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Mutation and genomic amplification of the *PIK3CA* proto-oncogene in pituitary adenomas

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Abstract

The tumorigenesis of pituitary adenomas is poorly understood. Mutations of the *PIK3CA* proto-oncogene, which encodes the p110- α catalytic subunit of PI3K, have been reported in various types of human cancers regarding the role of the gene in cell proliferation and survival through activation of the PI3K/Akt signaling pathway. Only one Chinese study described somatic mutations and amplification of the *PIK3CA* gene in a large series of pituitary adenomas. The aim of the present study was to determine genetic alterations of *PIK3CA* in a second series that consisted of 33 pituitary adenomas of different subtypes diagnosed by immunohistochemistry: 6 adrenocorticotrophic hormone-secreting microadenomas, 5 growth hormone-secreting macroadenomas, 7 prolactin-secreting macroadenomas, and 15 nonfunctioning macroadenomas. Direct sequencing of exons 9 and 20 assessed by qPCR was employed to investigate the presence of mutations and genomic amplification defined as a copy number ≥ 4 . Previously identified *PIK3CA* mutations (exon 20) were detected in four cases (12.1%). Interestingly, the Chinese study reported mutations only in invasive tumors, while we found a *PIK3CA* mutation in one noninvasive corticotroph microadenoma. *PIK3CA* amplification was observed in 21.2% (7/33) of the cases. This study demonstrates the presence of somatic mutations and amplifications of the *PIK3CA* gene in a second series of pituitary adenomas, corroborating the previously described involvement of the PI3K/Akt signaling pathway in the tumorigenic process of this gland.

Key words: Pituitary adenomas; *PIK3CA* proto-oncogene; Genomic amplification; Somatic mutation

Introduction

The tumorigenesis of pituitary adenomas is poorly understood. The phosphatidylinositol 3-kinases (PI3Ks) are a group of heterodimeric lipid kinases composed of regulatory (85-kDa; p85) and catalytic (110-kDa; p110) subunits that play a key role in cell growth, proliferation, motility, and survival (1-3). The activation of PI3K results in phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol-3,4,5-triphosphate (PIP₃), which acts as an anchor for Akt serine/threonine kinase and 3-phosphoinositide-dependent protein kinase-1 (PDK1), and facilitates phosphorylation of Akt by PDK1, stimulating Akt activity and subsequent phosphorylation of several proteins involved in a variety of intracellular processes, including antiapoptosis (4,5). PIP₃ levels are tightly regu-

lated by the action of phosphatases such as phosphatase and tensin homologue (PTEN) (6).

Mutations of the *PIK3CA* proto-oncogene, which encodes the p110- α catalytic subunit of PI3K, have been reported in various types of human cancers (7-10) and more than 80% of *PIK3CA* mutations are clustered in the helical domain encoded by exon 9 and in the kinase domain encoded by exon 20 (11), as shown with high prevalence in endometrioid, esophageal and pancreatic carcinomas (12-15).

A recent Chinese study (16) has described somatic mutations and amplifications of the *PIK3CA* gene in a large series of pituitary adenomas, implicating the PI3K/Akt signaling pathway in the tumorigenic process of this

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gland. Since a diversity of mutational spectra in different populations had been described for certain neoplasias (17,18), in the present study we investigated genetic alterations in the *PIK3CA* proto-oncogene in a second series of pituitary adenomas.

Material and Methods

Tissue specimens and DNA isolation

Tissue collection was carried out in compliance with the Institutional Ethics Committee (CAPPesq) and in accordance to the Declaration of Helsinki, with informed and free written consent being required from each subject or subject's guardian. From 1994-2009, tumor tissues were obtained from patients diagnosed with pituitary adenomas. During surgery, tumor fragments were collected into sterile containers and immediately frozen in liquid nitrogen. Thirty-three sporadic pituitary adenomas were obtained after surgery. The anatomic-pathological characteristics of these adenomas are shown in Table 1. Pituitary adenomas consisted of 6 adrenocorticotrophic hormone (ACTH)-secreting microadenomas, 5 growth hormone (GH)-secreting macroadenomas, 7 prolactin (PRL)-secreting macroadenomas, and 15 nonfunctioning (NF) macroadenomas. As control, four normal pituitary tissues were obtained within 8 h *post mortem* from subjects without endocrine diseases. The tissue fragments were fragmented in a tissue pulverizer (Mikro-Dismembrator U, B. Braun Melsungen, Germany). Tumor DNA was extracted using the DNeasy kit (Qiagen, USA) according to manufacturer instructions.

Mutation analysis

Exons 9 and 20 of *PIK3CA* were analyzed by direct sequencing on an ABI 3130X Genetic Analyzer (Applied Biosystems, USA) after PCR amplification of genomic DNA. PCR was carried out in 50 μ L containing 100 ng genomic DNA, 0.2 mM of each primer, 200 μ M deoxynucleotides, 1X buffer and 1 U DNA Taq polymerase (GE Healthcare, USA). The nucleotide sequences for the primers were designed using the Primer3 software: exon 9 forward 5'-CAAAGCAATTTCTACACGAGATCC-3'; exon 9 reverse 5'-GTAAAAACATGCTGAGATCAGCCACAT-3'; exon 20 forward 5'-TGGAATGCCAGAACTACAATCTTT-3', and exon 20 reverse 5'-GGTCTTTGCCTGCTGAGAGTT-3' (Invitrogen, USA). Reactions were carried out under the following cycling conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, followed by 56°C (exon 9) or 62°C (exon 20) for 30 s and 72°C for 10 min. The PCR products were subjected to direct sequencing with the use of BigDye Terminator sequencing reagents (Applied Biosystems) with the following cycles: 96°C for 15 s, 50°C for 15 s and 60°C for 4 min for 35 cycles. All

mutated DNA sequences were confirmed by sequencing in the reverse direction.

Copy number analysis

PIK3CA gene copy number amplification was assessed by qPCR using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 100 ng DNA and primers for genomic sequences (*PIK3CA* forward: 5'-ATCTTTTCTCAATGATGCTTGGCT-3' and *PIK3CA* reverse: 5'-CTAGGGTGTTCGAATGTATG-3') and compared with the signal obtained from *COL7A1* as reference gene (*COL7A1* forward: 5'-ACCAGTACCGCATCATTGTG-3' and *COL7A1* reverse: 5'-TCAGCTGGAACCTCAGTGTG-3').

PCR was carried out on a Rotor-Gene 6000 instrument (Corbett Life Science, Australia). The reaction consisted of an initial incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. Fluorescence changes were monitored after each cycle. Melting curve analysis was performed at the end of each reaction to confirm PCR product's identity (72°C ramping to 99°C at 0.2°C/s with continuous fluorescence readings). Standard curves were constructed to assess the amplification efficiency of *PIK3CA* and *COL7A1* using duplicate serial dilutions with seven different DNA concentrations (400 to 6.25 ng) from a reference sample of DNA obtained from normal pituitary tissue. Since equal amplification efficiencies of target and reference genes were attained ($E > 0.9$), the mathematical model $2^{-\Delta\Delta C_t}$ was used to evaluate the relative genomic amplification of *PIK3CA*. All samples were run in duplicate. DNA obtained from a pool of four normal pituitary tissues was included in the assay as a calibrator sample and DNA extracted from the MCF-7 cell line was used as a positive control for *PIK3CA* amplification. DNA amplification was defined as values ≥ 4 (16).

Results

PIK3CA mutations

Previously reported *PIK3CA* mutations were detected in four cases (12%): 3 of 15 (20%) NF pituitary adenomas and 1 of 6 (16.6%) ACTH-secreting tumors, both in exon 20 (Table 2). No mutations were found in exon 9.

Table 1. Demographic data and anatomic-pathological findings of 33 patients with pituitary adenomas who participated in the present study.

Pituitary adenomas	N	Gender (male/female)	Age (years)	Tumor size (micro/macroadenoma)
ACTH-secreting	6	4/2	32.3 (13-61)	6/0
GH-secreting	5	2/3	30.2 (23-55)	0/5
PRL-secreting	7	1/6	31 (20-64)	0/7
Nonfunctioning	15	6/9	44 (21-70)	0/15

ACTH = adrenocorticotrophic hormone; GH = growth hormone; PRL = prolactin; microadenoma = ≤ 10 mm; macroadenoma = ≥ 10 mm.

Amplification of PIK3CA

The number of copies of the *PIK3CA* gene in each tumor sample is shown in Figure 1. Genomic amplification

was observed in 21.2% (7/33) of the cases; in 4 of 5 (80%) GH-secreting adenomas, 1 of 6 (16.6%) ACTH-secreting adenomas and in 2 of 15 (13.3%) NF pitu-

Table 2. *PIK3CA* mutations found in four pituitary adenomas.

Exon	Functional domain	<i>PIK3CA</i> DNA mutation	Amino acid	Subtype of tumor
20	Kinase domain	GCC > GAC, C2994A	Ala ⁹⁹⁵ Asp, A > D	ACTH
20	Kinase domain	GGC > GGT, C3031T	Gly ¹⁰⁰⁷ Gly, G > G	NF
20	Kinase domain	TCT > CCT, T3053C	Ser ¹⁰¹⁵ Pro, S > P	NF
20	Kinase domain	CAA > CGA, A3108G	Gln ¹⁰³³ Arg, Q > R	NF

ACTH = adrenocorticotrophic hormone-secreting adenoma; NF = nonfunctioning pituitary adenoma.

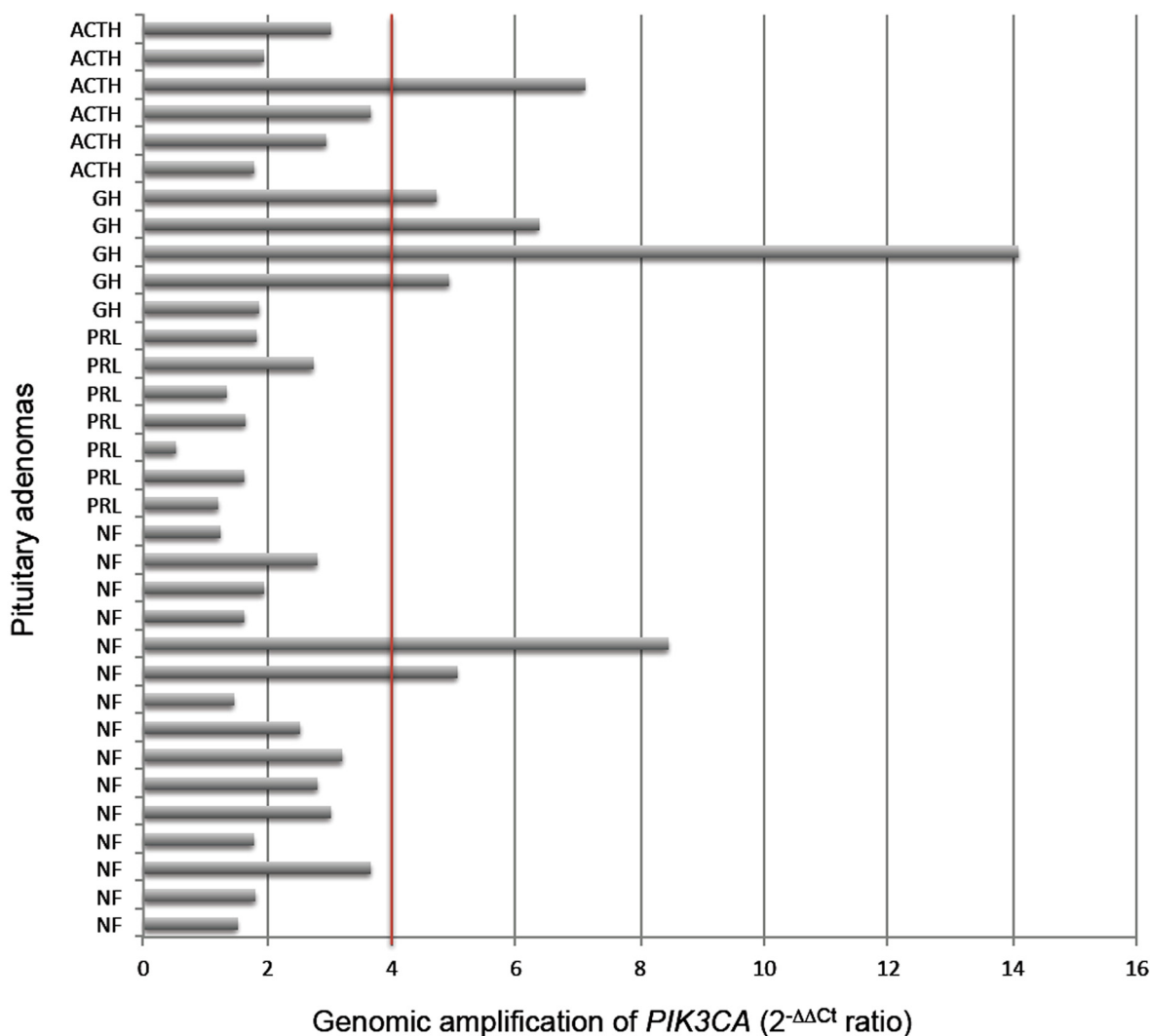


Figure 1. *PIK3CA* gene copy number detected by qPCR in different subtypes of pituitary adenomas. Positive amplification is defined as ≥ 4 copies. ACTH = adrenocorticotrophic hormone-secreting adenoma; GH = growth hormone-secreting macroadenoma; PRL = prolactin-secreting macroadenoma; NF = nonfunctioning pituitary adenoma.

itary adenomas. No genomic amplification was detected in PRL-secreting adenomas.

Discussion

Several studies have implicated *PIK3CA* somatic mutations and genomic amplification in the tumorigenic process of different human cancers (19-26). In the present study, we investigated mutations and genomic amplification of the *PIK3CA* proto-oncogene in 33 sporadic pituitary adenomas. During our experiments, another group of investigators evaluated the association of these genetic alterations in pituitary tumors (16). Given the diversity of mutational spectra in different populations for certain neoplasias (17,18), we decided to continue to examine the incidence of these mutations and amplifications in pituitary tumors of Brazilian patients.

The overall frequency of *PIK3CA* gene mutations and amplification in the Chinese study of 353 pituitary tumors was 2.3% (8.8% of the invasive tumors and 0% of the non-invasive ones) and 28% (26.3% of the noninvasive tumors and 32.9% of the invasive ones), respectively (16), while in the current series, *PIK3CA* mutations and amplification were found in 12.1 and 21.2% of the cases, respectively.

Although it is difficult to compare studies that differ so much in the size of the evaluated population, the present study corroborates the previous report (16) that genetic alterations in the *PIK3CA* gene are found in pituitary tumors. We only detected mutations in one ACTH-secreting and three NF pituitary adenomas, while Lin et al. (16) also found mutations in two PRL-secreting tumors. Mutations in GH-secreting tumors were not found in any of the two series. Interestingly, the Chinese study reported mutations only in invasive tumors, while we found a *PIK3CA* mutation in one noninvasive corticotroph microadenoma. Recent reports have shown that *PIK3CA* knockdown reduces proliferation and invasiveness in different cell lines (27-29).

Defining positive gene amplification as a copy number ≥ 4 , ACTH-secreting tumors (16.6%), NF pituitary tumors (13.3%) and particularly GH-secreting tumors (80%) were identified as harboring *PIK3CA* amplifications, with the highest number being 14 copies in a GH-secreting tumor. Adding to the previous Chinese study, *PIK3CA* amplification was more common than mutations. Amplification of *PIK3CA* has been demonstrated to be a mechanism of resistance for PI3K-targeted therapy in cancer (30).

The coexistence of *PIK3CA* mutations with amplifications in pituitary adenomas seems to be rare; only one case (NF tumor) harbored coexisting *PIK3CA* copy gain and a gene mutation. These findings suggest that *PIK3CA* mutation and amplification are independent oncogenic pathways in pituitary tumors (16), as mutually exclusive mechanisms, as already described for thyroid tumors (31); however, the coexistence of these genetic alterations has been reported in breast cancers (32).

The major effector of PI3K is Akt kinase, which phosphorylates target proteins regulating key processes such as cell proliferation and survival. This process is counteracted by the phosphatase encoded by the *PTEN* tumor suppressor gene, which opposes PI3K activity (33). A recent study has implicated phosphorylated Akt expression associated with *PIK3CA* mutations in low-stage colorectal cancers (34). In 2005, Musat et al. (6) reported a higher expression of phosphorylated Akt in pituitary tumors in comparison to the normal gland in the absence of *PTEN* mutations. Thus, it is reasonable to suppose that increased Akt phosphorylation in pituitary adenomas could result from activation of the PI3K pathway following *PIK3CA* mutation or amplification.

In summary, this study demonstrates the presence of somatic mutations and amplifications of the *PIK3CA* gene in a second series of pituitary adenomas, corroborating the previously described involvement of the PI3K/Akt signaling pathway in the tumorigenesis of this gland (16,35,36) and its molecular potential for targeted therapies (3,27-29,37)

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