

ABSENCE OF MUTATIONS IN THE P53 TUMOR SUPPRESSOR GENE IN  
NON-INVASIVE CUSHING ADENOMAS

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**Abstract** A lot of evidence supports the existence of a monoclonal origin for pituitary tumors, and several genetic alterations have already been confirmed as necessary or sufficient for unrestrained cellular growth and pituitary function. The p53 gene, a known tumor-suppressor gene (TSG), encodes a protein that exerts antiproliferative effects such as cell-growth arrest and apoptosis in response to several types of stimuli. In fact, several human cancers are believed to be caused by p53 mutations. In the case of pituitary tumors, p53 protein accumulation has been described in ACTH-secreting pituitary adenomas. Since increased amounts of the p53 protein are often related to mutations of its gene, we decided to explore the existence of p53 mutations in the tumor tissues of 9 patients bearing non-invasive corticotropinomas, excised by the transphenoidal route. We screened mutations in exons 5 to 8 of the p53 gene by the PCR-SSCP analysis. We were not able to find any mutation in the exons investigated. Our results are in close accordance with those obtained previously for other types of pituitary tumors.

**Resumen** *Ausencia de mutaciones en el gen supresor de tumores p53 en adenomas de Cushing no invasivos.* Existe abundante evidencia que prueba el origen monoclonal de los tumores pituitarios, y diversas alteraciones genéticas ya han sido determinadas como necesarias o suficientes para su iniciación y progresión. El gen supresor de tumores p53 codifica una proteína que, en respuesta a diversos estímulos, media efectos antiproliferativos que incluyen la detención del crecimiento celular y apoptosis. De hecho, varios tipos de cáncer podrían originarse a partir de mutaciones en el gen p53. En el caso de los tumores hipofisarios, se ha demostrado sobreexpresión de la proteína p53 en adenomas hipersecretorios de ACTH. Dado que cantidades aumentadas de p53 suelen vincularse a mutaciones del gen, resulta de interés investigar la eventual existencia de las mismas en adenomas de Cushing no invasivos. Por lo tanto, y con el fin de encontrar un marcador genético que brinde un pronóstico confiable acerca de su evolución clínica, se inició la búsqueda de mutaciones en el gen p53. Fueron analizados los exones 5 al 8 mediante la técnica de PCR-SSCP. Nuestros resultados concluyen en la ausencia de mutaciones en estos exones en adenomas corticotrópicos, y coinciden con lo observado en tumores pituitarios de otras estirpes celulares.

**Key words:** p53 gene, PCR/SSCP, corticotropinoma

Pituitary function strongly depends on hypothalamic control because stimulatory and inhibitory secreting hormones not only regulate function but also growth and differentiation rates of pituitary cells. It has been suggested that an increased stimulation of pituitary cells by hypothalamic factors could be the basis of tumor development. Nevertheless, some experimental and clinical evidence have weakened this hypothesis<sup>1</sup>. On the other hand, the origin of pituitary tumors from polyclonal cell proliferation was at first supported by the existence of mixed plurihormonal tumors, which could not be caused

by the hyperfunction of a single hypothalamic factor. Later, X-chromosomal inactivation analysis demonstrated that most pituitary adenomas have a monoclonal origin<sup>2</sup>. Their etiology is clearly under intense investigation and, indeed, several mechanism have already been demonstrated as necessary and/or sufficient factors for unrestrained cellular growth and hormone production in pituitary cells<sup>3</sup>.

The p53 protein serves different functions inside the cell<sup>4,5</sup>. As a TSG it constitutively impedes the progression of the cell cycle towards DNA replication; in addition, it induces the programmed death of those cells that, after being damaged at the genetic level, become strong candidates to undergo neoplastic transformation. Indeed, there are many human cancers in which mutations of the p53 gene have been found<sup>6</sup>. However, in other cases, p53 might be mutated as a result of several rounds of DNA replication in cells undergoing unrestrained prolif-

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Received: 22-I-1999

Accepted: 23-VIII-1999

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eration caused by mutations in other cell cycle regulatory genes. Whatever the first mutated gene causing cell transformation, the detection of a mutated p53 allele in tumoral tissues could provide reliable prognostic information about what the clinical outcome of the tumor will be.

Pituitary non-invasive adenomas are a type of human tumor in which the status of the p53 gene has not been widely investigated. Therefore, it would be extremely informative to gather data about a possible prognostic value of p53 as a genetic marker linked to aggressive-ness.

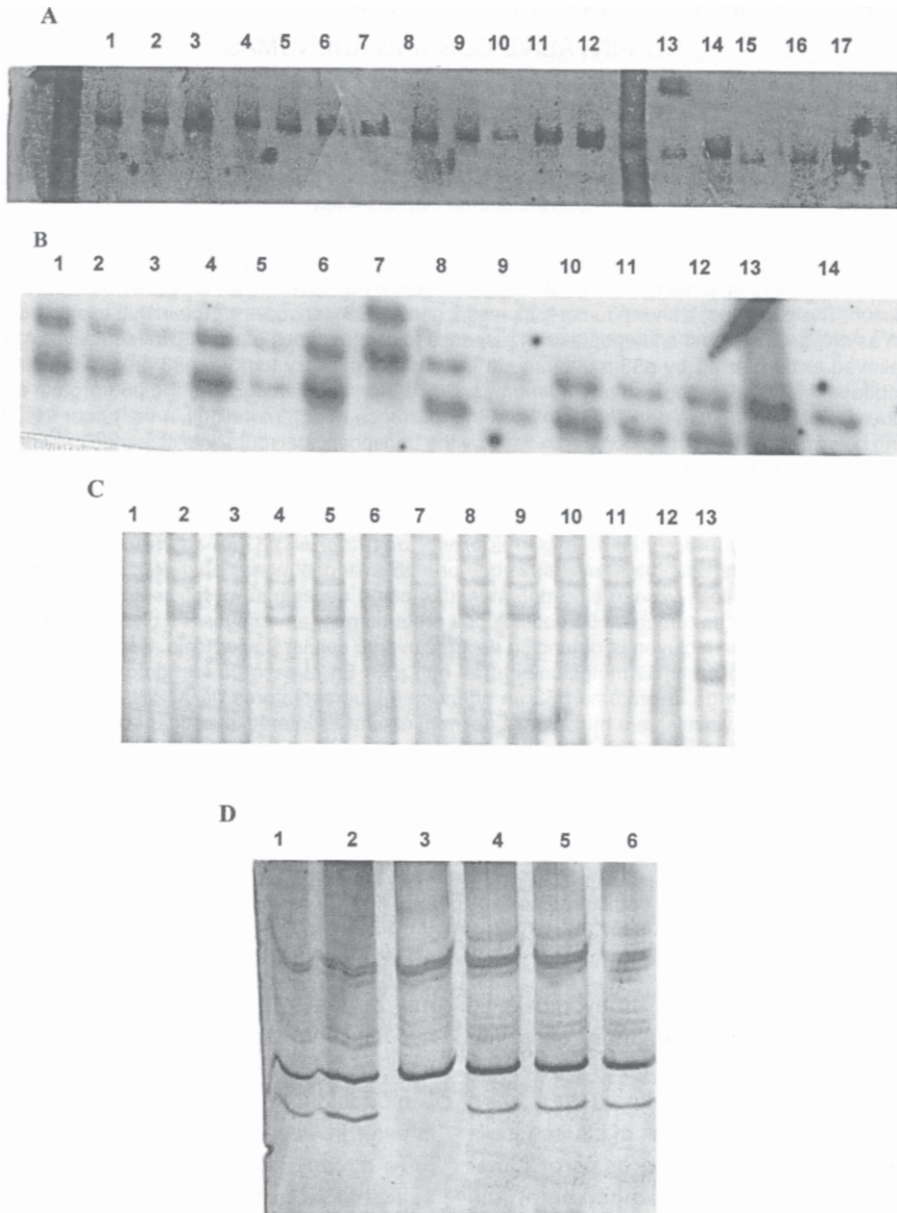


Fig. 1.— The figure shows the partial SSCP analysis by electrophoresis on non-denaturing 6% polyacrylamide gel containing 10% glycerol of exon 5 (A), exon 6 (B), exon 7 (C) and exon 8 (D) of p53 gene. 1-A, the DNA from leukocytes (1, 3, 5, 7, 9, 11, 15, 17) and tumoral tissue<sup>2</sup> 4, 6, 8, 10, 12, 16) was compared, in seven of the nine patients. 13 (tumor) and 14 (leukocytes) were positive control for a mutation in this exon. 1-B, the DNA from leukocytes (1, 3, 5, 9, 11, 13) and tumoral tissue (2, 4, 6, 10, 12) was compared, in six of the nine patients. 7 (tumor) and 8 (leukocytes) were positive control for a mutation in this exon. 1-C, the DNA from leukocytes (1, 2, 4, 6, 8, 10) and tumoral tissue (3, 5, 7, 9, 11) was compared, in five of the nine patients. 12 (leukocytes) and 13 (tumor) were positive control for a mutation in this exon. 1-D, shows the band pattern of DNA from leukocytes (1, 5) and tumoral tissue (2, 6), in two of the nine patients. 3 (tumor) and 4 (leukocytes) were positive control for a mutation in this exon. There was no band pattern alteration in the patients studied by this SSCP condition (1-A to 1-D).

Buckley et al.<sup>7</sup> described p53 protein accumulation in Cushing adenomas and invasive non-functional adenomas, strongly suggesting that mutations in p53 could have a role in tumor development.

Even if there are many different methods of detecting a mutation in a single gene, the combined technique of Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) is probably the most frequently applied in molecular diagnosis because it is fast, quite accurate and has a broad range of applicability. The PCR-SSCP technique has been widely applied to the analysis of the p53 gene. In accordance, the results obtained have often been correlated to the biological behavior of diverse human tumors, for instance esophageal cancer<sup>8</sup>. It is important to note that most of the present studies look for mutations in exons 5 to 8, because previous analysis have shown that mutations in p53 occur, in most of the cases, in these exons (i.e., exons 5 to 8 are mutational hot-spots of the gene).

The aim of our work was to analyze the presence of mutations in exons 5 to 8 of the p53 gene by PCR-SSCP in human corticotropic adenomas. We selected 9 patients who had pituitary Cushing syndrome (Cushing disease), which was confirmed in all cases after transphenoidal exeresis of micro (n = 7) or macroadenoma (n = 2) with positive immunohistochemistry for ACTH. Genomic DNA samples were obtained from tumoral tissue (resected at surgery) and from peripheral blood lymphocytes. The DNA samples were subjected to 35 cycles of amplification using oligoprimers corresponding to exons 5 to 8 as previously described<sup>9</sup>. The cycling reaction was performed in a programmable heat block (PTC-100 Thermal Cycler, MJ Research). A 5 µl aliquot of each PCR reaction was electrophoresed in a 1.5% agarose gel with TBE 0.5X buffer and examined by ethidium bromide staining in order to confirm the appropriate size of the template. Then, PCR-SSCP analysis was performed to screen for p53 gene mutations. PCR products of 290 bp (exon 5), 184 bp (exon 6), 209 bp (exon 7) and 237 bp (exon 8) were denatured and electrophoresed on non-denaturing polyacrilamide gel (MDE 0.5X) for 7 h at 4 °C and 150 V (data not shown). We also studied the PCR products (SSCP analysis) by electrophoresis on non-denaturing 6% polyacrilamide gel containing 10% glycerol (Fig. 1) and without glycerol (Fig. 2) for 4 h at room temperture and 150 volts. All the cases, and in the three conditions, were analyzed in the presence of a positive control for a mutation in each exon (colon tumor; manuscript in preparation). Finally, a modified silver stain method of staining for the gel was performed<sup>10</sup>. In all cases, paired leukocytes and tumor DNA samples were compared. Figures 1A to 1D and 2A to 2D show the absence of bands with alterations in exons 5 to 8 of the patients. These results strongly

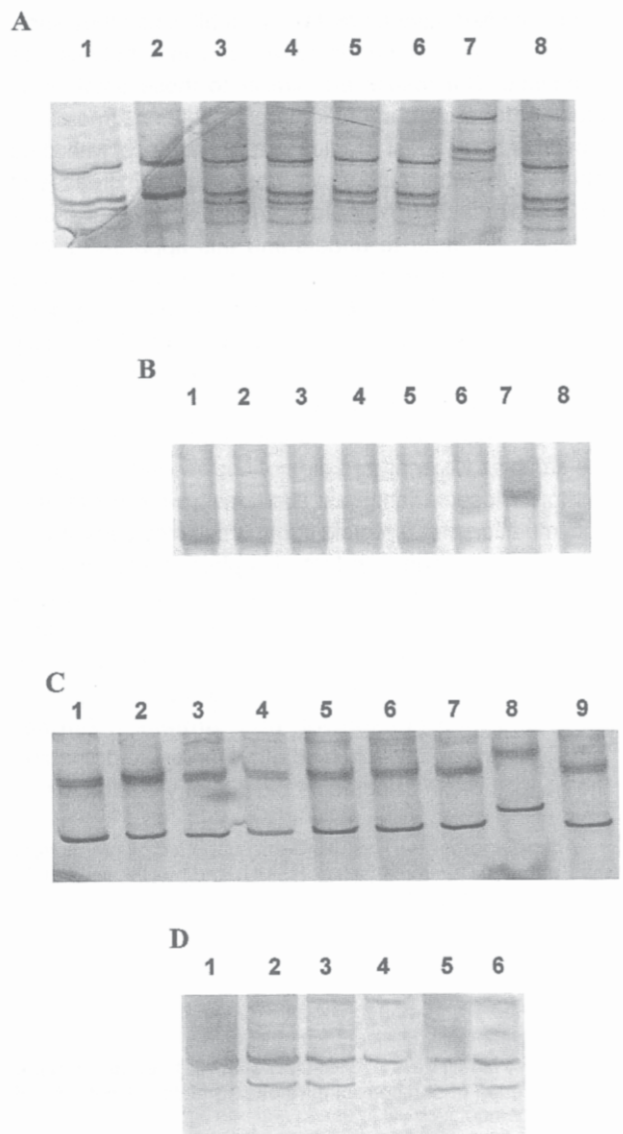


Fig. 2.— The figure shows the partial SSCP analysis by electrophoresis on non-denaturing 6% polyacrilamide gel without glycerol of exon 5 (A), exon 6 (B), exon 7 (C) and exon 8 (D) of p53 gene. 2-A, the DNA from leukocytes (1, 2, 4, 8) and tumoral tissue (3, 5) was compared, in two of the nine patients. 6 (leukocytes) and 7 (tumor) were positive control for a mutation in this exon. 2-B, the DNA from leukocytes (1, 3, 5, 9) and tumoral tissue (2, 4, 6) was compared, in three of the nine patients. 7 (tumor) and 8 (leukocytes) were positive control for a mutation in this exon. 2-C, the DNA from leukocytes (1, 3, 5, 9) and tumoral tissue (2, 4, 6) was compared, in three of the nine patients. 7 (leukocytes) and 8 (tumor) were positive control for a mutation in this exon. 2-D, shows the band pattern of DNA from leukocytes (1, 5) and tumoral tissue (2, 6), in two of the nine patients. 3 (leukocytes) and 4 (tumor) were positive control for a mutation in this exon. There was no band pattern alteration in the patients studied by this SSCP condition (2-A to 2-D).

suggest that the exons studied have no mutations at all, since they show no difference in the bands corresponding to both tissues examined. Our results are similar to those obtained in studies done on non-corticotropic pituitary tumors<sup>11, 12</sup>. However, these last studies are at variance with data obtained by other authors who have found increases of p53 expression in similar tumors with aggressive behavior. In order to understand this apparent discrepancy it is important to remember that p53 protein has a very short half-life (15-20 min), being normally degraded by peptide-sequence-specific mechanisms. Abnormally high protein levels may be due to the existence of mutations impairing the sequence-specific proteolytic process<sup>13</sup>. Nevertheless, it is important to consider that the samples we analyzed did not correspond to invasive but to enclosed adenomas; additionally, it is known that accumulation of the protein into the cell may coexist with a normal p53 gene<sup>14</sup>. In fact, this phenomenon may be explained by an increase of its synthesis or a decrease of its degradation, i.e., Mdm2 oncoprotein, a potent inhibitor of p53 blocks its ability to regulate its target genes, and a recent finding showed Mdm2 promoting the rapid degradation of p53 protein<sup>15</sup>. Further investigations should focus on other factors to determine the genetic origin of corticotropic pituitary adenomas.

**Acknowledgments:** This work was supported by grants from the European Community (D12 HSMU) and by Universidad de Buenos Aires (ME-010/T).

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*I look on that man as happy, who, when there is question of success, looks into his work for a reply.*

Yo considero dichoso a aquel que, cuando se habla de éxitos, busca la respuesta en su trabajo.

Ralph Waldo Emerson (1803-1882)

*Conduct of Life: Worship*