SHORT REPORT

A pleiomorphic GH pituitary adenoma from a Carney complex patient displays universal allelic loss at the protein kinase A regulatory subunit 1A (*PRKARIA*) locus

I Bossis, A Voutetakis, L Matyakhina, S Pack, M Abu-Asab, I Bourdeau, K J Griffin, N Courcoutsakis, S Stergiopoulos, D Batista, M Tsokos, C A Stratakis

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Carney complex (CNC) is a familial multiple endocrine neoplasia syndrome associated with GH-producing pituitary tumours and transmitted as an autosomal dominant trait. Mutations of the *PRKARIA* gene are responsible for approximately half the known CNC cases but have never found in sporadic pituitary tumours. Pituitary tissue was obtained from an acromegalic CNC patient heterozygote for a common (*PRKARIA*)₁-inactivating mutation. Both immunohistochemistry and electron microscopy showed a highly pleiomorphic pituitary adenoma. The cell culture population appeared morphologically heterogeneous and remained so after more than 30 passages. The mixture was comprised of cells strongly immunostained for GH, spindle-shaped myofibroblast-like cells, and cuboid cells with large axonal projections (negative for GH). The population appeared to have both epithelial and mesenchymal cells. Both at baseline and at passage 30, cytogenetic analysis indicated the presence of normal 46, XY diploid karyotype, whereas losses of the *PRKARIA*₁ locus were demonstrated in more than 98% of the cells by fluorescent in situ hybridisation, supporting this gene's involvement in pituitary tumorigenesis. Allelic loss may have occurred in a single precursor cell type that differentiated and clonally expanded into several phenotypes. Epithelial-to-mesenchymal transition may also occur in CNC-associated pleiomorphic pituitary adenomas.

Carney complex (CNC) has been described as the “complex of spotty skin pigmentation, myxomas, and endocrine overactivity”.^{1,2} A variety of endocrine and non-endocrine tumours have been reported in CNC patients.^{3,4} The pituitary gland is frequently affected in CNC and the clinical features are reminiscent of McCune-Albright syndrome (MAS): despite frequent abnormalities of growth hormone (GH) and prolactin (PRL) secretion, clinical acromegaly or significant hyperprolactinaemia and GH- or PRL-producing tumours, respectively, are relatively rare.^{4,5}

Two different genetic loci associated with CNC have been localised on chromosomes 2 (2p16) and 17 (17q22–24) by linkage analysis.^{6–8} We recently identified the responsible gene on 17q22–24,⁹ *PRKARIA*, which encodes type 1a regulatory subunit of the cAMP-dependent protein kinase A (PKA). Overall, inactivating mutations of this gene have been observed in approximately half of CNC patients.¹⁰ Most *PRKARIA* mutations result in premature termination of the predicted protein. In addition, *PRKARIA* mRNAs bearing a premature stop codon are unstable due to nonsense-mediated mRNA decay.¹⁰ The presence of inactivating mutations and/or the absence of the mutant protein and

the reported loss of the wild type allele in CNC lesions⁹ suggested that *PRKARIA* may function as a tumour-suppressor following Knudson's “two hit” theory.¹¹

In an effort to elucidate *PRKARIA*'s role in pituitary tumorigenesis, we studied a pleiomorphic GH-producing pituitary adenoma that was excised from a patient with CNC who carried a common *PRKARIA* mutation.⁹ We then established a primary culture and used the genetic status of the *PRKARIA* locus as a genotypic marker of tumour transformation. The proportion and phenotype of cells carrying *PRKARIA* changes was documented in subsequent passages. The normal allele of *PRKARIA* was lost in cells of variable morphology both at baseline and in late passages. The immunostaining features and genetic findings on this unique tumour point to a role for *PRKARIA* in pituitary tumorigenesis in CNC, and suggest the possibility of *PRKARIA*'s involvement in endocrine–mesenchymal pituitary interactions in this process.

METHODS

Subject and protocol

A male CNC patient, 35 years old, with acromegaly and a pituitary macroadenoma (identified by magnetic resonance imaging) underwent transsphenoidal surgery (TSS). The subject's photograph, his picture is included in fig 1 in the report of Kirschner *et al*⁹ family history, and peripheral blood sequencing data have been published before.⁹

The patient's blood and tumour samples were collected under a protocol approved by the NICHD Institutional Review Board, and an informed consent was obtained. Tissue from the patient's pituitary tumour was collected at surgery. Parts of the tumour were submitted for routine pathological analysis with immunostaining for all pituitary hormones, chromogranin A and reticulin, DNA extraction (for loss of heterozygosity (LOH) and sequencing analysis), and for cell culture.

Electron microscopy (EM)

Tissue pieces were removed from a paraffin block, deparaffinated in xylene, placed in absolute ethanol, and embedded in LR White (SPI, West Chester, PA). Ultrathin sections were mounted on 150 mesh uncoated nickel grids.

Abbreviations: BAC, bacterial artificial chromosome; CNC, Carney complex; EM, electron microscopy; EMT, epithelial-mesenchymal transition; FISH, fluorescence in situ hybridisation; FSH, follicular stimulating hormone; GH, growth hormone; LH, luteinising hormone; LOH, loss of heterozygosity; MAS, McCune-Albright syndrome; MEN 1, multiple endocrine neoplasia type 1; PKA, protein kinase A; *PRKARIA*, protein kinase A regulatory subunit type 1A; PRL, prolactin; TSH, thyroid stimulating hormone; TSS, transsphenoidal surgery

Grids were floated on blocking solution (PBS, 0.1% Tween 20, 0.5% cold-water fish gelatin [Ted Pella, Redding, CA]) for 20 min, incubated for 1 h with the primary antibody, rinsed in blocking buffer for 5 min, blocked with 2% goat serum, rinsed with blocking buffer, then incubated with 10 nm gold-conjugated secondary goat antibody (Ted Pella, Redding, CA), rinsed in PBS, and air dried. For double labelling, the same procedure was repeated for the second antibody, and the secondary antibody-conjugate was larger than the first. The dilution for the LH antibody (ASP55951889; obtained from the National Institute of Diabetes and Kidney Disorders, Bethesda, MD) was 1:1600 and was labelled with 10 nm secondary gold conjugate antibody. The dilution for the GH antibody (ASPC11981A; also obtained from the National Institute of Diabetes and Kidney Disorders, Bethesda, MD) was 1:4000; it was also labelled with 10 nm secondary gold conjugate antibody.

Sections were stained with aqueous uranyl acetate and examined with a Phillips CM10 electron microscope.

Cells in culture (see below) were processed for EM after detachment and centrifugation of each studied passage and stained as above.

Cell culture and immunostainings

For culture, the solid tumour was minced and after brief trypsin digestion, dispersed cells were plated in DMEM/F12 supplemented with 15% fetal bovine serum and 5% horse serum. Medium was changed every 3 days and cells were split once a week at 1.5–2 ratio. For immunocytochemistry, cells were grown on poly-L-lysine coated glass slides, and fixed in formalin and somatotrophs visualised using a polyclonal GH antibody and the ABC kit (Vector Labs, Burlingame, CA).

As controls, cell cultures from a heart myxoma and periadrenal tissue from two CNC patients, one with a previously described¹⁰ *PRKARIA*-inactivating germline mutation, and one without any *PRKARIA* coding sequence mutations, and from a breast biopsy from a normal 46, XY male with gynecomastia were cultured for an equal number of passages and analysed by the methods described below.

Karyotype, fluorescent in situ hybridisation (FISH), and DNA studies

Karyotyping and FISH were performed on chromosome preparations made from exponentially growing cultures treated for 1.5 h with 0.1 µg/ml colcemid. After harvesting and treatment with hypotonic solution, the cell pellet was fixed in methanol:glacial acetic acid (3:1) and dropped onto cold slides. For karyotyping, chromosome counts were done on well-spread metaphases stained for Giemsa bands.

FISH was performed as described elsewhere.¹² The probe that was used was a bacterial artificial chromosome (BAC) containing the *PRKARIA* gene, which has been described before.⁹ It was labelled by nick translation with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, IN) for 2.5 h at 15°C. A chromosome 17-specific centromeric α -satellite probe labelled with biotin-16-dUTP (Vysis, Donners Grove, IL) was used for chromosome identification. Fluorescence images were automatically captured and merged using IPLab Spectrum software (Scanalytics, Fairfax, VA) on a PowerPC 8500/150, as described before.¹²

For loss of heterozygosity (LOH) analysis, DNA from the tumour was analysed along with a paired DNA sample from peripheral blood. Seven microsatellite markers located on 17q22–24 were used as previously described.⁹ PCR was performed to amplify the region flanking 17q22–24 in the presence of [α -³²P]dCTP and analysed by electrophoresis through 6% polyacrylamide gel.

RESULTS

Genetic, immunostaining, EM, and FISH studies of the pituitary adenoma

A CNC patient (member of the CAR20 kindred)¹⁰ with the most common germ-line *PRKARIA*-inactivating mutation, c.578delTG, presented with acromegaly and a GH-producing pituitary adenoma. Parts of the tumour were used for DNA analysis and cell culture. LOH analysis (by 17q22–24 microsatellite markers) indicated that the tumour was composed of cells with only the mutant *PRKARIA* allele (data not shown), as has been shown before for a number of tumours from patients with CNC and germline *PRKARIA* mutations, including one pituitary tumour from a patient carrier of the c.578delTG mutation that belonged to another family.⁹

A highly variable picture was seen in the excised fragments of the pituitary gland after immunostaining for pituitary hormones. A tumour and several tumourlets were identified surrounded by areas of hyperplasia, as has been described in other somatotrophinomas from patients with CNC.¹³

Tumour cells stained mainly with antibodies for GH and LH β -subunit, although within the tumour there were areas of compact cells that did not stain for any hormone (fig 1). Within hyperplastic areas that surrounded the main tumour sections, all cells were stained for hormones; most cells in these areas stained for GH, PRL, LH β -subunit and weakly for α -subunit and the FSH β -subunit (fig 2).

Figure 1 Pleiomorphic pituitary tumour from a patient with CNC and the c.578delTG *PRKARIA*-inactivating mutation: (A) haematoxylin and eosin, (B) reticulin, (C) GH, (D) PRL, (E) β -subunit LH (luteinising hormone), (F) β -subunit FSH (follicular stimulating hormone), (G) α -subunit, and (H) β -subunit TSH ($\times 10$). The tumour includes several areas of compact cells that do not stain with any pituitary hormones.

Figure 2 Hyperplastic area from the areas proximal to the tumour shown in fig 1: (A) reticulin, (B) GH, (C) PRL, (D) β -subunit LH, (E) α -subunit, and (F) β -subunit FSH ($\times 40$); the β -subunit TSH staining was almost identical to that of FSH. Hyperplasia, unlike the tumour (seen in the upper right hand corner of each of these sections), does not contain any non-hormone staining cells, although GH-, PRL- and LH-positivity predominate.

FISH analysis of frozen tumour preparations (prepared at the time of resection and from within the adenoma) with a BAC containing the *PRKARIA* gene confirmed allelic loss of the 17q22–24 *PRKARIA* locus (data not shown; the image is similar to that in fig 6 showing the same result in cultured tumour cells).

EM studies also showed considerable morphologic and immunostaining variability (fig 3). Most tumour cells stained intensely for both GH and LH (fig 4), while other tumour cells did not stain for any hormones. The GH-producing cells had EM characteristics similar to those described before in patients with CNC,¹⁴ with prominent Golgi and rough endoplasmic reticulum, and typical granules. However, more than half of the cells examined by EM did not have that typical appearance and did not stain for GH, LH, or any other hormone. It is noteworthy that there was considerable variability in both the morphology and size of the secretory granules among the non-GH- and non-LH-staining cells. Normal pituitary cells were not seen.

Pituitary adenoma culture: genetic, EM, and immunostaining studies

A primary culture was successfully established from the tissue fragments obtained from within the tumour. On passage 2, the population was heterogeneous and composed of GH-immunoreactive cells (about 50% of all cells), spindle-shaped myofibroblast-like cells with peripheral fusiform densities and well-developed attachments (approximately 20% of the cells), and light-reflecting cuboid cells with large axonal projections resembling a neuronal phenotype (about 30%) (fig 5A). Thus, the population appeared to have cells of both epithelial and mesenchymal origin. After about 30 passages cellular morphology did not change; however, the number of cells that were immunoreactive for GH (fig 5B)

Figure 3 EM picture of pituitary tumour cells with variable morphology. There are secretory granules of different types in most cells, but not in all. Other cells have variable phenotypes, consistent with the morphology and immunostaining of the primary culture cells (see fig 5) ($\times 27\,500$).

declined to about 2%. The proportion of spindle-shaped myofibroblast-like cells was approximately 80%, while the remaining cells were those with a neuronal-like phenotype. These cells were chromogranin A-negative (data not shown).

Karyotypic analysis at passage 30 indicated normal karyotype (46, XY): at least 200 G-banded metaphase spreads were examined and no structural abnormalities were found (data not shown). However, FISH analysis showed allelic loss at the *PRKARIA* locus in more than 98% of the cells (fig 6). At about passage 40, replicative senescence occurred and the cell culture gradually deteriorated.

It is noteworthy that control cell cultures of fibroblasts and other mesenchymal cells established from a heart myxoma, perirenal tissue, and a breast biopsy, cultured for up to 40 passages and subjected to hybridisation studies with the *PRKARIA*-containing probe did not show spontaneous loss of the 17q22–24 locus regardless of the *PRKARIA* mutation status (fig 7).

DISCUSSION

The mechanisms underlying pituitary tumorigenesis are largely unknown. This is primarily due to the lack of human

Figure 4 Double immunogold staining with GH- and LH- β -subunit-specific antibodies showed that the tumour cells with the larger secretory granules were GH and LH co-secretors ($\times 4900$), consistent with the paraffin-embedded section immunostaining results (fig 1). Other cells were also positive for PRL and α -subunit (data not shown).

Figure 5 Morphology of cultured cells derived from the GH-producing pituitary tumour. (A) On the first passage of the culture, the population was heterogeneous and composed of round pituicytes, myofibroblast-like cells with well-developed attachments, and light-reflecting cuboid cells with large axonal projections (neuronal-like phenotype). (B) Immunocytochemical analysis indicated that about 50% of the cells were GH-producing.

pituitary cell lines and the difficulty in obtaining pre-tumourous pituitary tissue. Pituitary tumours are mainly benign adenomas, grow slowly, and can be rather small when they first form, and biopsies are not generally clinically indicated. These obstacles have hindered our efforts in understanding the early stages of tumorigenesis and assessing the clonal origin of pituitary tumours.

Earlier studies utilising allelotypes of microsatellite polymorphisms and X chromosome inactivation analysis have indicated that pituitary tumours are largely monoclonal.^{15–16} However, recent studies showed that some pituitary tumours may be polyclonal.^{17–18} The universal loss of the *PRKARIA*

Figure 6 FISH analysis of the cultured pituitary tumour cells. The picture shows allelic loss of BAC321-G-8 containing the *PRKARIA* gene (green, one signal), whereas two signals from the chromosome 17-specific centromeric α -satellite probe (red) are seen. A normal cell is also shown (on the left) that has the expected two signals from each probe.

allele in the present case indicated that this pleiomorphic tumour was most likely of monoclonal origin although definite proof of monoclonality could not be obtained by other means (for example X chromosome studies), since the subject was a male.

Other studies with cells derived from tumours such as Wilms' embryonic tumours, colonic neuroendocrine tumours, and multiple endocrine neoplasia type 1 (MEN 1)-associated pituitary adenomas have shown that homogeneous epithelial cells trans-differentiate to benign mesenchymal cells after several passages in culture.^{19–21} In the tumour that we studied, such epithelial-to-mesenchymal transition (EMT) was present in LOH-bearing, presumably tumour-derived cells, and it is possible that it occurred in vivo, since it was evident from the first passage. It is of course impossible to prove that EMT indeed occurred in vivo. The pleiomorphism of the tumour, however, in the initial, diagnostic immunohistochemistry and the EM and immunostaining studies of both the paraffin-embedded specimens and the cell line were supportive of a highly heterogeneous cellular population both at baseline and in the primary culture.

There are two other possibilities for the observed phenomena. First, it is possible that the capacity of mesenchymal cells for proliferation was at the outset greater than that of the somatotrophs. If this was the case, the transition that we observed was an early rather than a continuous phenomenon and in serial passages mesenchymal cells would outgrow the somatotrophs anyway. However, whether early or late, our

Figure 7 FISH analysis of primary cultures of mesenchymal cells that have retained both copies of the BAC321-G-8 containing the *PRKARIA* gene after several passages in culture: (A) periadrenal and (B) heart myxoma from two CNC patients, one with a previously described¹⁰ *PRKARIA*-inactivating germline mutation, and one with no *PRKARIA* coding sequence mutations, respectively, and (C) a breast biopsy from a normal 46, XY male with gynecomastia.

studies showed that these non-GH-producing pituitary cells had sustained *PRKARIA* losses. Second, could the cells in late passages be representative of other neuroendocrine populations that overgrew GH-producing tumour cells with time? The cultured cells in late passages not only did not stain with GH, LH, or any other pituitary hormone but also did not stain with chromogranin A (data not shown), a neuroendocrine marker that stains a number of similar, pleiomorphic pituitary tumours.^{22–24}

Tumour progression towards a more aggressive phenotype displaying characteristics of invasion, migration, and metastasis is often associated with the loss of the epithelial phenotype and the acquisition of a fibroblastic or mesenchymal one. This process, known as EMT, is more typical of aggressive carcinomas, occurs late in tumour progression, and correlates with metastasis. In addition, EMT is frequently accompanied by significant chromosomal rearrangements and a high degree of aneuploidy.^{25–26} Surprisingly, in the present case, EMT may have occurred in a benign pituitary tumour with normal diploid karyotype and a germline genetic aberration with the additional “hit” of 17q allelic losses.

In conclusion, we speculate that EMT and/or mesenchymal cell involvement may be a feature of tumours caused by the absence of a functional *PRKARIA* gene or may be more general and underlie tissue heterogeneity in pleiomorphic pituitary adenomas. EMT has been observed in at least one other set of pituitary somatotropinomas, those associated with MEN 1.¹⁹ The participation of mesenchymal cells in the altered genetic milieu of a benign tumour and/or EMT may indeed be representative of the uniqueness of endocrine neoplastic process,²⁷ as observed in conditions such as CNC or MEN 1.

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Authors' affiliations

I Bossis, A Voutetakis, L Matyakhina, S Pack, I Bourdeau, K J Griffin, N Courcousakis, S Stergiopoulos, D Batista, C A Stratakis, Section on Genetics and Endocrinology, Developmental Endocrinology Branch, National Institute of Child Health and Human Development, Bethesda, MD 20892, USA

M Abu-Asab, M Tsokos, Section of Ultrastructural Pathology, Laboratory of Pathology, National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD 20892, USA

A Voutetakis, Gene Therapy and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health (NIH), Bethesda, MD 20872, USA

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Correspondence to: Dr C A Stratakis, Section on Endocrinology and Genetics, DEB, NICHD, NIH, Building 10, Room 10N262, 10 Center Dr. 1862, Bethesda, MD 20892-1862, USA; stratakc@mail.nih.gov

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