A transgenic mouse bearing an antisense construct of regulatory subunit type 1A of protein kinase A develops endocrine and other tumours: comparison with Carney complex and other PRKAR1A induced lesions


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Background: Inactivation of the human type 1A regulatory subunit (R(1A)) of cyclic AMP dependent protein kinase (PKA) (PRKAR1A) leads to altered kinase activity, primary pigmented nodular adrenocortical disease (PPNAD), and sporadic adrenal and other tumours.

Methods and results: A transgenic mouse carrying an antisense transgene for Prkar1a exon 2 (X2AS) under the control of a tetracycline responsive promoter (the Tg(Prkar1a×x2as)1Stra, Tg(TAHCMV)3Uh or TgA/X2AS line) developed thyroid follicular hyperplasia and adenomas, adrenocortical hyperplasia and other features reminiscent of PPNAD, including late onset weight gain, visceral adiposity, and non-dexamethasone suppressible hypercorticosteronaemia, with histiocytic, epithelial hyperplasias, lymphomas, and other mesenchymal tumours. These lesions were associated with allelic losses of the mouse chromosome 11 Prkar1a locus, an increase in total type II PKA activity, and higher RIIβ protein levels; the latter biochemical and protein changes were also documented in Carney complex tumours associated with PRKAR1A inactivating mutations and chromosome 17 PRKAR1A locus changes.

Conclusion: We conclude that the TgA/X2AS mouse line with a downregulated Prkar1a gene replicates several of the findings in Carney complex patients and their affected tissues, supporting the role of R(1A) as a candidate tumour suppressor gene.

In the heterozygote state, PRKAR1A inactivating mutations cause adrenocorticotropic hormone (ACTH; corticotrophin) independent Cushing’s syndrome due to primary pigmented nodular adrenocortical disease (PPNAD) or Carney complex (CNC), a multiple neoplasia syndrome. Somatic mutations of the PRKAR1 gene have been found in sporadic PPNAD and other adrenocortical tumours and, rarely, in non-medullary thyroid cancer. In CNC associated tumours, and in adrenal and thyroid neoplasms with PRKAR1A down-regulation, allelic losses of the 17q22-24 PRKAR1A chromosomal locus are frequent and are associated with concomitant changes in cAMP stimulated protein kinase (PKA) activity.

PKA is a serine–threonine kinase that mediates cAMP regulation for a variety of cellular processes. The PKA regulatory subunits, PRKAR1A (R(1A)), PRKAR1B (R(1B)), PRKAR2A (R(1R)), and PRKAR2B (R(1β)), and catalytic subunits, PRKACA (Ca), PRKacb (Cb), and PRKARCG (Cγ) form two isoforms of the PKA holoenzyme, type I and type II, named according to their order of elution in DEAE chromatography and consisting of homodimers of either the R(1A) and R(1B), or the R(1R) and R(1β) subunits, respectively. Type I PKA is the physiological mediator of cAMP actions; in basal states, the catalytic subunits bind preferentially to type II regulatory subunits, whereas binding to type I subunits is favoured in stimulated states. Studies, mostly in cancer cell lines, have shown R(1A) overexpression, however, animal models with increased R(1A) levels, such as the Prkar1b-/-, Prkar2a-/-, and Prkar2b-/- mice, have not shown an increased frequency of tumours. Although young heterozygous mice of a mixed genetic background did not have an abnormal phenotype, we recently showed that Prkar1a-/- mice develop mesenchymal tumours at an older age. We also reported development of a variety of tumours in a transgenic (Tg) mouse carrying an antisense sequence for exon 2 of the Prkar1a gene (X2AS-R(1A)) under the control of a tetracycline responsive promoter regulated by the tetracycline transactivator (T) (the Tg(Prkar1a×x2as)1Stra, Tg(TAHCMV)3Uh mouse line, or the TgA/X2AS mice, which we used as a model of Prkar1a downregulation).

In the present investigation, we extend the above observations by presenting a complete phenotyping and biochemical characterisation of the TgA/X2AS mouse and comparing it with histopathology and similar analysis of tissues from patient with Carney complex and PRKAR1A inactivating mutations.

METHODS

Protocols for human and animal research on Carney complex

The institutional review board (IRB) and animal care and use committee (ACUC) of the National Institute for Child Health and Human Development (NICHD) have approved protocols 95CH0059 and ASP 01–003 for human and animal studies.

Abbreviations: ACTH, adrenocorticotropic hormone; BAC, bacterial artificial chromosome; bw, bodyweight; CNC, Carney complex; FISH, fluorescent in situ hybridisation; NIH, National Institutes of Health (USA); PKA, protein kinase A; PPNAD, primary pigmented nodular adrenocortical disease.

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respectively, on CNC. All human specimens studied in the present investigation were obtained under 95CH0059 after obtaining informed consent from CNC patients. The clinical profile of the patients from families CAR01 (YC01) and CAR110 have been reported previously.1,2 The mouse construct has been described elsewhere.20

Mouse dissection and phenotype analysis

For all analyses, the tTA mice and the corresponding MEF lines were considered the control group. All mice were fed and maintained similarly in an inhouse animal facility; they were weighed weekly. For phenotyping, age and sex matched tTA and tTA/X2AS mice were killed approximately every 2 months, from 4 to 18 months of age. Tissue fragments were snap frozen and stored at −70°C until processed for PKA assay activity, and mRNA and protein studies. Most of the tissue from each organ was fixed and paraffin embedded for routine histological analysis using haematoxylin and eosin staining and PKA subunit immunostaining, following protocols described elsewhere.3,4 Pathological lesions were further characterised by staining with appropriate tissue markers. Most histopathological analysis was performed in our laboratory and at Molecular Histology, Inc. (Gaithersburg, MD, USA) or at animal facilities at the NIH (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD, USA and the Division of Veterinary Resources, NIH, Bethesda, MD, USA). Tumour cells were prepared from freshly obtained or frozen specimens (touch preparations) and fixed on siliconised slides for genetic analysis (see below).

Dexamethasone administration and corticosterone assays

From the two groups (tTA/X2AS, n = 10 and control tTA, n = 10) 6 month old mice underwent a 6 day long, low and high dose dexamethasone suppression test based on Liddle’s protocol in humans19 with daily collection at 11.00 of serum for determination of corticosterone. Basal levels of the hormone were collected for 2 days, then dexamethasone (Sigma-Aldrich Inc., St. Louis, MO, USA) was administered following the manufacturer’s instructions. Adrenal glands were removed, dissected, and fixed for 90 minutes (2% OsO4 in 0.1 mol/l cacodylate buffer, 0.1 mol/l phosphate buffer, pH 7.3. Tissue slices were fixed at 70°C until processed for PKA activity determinations. PKA activity determinations

For all analyses, mice expressing tTA only were the control group. Weight comparisons over time were performed by one way analysis of variance followed by Tukey’s multiple comparisons test; p<0.05 was considered significant. PKA assay data from all tissues were compared with Statistica software (StatSoft, Inc., Cary, NC, USA) using Student’s t test for individual comparisons between the two mouse lines. All data are shown as mean (SEM). For PKA subtyping, the activity of individual chromatographic fractions was compared by t test. In all experiments, p<0.05 was considered significant; p<0.01 was interpreted as showing a tendency towards a significant difference.
RESULTS

PKA protein levels in tTA/X2AS mice decreased by 60% and 40% in liver and adrenal tissue, respectively (fig 1A); Western blot analysis from kidney and adrenal tissues were also consistent with an approximately 50% decrease of RIIx protein levels in these mice compared with the same tissues from matched tTA controls (fig 1). The RIIβ protein was increased in tissue lysates from the tTA/X2AS mice, whereas RIIα, RIIα, and Cα levels were all significantly decreased (fig 1A). These changes in the protein levels of PKA subunits in tTA/X2AS mice were comparable with those in adrenal tumours from patients with CNC and PRKAR1A inactivating mutations; these lesions also exhibited an increase in RIIβ (fig 1B). Tissues from the tTA/X2AS mice showed significant PKA activity changes. Overall, there was a marked increase in type II PKA activity (fig 2) with a corresponding increase in the ratio of type II to type I activity (fig 1C). This observation was similar to the type I to type II PKA activity switch that was observed in human adrenal tissue from CNC patients and c.578delTG PRKAR1A inactivating mutation carriers (fig 1D).

At birth, tTA/X2AS mice were not physically different from control animals; they gained weight and length normally. Although as a group, tTA/X2AS mice weighed less than their age and sex matched controls during their first weeks of life (p<0.05) (fig 3A), there were no weight differences between the two groups during the ensuing 12 months. Control mice reached a plateau in their weight gain by approximately 6 months of age, while tTA/X2AS mice continued to gain weight and length normally. Although as a group, tTA/X2AS mice weighed less than their controls, they gained weight and length normally. At birth, tTA/X2AS mice were not physically different from control animals; they gained weight and length normally. Although as a group, tTA/X2AS mice weighed less than their age and sex matched controls during their first weeks of life (p<0.05) (fig 3A), there were no weight differences between the two groups during the ensuing 12 months. Control mice reached a plateau in their weight gain by approximately 6 months of age, while tTA/X2AS mice continued to gain weight and length normally.

Histological abnormalities in the thyroid and adrenal glands, lymphoproliferative disease, and mesenchymal tumours developed in some mice as early as 4–6 months of age.\(^\text{16}\) Death rate differences became significant after 16 months of age;\(^\text{16}\) the most frequent cause of death was a pulmonary or kidney condition related to a lymphoma, histiocytic sarcoma, another lymphoproliferative or histioproliferative syndrome, or a mesenchymal tumour.

Thyroid follicular hyperplasia, adenomas, and cysts were present in 40% of the tTA/X2AS mice but in none of the controls. Mouse thyroid adenoma cells demonstrated loss of the chromosome Prkar1a 11 locus and decreased RIIx immunostaining (fig 4A–K). These histological and genetic changes are similar to those seen in the thyroids of CNC patients with the c578delTG and 769C→T PRKAR1A inactivating mutations (fig 4L–P).

Adrenocortical abnormalities in the transgenic animals were of two types: although pigment alone was not more frequent in tTA/X2AS mice, cortical hyperplasia with pigment deposits and congestion were seen more frequently in tTA/X2AS mice (p<0.05). X zone persistence and hyperplasia, after puberty in males, or multiple pregnancies in females, occurred exclusively in the tTA/X2AS animals and was more prominent in female mice (fig 5A, B). These changes are reminiscent of, but not identical to those of human adrenal glands affected by PPND (fig 5C, D). Mouse and human adrenal cells from tTA/X2AS mice and c.578delTG PRKAR1A inactivating mutation carriers demonstrated allelic losses of the mouse chromosome 11 Prkar1a and human chromosome 17q22-24 PRKAR1A loci, respectively (fig 5E–I).

Sections of the adrenal cortex from tTA/X2AS mice with abnormalities such as hyperplasia, congestion, and X zone vacuolisation (fig 5J, K) were examined by EM. Compared with those from tTA mice, tTA/X2AS adrenocortical cells showed cytoplasm with ample smooth endoplasmic reticulum, an increased number of round mitochondria with characteristic tubulovesicular internal membranes that often

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**Figure 1** PKA subunit and activity levels after Prkar1a downregulation. Decrease in RIIx and increase in RIIβ protein levels in mouse (A) and human (B) adrenals from mice with downregulated Prkar1a (tTA/X2AS), and from CNC patients with c.578delTG PRKAR1A inactivating mutations, respectively. This was associated with an increase in the ratio of type II to type I PKA activities in mouse (C) and human (D) adrenals from tTA/X2AS mice and CNC patients with c.578delTG PRKAR1A inactivating mutations, respectively. *p<0.05; **p<0.001.
bridged the mitochondrial matrix, and a reduced number of cholesterol storing liposomes (fig 5L, M, and fig 6). Cells from the tTA/X2AS mice also had frequent and large pigment deposits and often other, vesicle-like structures (fig 6B). In addition, in tTA/X2AS adrenal glands, extra-epithelial pigment cells containing melanosomes were found in direct cellular contact with adrenocortical cells (fig 5M, arrows). Immunostaining of tTA/X2AS adrenocortical cells from areas with the above abnormalities showed a decrease in RIIα (fig 7A, B, P, R) and an increase in RIIβ (fig 7K, L, Q, S), but...
no changes in RIIβ expression (fig 7F, G); these alterations corresponded to the PKA subunit staining of adrenal glands with PPNAD from c.578delTG carrying CNC patients (fig 7E, J, O, X, Y); this pattern was not observed in either normal human adrenocortical tissue (fig 7C, H, M, T, U) or ACTH induced hyperplastic adrenal cortex (Fig 7D, I, N, V, W).

Histiocytic hyperplasia, sarcomas, and lymphomas also developed in tTA/X2AS mice.16 Although lymphomas or leukaemias have not been recorded in the US National Institutes of Health (NIH) and Mayo Clinic registry of more than 400 patients with CNC, we were prompted by the tTA/X2AS mouse data to investigate this, and identified two cases.
of ectopic, hyperplastic thymic tissue among the four patients with CNC and thyroid tumours who underwent surgery at the NIH (fig 8). In addition, large, macroscopically visible, and occasionally metastatic tumours grew in tTA/X2AS mice. Most of these lesions were of mesenchymal origin and corresponded to tumours seen in CNC patients (fig 9).

**DISCUSSION**

Our study extends our previous findings, and those of Amieux et al. and Kirschner et al. Overall, there were significant similarities between the phenotype of tTA/X2AS mice and CNC patients. Firstly, there was a high incidence of thyroid lesions in the tTA/X2AS mice, which are extraordinarily rare in wild type...
similar to thyroid tumours with PRKAR1A mutations and loss of the corresponding chromosome 17q22-24 PRKAR1A region, thyroid lesions from tTA/X2AS mice demonstrated loss of the Prkar1a locus on mouse chromosome 11 (fig 4).

Secondly, the tTA/X2AS mice had mild hypercorticoestrogenaemia that led to a chronic Cushing's syndrome-like

Figure 6  Electron micrographs of adrenocortical cells from tTA/X2AS mice. (A) Significantly more mitochondria than liposomes (LIP) are present in tTA/X2AS adrenocortical cells. The internal membranes of mitochondria (MIT) frequently bridge the mitochondrial matrix (arrows). (B) In close proximity to the plasma cell membrane, a subset of adrenocortical cells in tTA/X2AS demonstrate pigment deposition and vesicle-like structures (arrow). Bar=1 μm.

Figure 7  PKA subunit immunostaining of adrenal cortex from control mice (A, F, K; ×20), tTA/X2AS mice (B, G, L; ×20), normal human adrenal cortex (C, H, M; ×10), ACTH induced hyperplasia (D, I, N; ×10), and primary pigmented nodular adrenocortical disease (PPNAD) from a patient with the c.578delTG PRKAR1A mutation (E, J, O; ×10). (A–E) RIIα, (F–J) RIIβ, and (K–O) RIIι. RIIι stains more intensely in hyperplastic or nodular tissue. Higher magnifications are shown, stained for RIIα (P, Q, R, T, V, X) and RIIι (O, S, U, W, Y) from tTA/X2AS mice (P, Q; R, S; ×40), human normal adrenal cortex (T, U, ×20), cortex from ACTH induced hyperplasia (V, W; ×20), and PPNAD (X, Y; ×20). The arrows point to nodules forming in tTA/X2AS mouse adrenocortical tissue (P, R), which become apparent with the relative decrease in RIIα staining, whereas RIIι staining is either normal or increased. In normal or hyperplastic human adrenal cortex, there were no changes in RIIα or RIIι staining; in contrast, in PPNAD, arrows point to an area of decreased RIIα staining (X) that corresponds to an adrenocortical nodule clearly identified by the increased RIIι staining (Y).

Figure 8  Haematoxylin and eosin staining of ectopic thymic tissue (×40) found in the thyroid of a patient with CNC, a carrier of the c.769C→T PRKAR1A inactivating mutation; his thyroid tumour is shown in fig 7L.
Fourthly, the pattern of alterations of PKA activity in liver, testes, and adrenals from tTA/X2AS mice were generally similar to those seen in tumours from CNC patients, or with spontaneous mutations in the PRKAR1A gene (figs 1 and 2).

The greatest difference between the phenotypes of CNC patients and tTA/X2AS mice was that of the frequent lymphoid hyperplasia and lymphomas in the latter. However, laboratory mice of many backgrounds (including those of the C57BL/6 background that was used here) tend to develop lymphomas and other haematological pathology more frequently than other types of tumours. Indeed, Tp53 mutant mice develop lymphoproliferative disease prior to findings suggestive of Li-Fraumeni or other human syndromes caused by TP53 mutations. While CNC patients are not known to develop lymphomas (in a database that we maintain of more than 400 patients with the disease, none has died of a haematological malignancy), a number of patients with Cushing’s syndrome due to PPNA and evaluated at the NIH Clinical Center have been noted to have enlarged thymus on chest MRI (unpublished observations). Fig 8 shows the histology of hyperplastic thymic tissue from the thyroid of the patient that had the tumour shown in fig 4L and was a carrier of the c.769C→T PRKAR1A mutation. Of the four CNC patients with thyroid tumours that have been operated at the NIH Clinical Center over the last 20 years, two had ectopic thymic tissue such as the one shown in figure 8. This is an otherwise extraordinarily rare event. It should be noted that both patients also had PPNA and subclinical Cushing’s syndrome at the time of their thyroid operation, which should have made their thymic tissue atrophic.

There are also some subtle differences in the amounts of cAMP PKA activities between this mouse model and CNC tumours. While there is a minimal decrease in the type I peak in the tTA/X2AS mouse, there is a dramatic increase in the type II activity (fig 2). One of the significant problems of any effort to reproduce the CNC PKA biochemical phenotype in rodents is the apparent greater percentage of type II PKA activity in murine cells. This may make the decrease in type I activity more difficult to detect, particularly as free catalytic subunits, which would be expected to increase in any state of Ptkarl down-regulation, co-elute with the type I peak. Despite these problems, the tTA/X2AS mice showed the expected further increase in type II PKA activity in almost all tissues (figs 1 and 2).

What is the molecular mechanism leading to tumours in these mice? Their tissues showed an abnormal cAMP response, a switch to mostly type II PKA activity and an increase in RIIβ subunit. It has been suggested that type I PKA is associated with growth and proliferation, whereas type II PKA is associated with increased differentiation and decreased proliferation. However, primary cultures of melanocytes and mammary cells (with mostly type II PKA) are stimulated by cAMP, whereas the mouse Cloudman melanoma and human breast carcinoma lines (with mostly type I PKA) are inhibited by cAMP. Furthermore, the switch to type I PKA that was recorded in proliferating cancer cell lines was dependent on high, pharmacologically induced levels of cellular cAMP. Indeed, most cells respond to high cAMP levels with inhibition of growth, but some, such as lymphocytes and melanoma cells, are actually stimulated by low cAMP levels. Thus, it is not premature to say that the dysregulated cAMP response of PKA activity in Ptkarl and tTA/X2AS cells is at least in part responsible for the pathology we observed. Additionally, there may also be some PKA independent effects of Ptkarl that contributed to the phenotype.
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