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The murine gene p27 *Kip1* is haplo-insufficient for tumour suppression

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Abstract

p27^{Kip1} is a candidate human tumour-suppressor protein, because it is able to inhibit cyclin-dependent kinases and block cell proliferation[1-5]. Abnormally low levels of the p27 protein are frequently found in human carcinomas, and these low levels correlate directly with both histological aggressiveness and patient mortality[6-10]. However, it has not been possible to establish a causal link between p27 and tumour suppression, because only rare instances of homozygous inactivating mutations of the p27 gene have been found in human tumours[11-14]. Thus, p27^{Kip1} does not fulfill Knudson's 'two-mutation' criterion for a tumour suppressor gene[15]. Here we show that both p27 nullizygous and p27 heterozygous mice are predisposed to tumours in multiple tissues when challenged with g-irradiation or a chemical carcinogen. Therefore p27 is a multiple-tissue tumour suppressor in mice. Molecular analyses of tumours in p27 heterozygous mice show that the remaining wild-type allele is neither mutated nor silenced. Hence, p27 is haplo-insufficient for tumour suppression. The assumption that null mutations in tumour-suppressor genes are recessive excludes those genes that exhibit haplo-insufficiency.

Tumour-suppressor proteins affect several cellular pathways, such as those controlling proliferation, apoptosis, differentiation and genomic integrity. Consequently, the identification of a tumour-suppressor gene is usually established genetically rather than by any particular functional criterion. The common operational definition of a tumour-suppressor gene requires the demonstration of mutations of both copies of a candidate gene in tumours[15]. Genetic and physical mapping of bi-allelic tumour specific mutations allows localization of tumour-suppressor genes, and provides evidence for a causal link between mutations in those genes and tumorigenesis. This approach has successfully demonstrated the tumour-suppressor function of the retinoblastoma protein (Rb), p53, INK4a, and others[16-18]. However, we show here that this definition is too restrictive because it excludes tumour suppressors such as p27^{Kip1} that exhibit haplo-insufficiency.

We studied the tumour-suppressor function of p27^{Kip1} by examining the susceptibility of p27-deficient mice to tumorigenesis induced by two different DNA-damaging agents, g-

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irradiation and the chemical carcinogen N-ethyl-N-nitrosourea (ENU). $p27^{-/-}$ mice showed decreased tumour-free survival following g-irradiation compared with $p27^{+/+}$ controls (Fig. 1a). $p27^{+/-}$ mice showed an intermediate sensitivity (see below). Thymic lymphomas were the most frequent tumours in wild-type mice (incidence 0.07), but their frequency was not significantly increased in $p27^{-/-}$ animals (incidence 0.17). The increased mortality of p27-null mice was mainly due to the development of pituitary tumours and intestinal adenomas (Fig. 2). A separate group of mice was injected with a single dose of ENU, and again p27-null animals showed increased tumour-related mortality (Fig. 1b). In $p27^{+/+}$ mice, lymphoma incidence (0.26) and liver adenomas were the most frequent causes of tumour morbidity. The frequency of these tumour types was not increased in $p27^{-/-}$ animals (lymphoma incidence 0.23). Instead, the increased tumour-related mortality in $p27^{-/-}$ mice following ENU treatment was mainly attributable to increased numbers of adenomas of the intestine and pituitary (Fig. 2). Not all tumours caused morbidity: some were identified concurrently at necropsy or at the termination of the experiments (see Methods). Concurrent tumours had less time to develop in p27-deficient animals compared with controls because of their shorter lifespan. Hence, the experimental design underestimated the contribution of p27 deficiency to tumorigenesis.

Lesions of female reproductive organs also occurred more frequently in p27-deficient mice (Fig. 2), and were usually associated with histological evidence of neoplasia. These lesions included granulosa cell tumours of the ovary, endometrial adenocarcinomas, endometrial polyps, angiosarcomas, and fibromas. Adrenal tumours, both medullary and cortical, occurred at higher frequency in p27deficient animals (one $p27^{+/+}$, four $p27^{+/-}$, and four $p27^{-/-}$ mice in both experiments combined).

In $p27^{-/-}$ mice, the incidence of adenomas in both the small and the large intestine was increased (Fig. 2), as was the number of malignant tumours; 7 out of 46 (15%) of the p27-null animals but just 1 out of 63 (1.6%) of $p27^{+/+}$ controls ($P<0.05$, chi-squared test) developed locally invasive intestinal tumours, defined as invasion of neoplastic epithelia through the full thickness of the muscularis, indicating progression to adenocarcinoma. A smaller number of intestinal stromal sarcomas also occurred late in the ENU-exposed animals, but these were independent of p27 genotype.

The number of lung adenomas was increased in $p27^{-/-}$ animals following both irradiation and ENU treatment (Fig. 2) and ranged in size from 1 to 5 mm. In three p27-null animals, but in none of the wild-type controls, the lung tumours completely consolidated one or more lung lobes and exhibited dysplasia typical of adenocarcinomas. Pituitary tumours in p27-deficient mice were intermediate melanotroph adenomas, similar to those seen in untreated p27 knockouts[5].

$p27^{+/-}$ mice were also significantly more susceptible than wildtype mice to γ-irradiation and ENU-induced tumorigenesis of the lung, intestine, and pituitary, but at intermediate rates (Fig. 2). The tumours were histologically similar to those in $p27^{-/-}$ animals. Increased tumorigenesis is often observed in mice heterozygous for a germline deletion of a tumour-suppressor gene, and in these cases, in keeping with the two-mutation model, the remaining wildtype allele is inactivated[19-22]. In contrast, no deletions or rearrangements of the wild-

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type p27 allele were found in 33 tumours from p27 heterozygotes, including 15 samples of lung, intestine and pituitary obtained after both X-ray and ENU treatments (Fig. 3a, and results not shown). Of 13 tumours assessed further by DNA sequencing, no point mutations were present in the p27 gene's coding region. Moreover, the p27 protein was present at significant levels in 23 p27-heterozygote tumours analysed by immunoblot (Fig. 3b-d, and results not shown). Five lung and four pituitary tumours that had been analysed by Southern blotting and DNA sequencing were also analysed by quantitative immunoblotting, which showed that the amount of p27 protein closely approximated that found in normal heterozygous tissue, which is 50% of the level in wild-type tissue (Fig. 3c, d). Immunostaining for p27 protein showed that p27 protein was expressed in the nuclei of the tumour cells (Fig. 3e). Intestinal tumours contained amounts of p27 protein that were greater than those in normal $p27^{+/-}$ intestine (Fig. 3b), indicating that expression of p27 in the intestine as a whole is not representative of its expression in the cell of origin of these tumours. However, the amount of p27 protein in these tumours was half of that in an intestinal tumour from a wild-type mouse, indicating that expression from the intact allele in the $p27^{+/-}$ tumors was not downregulated (results not shown).

p27 was also haplo-insufficient for suppressing spontaneous tumorigenesis. $p27^{+/-}$ mice spontaneously developed melanotroph tumours in the pituitary with a penetrance of 32% (n=38) and a median latency of 19 months, whereas wild-type littermates developed no pituitary tumours (n=42). We found no deletions or silencing of the wild-type p27 allele when we analysed four of these tumours from heterozygous mice (Fig. 3f, g). We sequenced the coding region of the p27 gene from one of these tumours, and found no mutations. Rb+/ – mice also spontaneously develop melanotroph tumours in the pituitary; however, in those tumours the remaining wild-type Rb allele was always lost[21,22]. p27 contrasts with Rb in this regard; our results show that p27 does not conform to the paradigm of two-hit inactivation in tumours.

Our results suggest that decreased p27 protein expression and human tumour progression are causally related. The sensitivity to p27 dosage suggests that decreased p27 function may be a common event in sporadic human tumours. A twofold drop in p27 expression could occur either by heterozygous gene mutation or by changes in extragenic pathways that modulate p27 protein levels. Evidence from studies of humans supports both of these possibilities. More than 50% of primary breast cancers expressed low amounts of the p27 protein, and similar results have been obtained for colon, gastric and prostate tumours6-10. This is likely to be the result of defects in pathways that regulate p27[7]. Hemizygous deletions at chromosome 12p13, which include the $p27^{Kip1}$ gene, have also been seen in primary human tumours[11,14,23-25]. Inactivation of both p27 alleles would be relatively rare, and may not provide sufficient advantage over the heterozygous state to be strongly selected in tumours.

The molecular basis for p27 haplo-insufficiency is not yet understood. Decreased amounts of p27 may alter the stoichiometric relationship between itself and cyclin-dependent kinases (CDKs), and this could underlie the proliferative abnormalities in p27deficient cells. This suggests that the haplo-insufficient phenotypes of p27 could be enhanced by mutations in genes that modulate cyclin expression and CDK activity. The importance of haploinsufficiency in tumorigenesis is also shown by the tumour predisposition of mice

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heterozygous for a transforming growth factor-β (TGF-β) deletion[26]. However, because TGF-β is a secreted protein, its tumour-inhibitory effect depends on its expression in the whole animal, not by the tumour cell (that is, it does not produce a cell-autonomous phenotype). Therefore, the significance of this example lies in understanding tumour-prone phenotypes in the animal, as opposed to the genetic changes that occur within the tumour cell.

Our results establish a new class of tumour-suppressor genes that are haplo-insufficient, $p27^{Kip1}$ being one example. Inheritance of just one p27 allele resulted in a tumour-prone phenotype, and tumour cells arising in $p27^{+/-}$ mice did not show mutation or silencing of expression of the intact p27 gene. Genes exhibiting haplo-insufficiency may be frequent targets during the evolution of tumour cells, because inactivation of only one allele or a moderate decrease in protein expression is sufficient to predispose to tumorigenesis. Deletion of one p53 allele has been shown to compromise its biological functions in vivo, indicating that haplo-insufficient phenotypes may be widespread[27-29].

A potentially important strategy for cancer therapeutics will be to restore expression of tumour-suppressor proteins. Haplo-insufficient tumour suppressors such as p27 may be useful in achieving this goal, because they retain a functional allele in tumours.

Methods

Mice

All of the animals studied were littermates produced from heterozygous crosses. An untreated cohort including each genotype was observed for 2 years and comprised 25 F1 and F2 generation C57B6/J X 129/Sv hybrids, plus 15 more inbred animals (congenic to the founding 129/Sv embryonic cell line). For the X-ray study, C57BL6/J X 129/Sv F2 generation hybrids were used as breeders. Mice for the experiment with ENU were produced by first backcrossing the original p27 chimaera to C57BL/6J mice for six generations. These were bred to $p27^{+/-}$ 129/Sv inbred mice to produce F1 hybrids. All mice were genotyped by the polymerase chain reaction (PCR) as described[9].

Treatment

The 94 mice included in the X-ray experiment were treated with 4 Gy whole-body γ-irradiation (with a ^{137}Cs source, 330 cGy per second) at the age of 14±2 days, and were weaned at 3-4 weeks. Twenty nine +/+, thirty-two +/–, and twenty-five -/mice were included in the experiment. There was no increase in early mortality associated with the radiation treatment. In a separate experiment, 109 littermates (34 $p27^{+/+}$, 51 $p27^{+/-}$ and 24 $p27^{-/-}$) were injected intraperitoneally with ENU (0.5 mmol per g mouse) at the age of 15±2 days, and were weaned at 3-4 weeks.

Histological analysis

Complete necropsies were done at the first sign of morbidity, or when visible tumours were 1 cm in size. All remaining animals were necropsied at the termination of the experiment (16 months following irradiation, or 12 months following ENU treatment). Mortality was

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scored as tumour-related if the tumour was large (1 g) or if secondary pathophysiology was present. Non-tumour-related morbidity occurred in ~10% of animals, independent of genotype, and consisted primarily of ocular and skin lesions. Tumours greater than 1 mm in size were quantified. Haematoxylin-and-eosin stained sections were separately assessed by M.L.F. and C.J.K. and discrepancies of interpretation were resolved through an independent pathologist's review. Separate sections were immunostained after antigen retrieval over a steaming citrate (0.01 M, pH6) bath, followed anti-p27 monoclonal antibody (1:50; NeoMarkers), biotinylated anti-IgG1 secondary antibody (1:200; Southern Biotech), StreptABC-HRP (DAKO), and colour development using DAB/NiCl.

Biochemical analysis

Tumours were selected for biochemical analysis if they were of sufficient size and histological homogeneity (>95% homogeneity for lung and pituitary adenomas). Intestinal tumours selected for analysis consisted of pedunculated polyps which could be easily excised away from their stalk of normal tissue. Protein extracts were prepared from frozen specimens by homogenization in buffer containing protease inhibitors. Protein content was standardized between samples by Bradford assay (BioRad) and by staining the gel with Coumassie blue. p27 protein expression was determined through immunoblot using a rabbit polyclonal anti-p27 antisera as described8. Autoradiographs of p27 immunoblots were scanned and quantified (NIH Image). A p27 standard curve generated by serial dilution was used to ensure that all samples were within the linear range of the assay. p27 protein levels were then normalized to the amount of tubulin protein present on the same filters (anti-tubulin monoclonal antibody; BAbCo). Tumours were genotyped by Southern blot with the 3W p27 genomic probe, using XbaI and XhoI restriction digests, as described9. Tumours were also sequenced using PCR to amplify and the two coding exons of p27 from 20 ng tumour DNA with nested primers. Fifteen cycles were performed using primers K15 (5'-TTCGAAGAGGGTTTTGCGCTCCAT-3W) and K26 (5'-CCAGATGGGGTGTCAGTTTTGTGT3'), followed by 25 cycles with K16 (5'-GAGAGGCGAGGCGGTGGTCC-3') plus K25 (5'-GCTGTTTACGTCTGGCGTCGA-3'). Dye-terminator cycle sequencing (Perkin Elmer) was used with primers for both coding exons. Sequences were compared with the published complementary DNA sequence for mouse p27 and with sequence data we obtained from wild-type 129/Sv and C57BL/6J mice30. C57BL/6J wild-type mice had the following p27 polymorphism: 66T→G, D22E; 422A→C, Q141P.

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References

1. Polyak K, et al. $p27^{Kip1}$, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. 1994; 8:9–22. [PubMed: 8288131]
2. Nourse J, et al. Interleukin-2-mediated elimination of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. Nature. 1994; 372:570–573. [PubMed: 7990932]

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3. Kato JY, Matsuoka M, Polyak K, Massague J, Sherr CJ. Cyclin AMP-induced G1 phase arrest mediated by an inhibitor ($p27^{Kip1}$) of cyclin-dependent kinase 4 activation. Cell. 1994; 79:487–496. [PubMed: 7954814]
4. Coats S, Flanagan WM, Nourse J, Roberts JM. Requirement of $p27^{Kip1}$ for restriction point control of the fibroblast cell cycle. Science. 1996; 272:877–880. [PubMed: 8629023]
5. Fero ML, et al. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in $p27^{Kip1}$-deficient mice. Cell. 1996; 85:733–744. [PubMed: 8646781]
6. Porter PL, et al. Expression of cell-cycle regulators $p27^{Kip1}$ and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nature Med. 1997; 3:222–225. [PubMed: 9018243]
7. Loda M, et al. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nature Med. 1997; 3:231–234. [PubMed: 9018245]
8. Esposito V, et al. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. Cancer Res. 1997; 57:3381–3385. [PubMed: 9270000]
9. Yang RM, et al. Low p27 expression predicts poor disease-free survival in patients with prostate cancer. J. Urol. 1998; 159:941–945. [PubMed: 9474188]
10. Yasui W, Kudo Y, Semba S, Yokozaki H, Tahara E. Reduced expression of cyclin-dependent kinase inhibitor $p27^{Kip1}$ is associated with advanced stage and invasiveness of gastric carcinomas. Jpn. J. Cancer Res. 1997; 88:625–629. [PubMed: 9310133]
11. Pietenpol JA, et al. Assignment of the human $p27^{Kip1}$ gene to 12p13 and its analysis in leukemias. Cancer Res. 1995; 55:1206–1210. [PubMed: 7882309]
12. Kawamata N, et al. Molecular analysis of the cyclin-dependent kinase inhibitor gene $p27^{Kip1}$ in human malignancies. Cancer Res. 1995; 55:2266–2269. [PubMed: 7757974]
13. Morosetti R, et al. Alterations of the $p27^{Kip1}$ gene in non-Hodgkin's lymphomas and adult T-cell leukemia/lymphoma. Blood. 1995; 86:1924–1930. [PubMed: 7655021]
14. Spirin KS, et al. p27/Kip1 mutation found in breast cancer. Cancer Res. 1996; 56:2400–2404. [PubMed: 8625318]
15. Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl Acad. Sci. USA. 1971; 68:820–823. [PubMed: 5279523]
16. Dryja TP, et al. Homozygosity of chromosome 13 in retinoblastoma. N. Engl. J. Med. 1984; 310:550–553. [PubMed: 6694706]
17. Baker SJ, et al. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science. 1989; 244:217–221. [PubMed: 2649981]
18. Kamb A, et al. A cell cycle regulator potentially involved in genesis of many tumor types. Science. 1994; 264:436–440. [PubMed: 8153634]
19. Harvey M, et al. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nature Genet. 1993; 5:225–229. [PubMed: 8275085]
20. Serrano M, et al. Role of the INK4a locus in tumor suppression and cell mortality. Cell. 1996; 85:27–37. [PubMed: 8620534]
21. Hu N, et al. Heterozygous Rb-1/+ mice are predisposed to tumors of the pituitary gland with a nearly complete penetrance. Oncogene. 1994; 9:1021–1027. [PubMed: 8134105]
22. Jacks T, et al. Effects of an Rb mutation in the mouse. Nature. 1992; 359:295–300. [PubMed: 1406933]
23. Takeuchi S, et al. Frequency loss of heterozygosity in region of the KIP1 locus in non-small cell lung cancer: evidence for a new tumor suppressor gene on the short arm of chromosome 12. Cancer Res. 1996; 56:738–740. [PubMed: 8631006]
24. Stegmaier K, et al. Mutational analysis of the candidate tumor suppressor genes TEL and KIP1 in childhood acute lymphoblastic leukemia. Cancer Res. 1996; 56:1413–1417. [PubMed: 8640833]
25. Hayette S, et al. Molecular analysis of cyclin-dependent kinase inhibitors in human leukemias. Leukemia. 1997; 11:1696–1699. [PubMed: 9324291]
26. Tang B, et al. Transforming growth factor-B1 is a new form of tumor suppressor with true haploid insufficiency. Nature Med. 1998; 4:802–807. [PubMed: 9662371]

27. Bouffler SD, Kemp CJ, Balmain A, Cox R. Spontaneous and ionizing radiation-induced chromosomal abnormalities in p53-deficient mice. Cancer Res. 1995; 55:3883–3889. [PubMed: 7641208]

28. Gottlieb E, et al. Transgenic mouse model for studying the transcriptional activity of the p53 protein: ageand tissue dependent changes in radiation-induced activation during embryogenesis. EMBO J. 1997; 16:1381–1390. [PubMed: 9135153]

29. Clarke AR, et al. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature. 1993; 362:849–852. [PubMed: 8479523]

30. Polyak K, et al. Cloning of p27^{Kip1}, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell. 1994; 78:59–66. [PubMed: 8033212]

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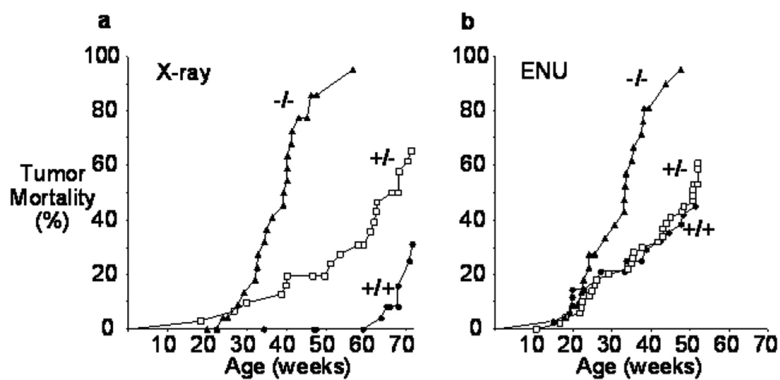

Figure 1.

Tumour-induced mortality according to p27 genotype. **a,** γ-irradiation- induced tumour mortality (all tumour types) is increased in both $p27^{-/-}$ and $p27^{+/-}$mice. **b,** ENU-induced tumour mortality is increased in $p27^{-/-}$ mice. Non-tumour-related mortality was censored at the time of death (see Methods).

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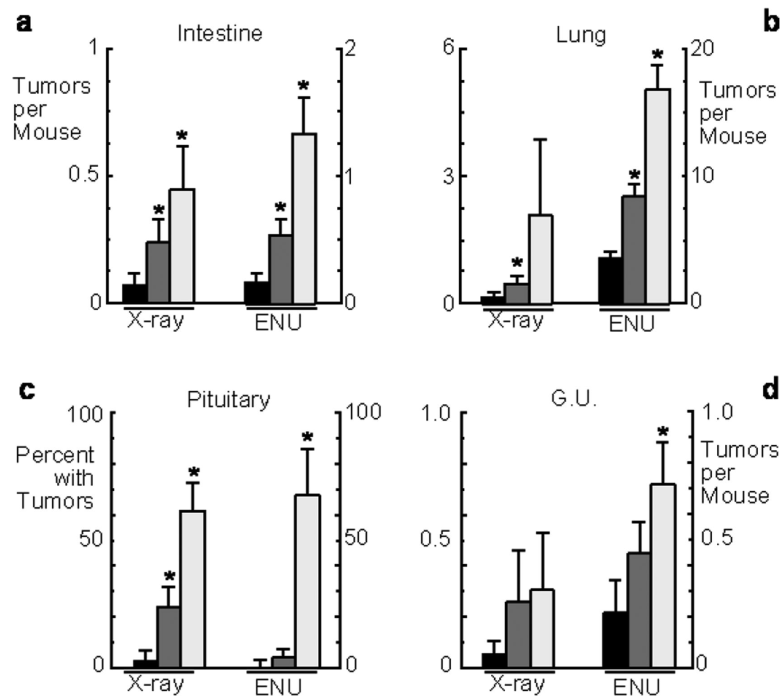

Figure 2.

Cumulative occurrence of specific tumour types. The mean number of tumours (± s.e.m.) per mouse (tumour multiplicity) following treatment with either γ-irradiation or ENU is plotted for $27^{+/+}$ (black bars), $p27^{+/-}$ (grey bars), and $p27^{-/-}$(striped bars) mice. **a,** Intestinal adenomas; **b,** lung adenomas; and d, tumours of the female reproductive tract. (Asterisks indicate □ , 0X05 compared with wild- type by Wilcoxon rank sum test.) **c,** The occurrence of pituitary tumours is plotted as percentage incidence. (Asterisk indicates P< 0.05 compared with wild-type controls by chi-squared test.)

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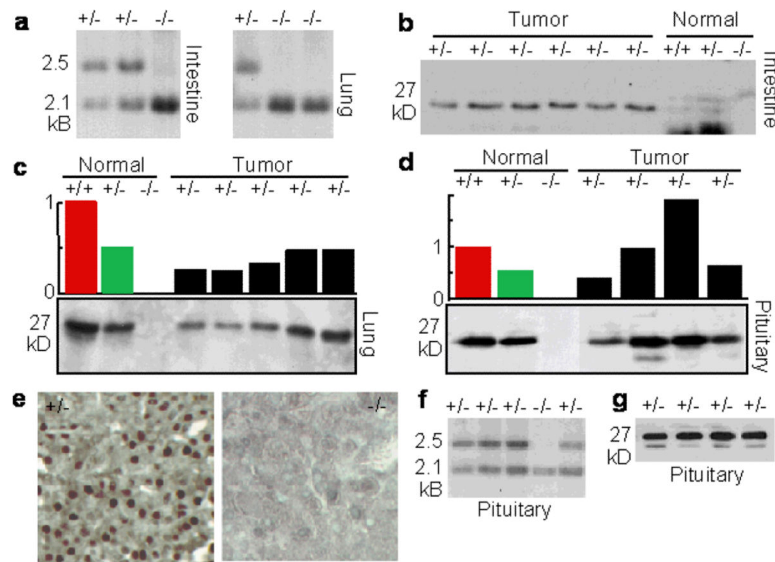

Figure 3.

Analysis of tumours from p27$^{+/-}$ mice. **a,** Representative Southern blots of ENU-induced intestinal and lung adenomas are shown with the genotype of the mouse listed above. The wild-type allele corresponds to the 2.5-kb band, and the knockout allele to the 2.1-kb band. No evidence of allelic loss is seen in p27$^{+/-}$ tumours. **b-d,** Immunoblots of p27 protein expression in normal intestine, lung and pituitary, compared with p27 expression in tumours arising in these tissues following ENU treatment (intestine and lung) and γ-irradiation (pituitary). In **c, d,** the expression of p27 was quantified by densitometry followed by normalization to the amount of tubulin present in each sample (see Methods). Plotted are levels of protein in tumours relative to levels in normal, wild-type tissue. kD (kiloDalton), apparent relative molecular mass. **e,** Immunostaining of p27 protein in a p27$^{+/-}$ pituitary tumour induced by g-irradiation (compared with p27–/– control). The p27 protein is localized to the nuclei of the tumour cells. **f,** Southern blot of spontaneous pituitary adenomas, with the corresponding genotype of the mouse listed above. **g,**Immunoblot of p27 protein expression in the same p27$^{+/-}$ tumours as in f. The amount of p27 protein is similar to that present in normal p27$^{+/-}$ pituitary tissue.