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


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 been found in sporadic pituitary tumours. Pituitary tissue was obtained from an acromegalic CNC patient heterozygote for a common (PRKARIA)-inactivating mutation. Both immunohistochemistry and electron microscopy showed a highly pleomorphic 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 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.

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 family history, and peripheral blood sequencing data have been published before.

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

A CNC patient (member of the CAR20 kindred) with the most common germ-line PRKAR1A-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 PRKAR1A allele (data not shown), as has been shown before for a number of tumours from patients with CNC and germline PRKAR1A mutations, including one pituitary tumour from a patient carrier of the c.578delTG mutation that belonged to another family. 7

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 somatomammotropinomas 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 PRKAR1A-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 (×10). The tumour includes several areas of compact cells that do not stain with any pituitary hormones.
FISH analysis of frozen tumour preparations (prepared at the time of resection and from within the adenoma) with a BAC containing the PRKAR1A gene confirmed allelic loss of the 17q22–24 PRKAR1A 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) 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, periadrenal 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...
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 asses-
sing the clonal origin of pituitary tumours.

Earlier studies utilising allelotypes of microsatellite poly-
morphisms 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 \textit{PRKARIA}
allele in the present case indicated that this pleomorphic
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 immuno-
histochemistry 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 phenom-
ena. 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

\begin{figure}
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\includegraphics[width=0.5\textwidth]{figure5}
\caption{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.}
\end{figure}

\begin{figure}
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\includegraphics[width=0.5\textwidth]{figure6}
\caption{FISH analysis of the cultured pituitary tumour cells. The picture
shows allelic loss of BAC321-G-8 containing the \textit{PRKARIA} gene (green,
one signal), whereas two signals from the chromosome 17-specific
centromeric \textalpha-\textit{satellite} probe (red) are seen. A normal cell is also shown
(on the left) that has the expected two signals from each probe.}
\end{figure}

\begin{figure}
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\includegraphics[width=0.5\textwidth]{figure7}
\caption{FISH analysis of primary cultures of mesenchymal cells that have retained both copies of the BAC321-G-8 containing the \textit{PRKARIA} gene
after several passages in culture: (A) periadrenal and (B) heart myxoma from two CNC patients, one with a previously described 10 \textit{PRKAR1A}-
inactivating germline mutation, and one with no \textit{PRKAR1A} coding sequence mutations, respectively, and (C) a breast biopsy from a normal 46, XY
male with gynaecomastia.}
\end{figure}
studies showed that these non-GH-producing pituitary cells had sustained PRKAR1A losses. Second, could the cells in late passages be representative of other neuroendocrine populations that overexpress 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, pleomorphic pituitary tumours.27–29

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 PRKAR1A gene or may be more general and underlie tissue heterogeneity in pleomorphic pituitary adenomas. EMT has been observed in at least one other set of pituitary somatotropinomas, those associated with MEN 1.10 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, as observed in conditions such as CNC or MEN 1.

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