. 4	Chr. 6	Chr. 7	Chr. 8	Chr. 9	Chr. 13	Chr. 14	Chr. 15	Chr. 16	Chr. 19
3	*Pten*$^{loxP/loxP}$								WC −	62–103 Mb +		
4	*Pten*$^{loxP/loxP}$									WC +		WC −
6	*Pten*$^{loxP/loxP}$	42–181 Mb +		27–150 Mb −	79–153 Mb −				WC −			
10	*Pten*$^{loxP/loxP}$			WC −	51–153 Mb −							WC −
13	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/+}$								WC −	WC +		WC −
14	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/+}$									WC +		WC −
15	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/+}$					30–61 Mb +				88–103 Mb +		WC −
30	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/+}$									WC +		WC −
31	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/+}$				112–153 Mb −					WC +		WC −
32	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/+}$					WC +			WC −	WC +	WC +	WC −
33	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/+}$									WC+		WC −
18	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/loxP}$			WC −	100–153 Mb −		WC −	WC −	0–104 Mb −			WC −
28	*Pten*$^{loxP/loxP}$; *Trp53*$^{loxP/loxP}$			WC −						WC +		WC −
29	*Pten*$^{loxP/+}$; *Trp53*$^{loxP/loxP}$		0–18 Mb −	WC −			WC −		WC −			WC −

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.

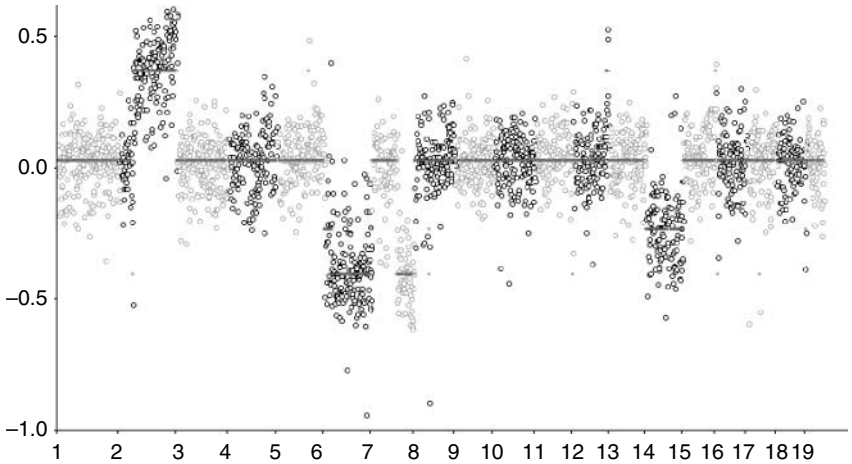

Figure 2 Example of a CGH result of mouse chromosome 6, showing gain of chromosome 2, loss of whole chromosomes 6 and 14 and a part of chromosome 7.

different between the two groups, this difference was only significant in case of chromosome 15.

The *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 AKT and its downstream targets (Di Cristofano & Pandolfi 2000). Although *PTEN* mutations have not been found in human PCCs, overexpression of its downstream target AKT has been associated with human PCCs (Fassnacht *et al*. 2005). To investigate the effect of *PTEN* inactivation in tumourigenesis, many *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 *Pten* KO mouse models, *Pten* nullizygosity leads to embryonic lethality, whereas *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 *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 $Pten^{loxP/loxP}$ 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 $Pten^{loxP/loxP}$ 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.

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 *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 *Trp53* resulted in an earlier tumour presentation in the *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 *Pten* inactivation. It must be noted that numbers were too small to perform statistical analysis. No *TP53* mutations have been found in human PCCs, although chromosomal loss of the *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 *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) *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 $Pten^{loxP/loxP}$ mice of the this and previous study (as they are genotypically identical; Korpershoek *et al*. 2009), and group 2 included $Pten^{loxP/+}$;$Trp53^{loxP/loxP}$, $Pten^{loxP/loxP}$;$Trp53^{loxP/+}$ and $Pten^{loxP/loxP}$;$Trp53^{loxP/loxP}$ 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 $Pten^{loxP/loxP}$;$Trp53^{loxP/+}$ 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 *et al*. 2004, Gimenez-Roqueplo *et al*. 2006, Amar *et al*. 2007, Timmers *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 *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 $Pten^{loxP/loxP}$ mice (Korpershoek *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 $Pten^{loxP/loxP}$;$Trp53^{loxP/loxP}$ and the $Pten^{loxP/lox}$;$Trp53^{loxP/+}$ mice, compared with the $Pten^{loxP/loxP}$ 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 Trapman 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.

References

Adjalle R, Plouin PF, Pacak K & Lehnert H 2009 Treatment of malignant pheochromocytoma. *Hormone and Metabolic Research* **41** 687–696. (doi:10.1055/s-0029-1231025)

Amar L, Baudin E, Burnichon N, Peyrard S, Silvera S, Bertherat J, Bertagna X, Schlumberger M, Jeunemaitre X, Gimenez-Roqueplo AP *et al.* 2007 Succinate dehydrogenase B gene mutations predict survival in patients with malignant pheochromocytomas or paragangliomas. *Journal of Clinical Endocrinology and Metabolism* **92** 3822–3828. (doi:10.1210/jc.2007-0709)

Bai F, Pei XH, Pandolfi PP & Xiong Y 2006 p18 Ink4c and Pten constrain a positive regulatory loop between cell growth and cell cycle control. *Molecular and Cellular Biology* **26** 4564–4576. (doi:10.1128/MCB.00266-06)

Benn DE, Gimenez-Roqueplo AP, Reilly JR, Bertherat J, Burgess J, Byth K, Croxson M, Dahia PL, Elston M, Gimm O *et al.* 2006 Clinical presentation and penetrance

of pheochromocytoma/paraganglioma syndromes. *Journal of Clinical Endocrinology and Metabolism* **91** 827–836. (doi:10.1210/jc.2005-1862)

Brouwers FM, Eisenhofer G, Tao JJ, Kant JA, Adams KT, Linehan WM & Pacak K 2006 High frequency of SDHB germline mutations in patients with malignant catecholamine-producing paragangliomas: implications for genetic testing. *Journal of Clinical Endocrinology and Metabolism* **91** 4505–4509. (doi:10.1210/jc.2006-0423)

Burnichon N, Briere JJ, Libe R, Vescovo L, Riviere J, Tissier F, Jouanno E, Jeunemaitre X, Benit P, Tzagoloff A *et al.* 2010 SDHA is a tumor suppressor gene causing paraganglioma. *Human Molecular Genetics* **19** 3011–3020. (doi:10.1093/hmg/ddq206)

Comino-Mendez I, Gracia-Aznarez FJ, Schiavi F, Landa I, Leandro-Garcia LJ, Leton R, Honrado E, Ramos-Medina R, Caronia D, Pita G *et al.* 2011 Exome sequencing identifies MAX mutations as a cause of hereditary pheochromocytoma. *Nature Genetics* **43** 663–667. (doi:10.1038/ng.861)

Chung YJ, Jonkers J, Kitson H, Fiegler H, Humphray S, Scott C, Hunt S, Yu Y, Nishijima I, Velds A *et al.* 2004 A whole-genome mouse BAC microarray with 1-Mb resolution for analysis of DNA copy number changes by array comparative genomic hybridization. *Genome Research* **14** 188–196. (doi:10.1101/gr.1878804)

Dannenberg JH, Schuijff L, Dekker M, van der Valk M & te Riele H 2004 Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. *Genes and Development* **18** 2952–2962. (doi:10.1101/gad.322004)

Di Cristofano A & Pandolfi PP 2000 The multiple roles of PTEN in tumor suppression. *Cell* **100** 387–390. (doi:10.1016/S0092-8674(00)80674-1)

Di Cristofano A, De Acetis M, Koff A, Cordon-Cardo C & Pandolfi PP 2001 Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. *Nature Genetics* **27** 222–224. (doi:10.1038/84879)

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS & Bradley A 1992 Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356** 215–221. (doi:10.1038/356215a0)

Fassnacht M, Weismann D, Ebert S, Adam P, Zink M, Beuschlein F, Hahner S & Allolio B 2005 AKT is highly phosphorylated in pheochromocytomas but not in benign adrenocortical tumors. *Journal of Clinical Endocrinology and Metabolism* **90** 4366–4370. (doi:10.1210/jc.2004-2198)

Franklin DS, Godfrey VL, O'Brien DA, Deng C & Xiong Y 2000 Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity. *Molecular and Cellular Biology* **20** 6147–6158. (doi:10.1128/MCB.20.16.6147-6158.2000)

Freeman D, Lesche R, Kertesz N, Wang S, Li G, Gao J, Groszer M, Martinez-Diaz H, Rozengurt N, Thomas G *et al.* 2006 Genetic background controls tumor development in PTEN-deficient mice. *Cancer Research* **66** 6492–6496. (doi:10.1158/0008-5472.CAN-05-4143)

Gimenez-Roqueplo AP, Lehnert H, Mannelli M, Neumann H, Opocher G, Maher ER & Plouin PF 2006 Phaeochromocytoma, new genes and screening strategies. *Clinical Endocrinology* **65** 699–705. (doi:10.1111/j.1365-2265.2006.02714.x)

Hao HX, Khalimonchuk O, Schraders M, Dephoure N, Bayley JP, Kunst H, Devilee P, Cremers CW, Schiffman JD, Bentz BG *et al.* 2009 SDH5, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma. *Science* **325** 1139–1142. (doi:10.1126/science.1175689)

Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT & Weinberg RA 1994 Tumor spectrum analysis in p53-mutant mice. *Current Biology* **4** 1–7. (doi:10.1016/S0960-9822(00)00002-6)

Korpershoek E, Loonen AJ, Corvers S, van Nederveen FH, Jonkers J, Ma X, Ziel-van der Made A, Korsten H, Trapman J, Dinjens WN *et al.* 2009 Conditional Pten knock-out mice: a model for metastatic phaeochromocytoma. *Journal of Pathology* **217** 597–604. (doi:10.1002/path.2492)

Korpershoek E, Pacak K & Martiniova L 2012 Murine models and cell lines for the investigation of pheochromocytoma: applications for future therapies? *Endocrine Pathology* **23** 43–54. (doi:10.1007/s12022-012-9194-y)

Lee TK, Murthy SR, Cawley NX, Dhanvantari S, Hewitt SM, Lou H, Lau T, Ma S, Huynh T, Wesley RA *et al.* 2011 An N-terminal truncated carboxypeptidase E splice isoform induces tumor growth and is a biomarker for predicting future metastasis in human cancers. *Journal of Clinical Investigation* **121** 880–892. (doi:10.1172/JCI40433)

Lenders JW, Eisenhofer G, Mannelli M & Pacak K 2005 Phaeochromocytoma. *Lancet* **366** 665–675. (doi:10.1016/S0140-6736(05)67139-5)

Lesche R, Groszer M, Gao J, Wang Y, Messing A, Sun H, Liu X & Wu H 2002 Cre/loxP-mediated inactivation of the murine Pten tumor suppressor gene. *Genesis* **32** 148–149. (doi:10.1002/gene.10036)

Ma X, Ziel-van der Made AC, Autar B, van der Korput HA, Vermeij M, van Duijn P, Cleutjens KB, de Krijger R, Krimpenfort P, Berns A *et al.* 2005 Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis. *Cancer Research* **65** 5730–5739. (doi:10.1158/0008-5472.CAN-04-4519)

Maher ER & Eng C 2002 The pressure rises: update on the genetics of phaeochromocytoma. *Human Molecular Genetics* **11** 2347–2354. (doi:10.1093/hmg/11.20.2347)

Marino S, Vooijs M, van Der Gulden H, Jonkers J & Berns A 2000 Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes and Development* **14** 994–1004. (doi:10.1101/gad.14.8.994)

Muller PA, Vousden KH & Norman JC 2011 p53 and its mutants in tumor cell migration and invasion. *Journal of Cell Biology* **192** 209–218. (doi:10.1083/jcb.201009059)

van Nederveen FH, Perren A, Dannenberg H, Petri BJ, Dinjens WN, Komminoth P & de Krijger RR 2006 PTEN gene loss, but not mutation, in benign and malignant phaeochromocytomas. *Journal of Pathology* **209** 274–280. (doi:10.1002/path.1968)

van Nederveen FH, Gaal J, Favier J, Korpershoek E, Oldenburg RA, de Bruyn EM, Sleddens HF, Derkx P, Riviere J, Dannenberg H *et al.* 2009 An immunohistochemical procedure to detect patients with paraganglioma and phaeochromocytoma with germline SDHB, SDHC, or SDHD gene mutations: a retrospective and prospective analysis. *Lancet Oncology* **10** 764–771. (doi:10.1016/S1470-2045(09)70164-0)

Neumann HP, Hoegerle S, Manz T, Brenner K & Iliopoulos O 2002 How many pathways to pheochromocytoma? *Seminars in Nephrology* **22** 89–99. (doi:10.1053/snep.2002.30207)

Neumann HP, Pawlu C, Peczkowska M, Bausch B, McWhinney SR, Muresan M, Buchta M, Franke G, Klisch J, Bley TA *et al.* 2004 Distinct clinical features of paraganglioma syndromes associated with SDHB and SDHD gene mutations. *Journal of the American Medical Association* **292** 943–951. (doi:10.1001/jama.292.8.943)

Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P *et al.* 1989 Mutations in the p53 gene occur in diverse human tumour types. *Nature* **342** 705–708. (doi:10.1038/342705a0)

Nikitin AY, Juarez-Perez MI, Li S, Huang L & Lee WH 1999 RB-mediated suppression of spontaneous multiple neuroendocrine neoplasia and lung metastases in Rb+/− mice. *PNAS* **96** 3916–3921. (doi:10.1073/pnas.96.7.3916)

Nolting S, Garcia E, Alusi G, Giubellino A, Pacak K, Korbonits M & Grossman AB 2012 Combined blockade of signalling pathways shows marked anti-tumour potential in phaeochromocytoma cell lines. *Journal of Molecular Endocrinology* **49** 79–96. (doi:10.1530/JME-12-0028)

Petri BJ, Speel EJ, Korpershoek E, Claessen SM, van Nederveen FH, Giesen V, Dannenberg H, van der Harst E, Dinjens WN & de Krijger RR 2008 Frequent loss of 17p, but no p53 mutations or protein overexpression in benign and malignant pheochromo cytomas. *Modern Pathology* **21** 407–413. (doi:10.1038/modpathol.3801013)

Plouin PF, Fitzgerald P, Rich T, Ayala-Ramirez M, Perrier ND, Baudin E & Jimenez C 2012 Metastatic pheochromocytoma and paraganglioma: focus on therapeutics. *Hormone and Metabolic Research* **44** 390–399. (doi:10.1055/s-0031-1299707)

Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE & Parsons R 1999 Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *PNAS* **96** 1563–1568. (doi:10.1073/pnas.96.4.1563)

Powers JF, Evinger MJ, Tsokas P, Bedri S, Alroy J, Shahsavari M & Tischler AS 2000 Pheochromocytoma cell lines from heterozygous neurofibromatosis knockout mice. *Cell and Tissue Research* **302** 309–320. (doi:10.1007/s004410000290)

Qin Y, Yao L, King EE, Buddavarapu K, Lenci RE, Chocron ES, Lechleiter JD, Sass M, Aronin N, Schiavi F *et al.* 2010 Germline mutations in TMEM127 confer susceptibility to pheochromocytoma. *Nature Genetics* **42** 229–233. (doi:10.1038/ng.533)

Suzuki A, de la Pompa JL, Stambolic V, Elia AJ, Sasaki T, del Barco Barrantes I, Ho A, Wakeham A, Itie A, Khoo W *et al.* 1998 High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Current Biology* **8** 1169–1178. (doi:10.1016/S0960-9822(07)00488-5)

Timmers HJ, Kozupa A, Eisenhofer G, Raygada M, Adams KT, Solis D, Lenders JW & Pacak K 2007 Clinical presentations, biochemical phenotypes, and genotype–phenotype correlations in patients with succinate dehydrogenase subunit B-associated pheochromocytomas and paragangliomas. *Journal of Clinical Endocrinology and Metabolism* **92** 779–786. (doi:10.1210/jc.2006-2315)

You MJ, Castrillon DH, Bastian BC, O'Hagan RC, Bosenberg MW, Parsons R, Chin L & DePinho RA 2002 Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice. *PNAS* **99** 1455–1460. (doi:10.1073/pnas.022632099)

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