Loss of heterozygosity of the *MEN1* gene in a large series of TSH-secreting pituitary adenomas

C. Asteria*, M. Anagni**, L. Persani***, and P. Beck-Peccoz*

*Institute of Endocrine Sciences, University of Milan; ***Istituto Auxologico Italiano IRCCS, Milan;
**Department of Internal Medicine, Bellaria Hospital, Bologna, Italy

ABSTRACT. Thyrotropin-secreting pituitary adenomas (TSH-omas) are rare tumors (0.5% of all pituitary adenomas) showing an invasive behavior and usually sporadic, although a few cases are associated with multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant inherited syndrome. This disorder is linked to loss of heterozygosity (LOH) on 11q13 and inactivating mutations of *MEN1* gene, which is located in the same chromosomal region. As other types of anterior pituitary adenomas, TSH-omas are the result of a monoclonal outgrowth where the intrinsic genetic defects involving oncogenes or tumor suppressor genes occur in a progenitor cell. However, so far no activating mutations of particular oncogenes or inactivating mutations of tumor suppressor genes have been identified. Starting from the observation that 3-30% of sporadic pituitary adenomas show LOH on 11q13, and that allelic losses on the long arms of chromosome 11, beside 10 and 13, are significantly associated with the transition from the non-invasive to the invasive phenotype, we decided to investigate LOH on 11q13 and mutations of menin in a large series of TSH-omas. Thirteen tumors were evaluated. DNA was extracted from tumors by standard methods and genomic DNA from peripheral blood leukocytes was used as control. LOH was screened by using 3 polymorphic markers on 11q13: D11S956, PYGM, INT-2. In 3 out of 15 cases we could demonstrate LOH on 11q13, but none of the tumors showed menin mutation after sequence analysis. These data strongly suggest that menin does not play a causative role in the development of TSH-omas, and are in agreement with other studies demonstrating a limited role of menin in pituitary sporadic tumorigenesis.

(J. Endocrinol. Invest. *24:* 796-801, 2001)

INTRODUCTION

Although the majority of pituitary tumors are benign, thyrotropin-secreting pituitary adenomas (TSH-omas) usually present an invasive behavior over time, as exemplified by the tendency to become macroadenomas and to suprasellar extension, so that symptoms and signs of expanding tumor mass may accompany those of thyroid hyperfunction (1). Nowadays, it is not possible to determine those tumors that are destined to show the invasive phenotype, using current routine histological criteria. Therefore, several studies have been performed in recent years in order to advance the knowledge of the underlying mechanisms responsible for both tumor initiation and progression. By the means of molecular biological techniques it was demonstrated that anterior pituitary adenomas are the result of a monoclonal outgrowth where the intrinsic genetic defects involving oncogenes or tumor suppressor genes occur in a progenitor cell (2, 3). In TSH-omas, X-chromosomal inactivating analysis in female patients has confirmed their monoclonal origin (4), but neither activation of cellular protooncogenes (G-protein α-subunit, TRH-receptor, Pit-1, c-myc, c-fos, c-myb) (5-14), nor loss of function of tumor suppressor genes, such as p53 (15), has been identified as responsible for the development of this subgroup of pituitary tumors.

Moreover, several lines of evidence have suggested that allelic loss on different chromosomes represents another important genetic event usually observed in pituitary tumors, especially in those presenting an aggressive behavior. In particular, loss of heterozygosity (LOH) on the long arms of chromosome 11, beside 10 and 13, seems to be significantly associated with the transition from the non-invasive to the

Key-words: TSH-secreting pituitary adenomas, menin, LOH, sequence analysis.

Correspondence: Prof. Paolo Beck-Peccoz, Istituto di Scienze Endocrine, Università di Milano, Ospedale Maggiore IRCCS, Via F. Sforza 35, 20122 Milano, Italy.

E-mail: paolo.beckpeccoz@galactica.it

Accepted May 29, 2001.

invasive phenotype (16, 17). More recently, the attention of the researchers has been focused on the newly discovered multiple endocrine neoplasia type 1 (*MEN1*) gene located at 11q13 because allelic deletion and inactivating mutations of this gene are implicated in the etiopathogenesis of a variable percentage of *MEN1*-associated (10-18%) as well as sporadic pituitary tumors (3-30%) (8, 18-20). However, until now only few cases of TSH-omas have been tested for LOH on 11q13/*MEN1* gene inactivating mutations and have been found to be negative (8, 19, 21). The aim of our study was to screen a large series of sporadic TSH-omas for menin inactivation, in order to evaluate whether this tumor suppressor gene may play a role in the initiation and transition to an aggressive phenotype of this particular subgroup of tumors.

MATERIALS AND METHODS

Patients and tumor characterization

A total of 13 patients affected with TSH-secreting pituitary tumors were studied. All the patients presented symptoms and signs of thyroid hyperfunction and 8 out of 13 showed headache and visual field defects. The biochemical phenotype was characterized by elevated serum thyroid hormone levels in the presence of measurable TSH, elevated concentrations of circulating free α-subunit, and an increased α-subunit/TSH molar ratio. TRH injection test (200 µg, iv) failed to increase TSH and α-subunit circulating levels in all the patients. Eight of 13 patients (61%) presented macroadenomas with an exstrasellar extension and 5 showed intrasellar microadenomas at the nuclear magnetic resonance (NMR) study of the pituitary region.

All the patients were subjected to adenomectomy by sphenoidal surgery. Fresh tumor tissue obtained at surgery was divided in separate parts. A part of the tissues was fixed in formalin and embedded in paraffin for histological and immunoistochemical analysis, which allowed the classification of the tumors as TSH-omas. For light microscopy, tumor sections were stained with hematossilin-eosine (H&E), periodic acid-Schiff, and the Gordon-Sweet silver reticulin stain. The avidin-biotin-peroxidase complex technique was used to localize β- and α-subunits of TSH.

The remaining part of the tumors was snap frozen and stored at –70 C for molecular analysis.

DNA isolation

For each patient DNA was isolated from frozen tissue of surgically resected tumor using TRIREAGENT kit (Molecular Research Center, Cincinnati, USA) and from 5 ml of peripheral blood lymphocytes using Nucleon DNA extraction kit (Amersham Life Science), according to the manufacturer's instructions. Peripheral blood samples were collected in order to obtain matched normal DNA from the patients.

PCR amplification

DNA extracted from the peripheral blood lymphocytes and from the tumors was amplified by PCR. Eleven intronic primer pairs were appropriately selected for the amplification of the coding exons (2-10) and the intron/exon boundaries of the *MEN1* gene. These primer pairs, their relative annealing temperature and PCR product sizes are shown in

Table 1 - *Primer pairs used to amplify by PCR the exons (2-10) and the intron/exon boundaries of the multiple endocrine neoplasia type 1 (*MEN1*) gene.*

Pair no.	Exon	Sequence of forward primer	Sequence of reverse primer	*AT C	Product size
1	2	TTGCCTTGCAGGCCGCCGCC	TGGTAGGGATGACGCGGTTG	62 C	203 bp
2	2	GGCTTCGTGGAGCATTTTCT	CTCGAGGATAGAGGGACAGG	62 C	203 bp
3	2	TTCACCGCCCAGATCCGAGG	TAAGATTCCCACCTACTGGG	58 C	185 bp
4	3	GGAGTGTGGCCCATCACTA	TGGAGTCCCTTGGGTGGCTTG	60 C	359 bp
5	4	CCATCACCACCCACATAGGA	TCAAGTCTGGCCTAGCCAG	60 C	291 bp
6	5-6	CGTGGCTCATAACTCTCTCC	TAGGGTCTCCCTTCTGCACC	56 C	279 bp
7	7	ATTTGTGCCAGCAGGGCAGC	CAGTCCTGGACGAGGGTGGTT	60 C	279 bp
8	8	CCTTCAGACCCTACAGAGAC	CCATGGCCCTGTGGAAGGGA	56 C	242 bp
9	9	AGAGACTGATCTGTGCCCTC	TCAGTCCCATCGGCACCGAAG	56 C	320 bp
10	10	CACTGGCCGGCAACCTTGCT	ACAGTCCCAGGAGGCTTCCG	66 C	260 bp
11	10	CGGAAGCCTCCTGGGACTGT	CCCACAAGCGGTCCGAAGTC	66 C	317 bp

*AT°C: annealing temperature of the primer pairs.

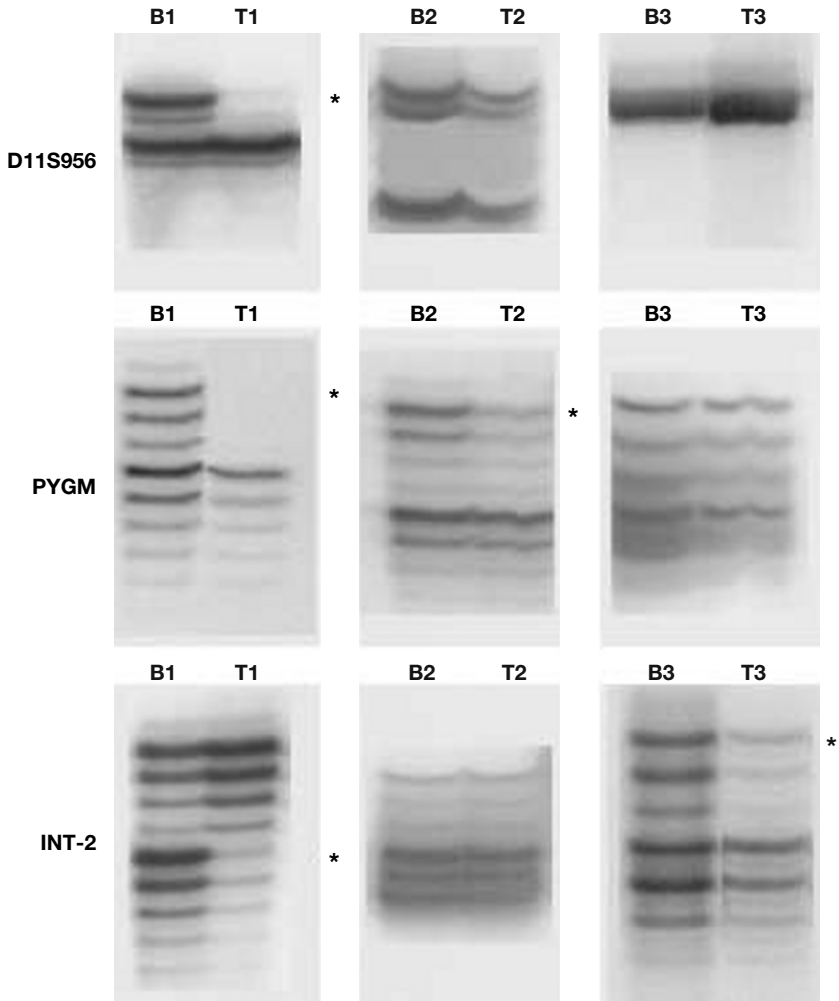

Fig. 1 - *Loss of heterozygosity in 3 TSH-secreting pituitary adenomas compared to matched blood sample at polymorphic markers D11S956, PYGM and INT-2 on chromosome 11q13. B: blood; T: tumor; *: deleted allele.*

Table 1. PCR conditions were as follows: 50 µl of reaction containing 150 ng genomic DNA, 1xPCR buffer, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPS, 10 pmol primers, and 2.5 U AmpliTaq (Perkin-Elmer). After incubation at 94 C for 3 min the mixture was subjected to 35 amplification cycles of 1 min at 94 C, 45 s at 56-66 C (depending on the primer pair used) and 1 min at 72 C.

Loss of heterozygosity

DNA from peripheral blood lymphocytes and tumor samples was screened for LOH using three polymorphic markers on 11q13 as described previously (22).

MEN1 *gene mutational analysis*

After verifying the amplification by agarose gel electrophoresis, each exon of the *MEN1* gene was subjected to double-stranded direct sequencing by automated method (PE Applied Biosystem, ABI PRISM 310 DNA sequencer, Perkin-Elmer) using specific primers.

RESULTS

One copy of the *MEN1* gene was found to be deleted in 3 out of13 sporadic TSH-omas evaluated by LOH analysis. As shown in Figure 1, a complete LOH with all three polymorphic markers tested was detected in tumor 1 (T1) as compared to normal blood DNA (B1), while tumors 2 (T2) and 3 (T3) showed a partial allelic loss, demonstrated by 50% reduction of the signal intensity, with one polymorphic marker: T2 with PYGM marker and T3 with INT-2 marker. D11S956 and INT-2 were not informative in tumors 3 and 2, respectively. All the other tumors, 10 cases, presented allelic re-

tention with all three polymorphic markers (data not shown).
As far the sequence analysis of the *MEN1* gene is concerned, none of the 13 TSH-omas showed mutations in the coding regions. In 2 of 13 tumors we found the previously described polymorphism (D418D) in exon 9 of the MEN1 gene (23, 24).

DISCUSSION

We tested 13 sporadic TSH-omas for allelic deletions and mutations at the *MEN1* gene (Table 2). LOH analysis with 3 polymorphic markers (D11S956, PYGM, INT-2) on 11q13 showed in 3 of 13 tumor tissues (2.3%) allelic loss of one copy of the *MEN1* gene. In particular a complete LOH was observed in one tumor (T1) and a partial allelic loss in two tumors (T2 and T3). No correlation between LOH on 11q13 and the aggressive behavior of the tumors, as exemplified by the tendency to become macroadenomas, has been observed in our series (Table 2). This finding suggests that other mechanisms are involved in tumor progression and transition from non-invasive to invasive phenotype.
As far as the partial allelic loss is concerned, this result could be explained by the fact that allelic deletions may be restricted to a subclone of neoplastic cells, thus representing a second event in the tumorigenesis. Alternatively, it is conceivable that tumor tissues used for DNA extraction contain a mixture of adenomatous and normal cells. No mutations of the *MEN1* gene were identified by sequence analysis in these 3 tumors with LOH. The same result was obtained in the remaining 10 tumors analyzed in the study, which were also negative for allelic deletion on 11q13.
Our results are consistent with previous reports suggesting that menin inactivation is a rare event in sporadic pituitary adenomas (19, 20, 25). Indeed, Zhuang *et al*. (19) using single strand conformational polymorphism analysis found only 2 missense mutations among 4 pituitary adenomas with LOH for *MEN1* in a sample of 39 sporadic tumors. Prezant *et al*. (20) using dideoxy fingerprinting analysis studied 45 sporadic tumors but failed to reveal any mutations in the coding sequence of *MEN1*. Tanaka *et al*. (25), in a series of 31 sporadic tumors detected only 1 nonsense mutation in a GH/PRL-secreting adenoma with LOH on 11q13.
These data are difficult to understand especially taking into account the discrepancy between allelic loss on 11q13 and mutations of the *MEN1* gene. At least three different hypotheses could be postulated in order to explain this discrepancy: 1) tumors may contain mutations in regions of the *MEN1* gene that were not screened, such as promoter, introns and untraslated regions; 2) the hypermetilation of CpG islands in the promoter region, as reported for other tumor suppressor genes

Table 2 - *Clinical and genetic features of 13 patients with TSH-secreting pituitary adenomas.*

Patients	Clinical phenotype	LOH on 11q13	*MEN1* gene mutation/polymorphism
1	HT[1]+macroadenoma	Yes with PYGM, INT-2, D11S956	None
2	HT+microadenoma	Yes with PYGM	D418D
3	HT+macroadenoma	Yes with INT-2	None
4	HT+macroadenoma	No	None
5	HT+microadenoma	No	None
6	HT+microadenoma	No	None
7	HT+macroadenoma	No	D418D
8	HT+macroadenoma	No	None
9	HT+microadenoma	No	None
10	HT+macroadenoma	No	None
11	HT+microadenoma	No	None
12	HT+macroadenoma	No	None
13	HT+macroadenoma	No	None

HT: hyperthyroidism; LOH: loss of heterozygosity; *MEN1*: multiple endocrine neoplasia type 1.

(26, 27), may inhibit the transcription of the second copy of the *MEN1* gene. However, Prezant *et al*. addressed this point by studying *MEN1* mRNA expression in pituitary tumors which was found to be normal, and Asa *et al*. (28) demonstrated by competitive reverse transcription-PCR that there was a lack of menin down-regulation in the majority of tumors with LOH on 11q13; 3) another unidentified tumor suppressor gene may be located at the *MEN1* locus; 4) other factors (growth factors, oncogenes, tumor suppressor genes, hypophysiotropic factors) different from *MEN1* may be altered.

However, over the past years several studies have been performed, but they failed to identify intrinsic genetic defects causing the initiation and the progression of TSH-omas. This issue remains still unexplained probably because a complicate interaction of factors and multiple steps is required for the pathogenesis of these tumors. Our results contribute to exclude menin as the unique gene responsible for such a complex process.

ACKNOWLEDGMENTS

This work was partially supported by the current research from Ospedale Maggiore IRCCS, Milan, and by MURST (9806243848), Rome, Italy.

REFERENCES

1. Beck-Peccoz P., Bruker-Davis F., Persani L., Smallridge R.C., Weintraub B.D.
Thyrotropin secreting pituitary tumors.
Endocr. Rev. 1996, *17:* 610-638.
2. Alexander J.M., Biller M.B.K., Bikkal H., Zervas N.T., Arnold A., Klibanski A.
Clinically nonfunctioning pituitary tumors are monoclonal in origin.
J. Clin. Invest. 1990, *86:* 336-340.
3. Herman V., Fagin J., Gonsky R., Kovacs K., Melmed S.
Clonal origin of pituitary adenomas.
J. Clin. Endocrinol. Metab. 1990, *71:* 1427-1433.
4. Mantovani S., Beck-Peccoz P., Saccomanno K., Spada A., Faglia G., Barbetti F.
TSH-secreting pituitary adenomas are monoclonal in origin. In Book of Abstracts of the 77th Annual Meeting of the Endocrine Society, June 14-17, 1995, Washington, DC, Abstract P2-485, p. 412.
5. Vallar L., Spada V., Giannattasio G.
Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas.
Nature 1987, *330:* 566-568.
6. Landis C., Masters S.B., Spada A., Pace A.M., Bourne H.R., Vallar L.
GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumors.
Nature 1989, 340: 692-696.
7. Lyons J., Landis C.A., Harsh G., Vallar L., Grünewald K., Feichtinger H., Duh Q.Y., Clark O.H., Kawasaki E., Bourne H.R, McCormick F.
Two G protein oncogenes in human endocrine tumors.
Science 1990, *249:* 655-659.
8. Boggil M.D., Jenkinson S., Pistorello M., Boscaro M., Scanarini M., Mc Ternan P., Perrett C.W., Thakker R.V., Clayton R.N.
Molecular genetic studies of sporadic pituitary tumors.
J. Clin. Endocrinol. Metab. 1994, *78:* 387-392.
9. Dong Q., Bruker-Davis F., Weintraub B.D., Smallridge R.C., Carr F.E., Battey J., Spiegel A.M., Shenker A.
Screening of candidate oncogenes in human thyrotroph tumors: absence of activating mutations of the Gaq, Ga11, Gas, or thyrotropin-releasing hormone receptor genes.
J. Clin. Endocrinol. Metab. 1996, *81:* 1134-1140.
10. Delhase M., Vergani P., Malur A., Velkeniers B., Teugels E., Trouillas J., Hooghe-Peters E.L.
Pit1/GHF-1 expression in pituitary adenomas. Further analogy between humans adenomas and rat tumors.
J. Mol. Endocrinol. 1993, *11:* 129-139.
11. Pellegrini I., Barlier A., Gunz G., Figarella-Branger D., Enjalbert A., Grisoli F., Jaquet P.
Pit-1 gene expression in the human pituitary and pituitary adenomas.
J. Clin. Endocrinol. Metab. 1994, *79:* 189-196.
12. Sanno N., Teramoto A., Matsuno A., Inada K., Itoh J., Osamura R.J.
Clinical and immunoistochemical studies on TSH-secreting pituitary adenoma: its multihormonality and expression of Pit-1.
Mod. Pathol. 1994, *7:* 893-899.
13. Sanno N., Teramoto A., Matsuno A., Takekoshi S., Osamura R.J.
GH and PRL gene expression by nonradioisotopic in situ hybridization in TSH-secreting pituitary adenomas.
J. Clin. Endocrinol. Metab. 1995, *80:* 2518-2522.
14. Woloschak M., Roberts J.L., Post K.
c-myc, c-fos, and c-myb gene expression in human pituitary adenomas.
J. Clin. Endocrinol. Metab. 1992, *79:* 253-257.
15. Sumi T., Stefaneanu L., Kovacs K., Asa S.L., Rindi G.
Immunohistochemical study of p53 protein in human and animal pituitary tumors.
Endocr. Pathol. 1993, *4:* 95-99.

16. Bates A.S., Talbot A.J., Buckley N., Bickness J., Farrell W.E., Clayton R.N.
Tumor suppressor genes as a marker of invasive behavior in pituitary tumors.
J. Endocrinol. 1995, (Suppl. 1) 144.

17. Bates A.S., Farrell W.E., Bicknell E.J., McNicol A.M., Talbot A.J., Broome J., Perret C.W., Thakker R.V., Clayton R.N.
Allelic deletion in pituitary adenomas reflects aggressive biological activity and has potential value as a prognostic marker.
J. Clin. Endocrinol. Metab. 1997, *82:* 818-824.

18. Dong Q., Debelenko L.V., Chandrasekharappa S.C., Emmert-Buck M., Zhuang Z., Guru S.C., Manickam P., Skarulis M., Lubensky I.A., Liotta L.A., Collins F.S., Marx S.J., Spiegel A.M.
Loss of heterozygosity at 11q13 analysis of pituitary tumors, lung carcinoids, lipomas, and other uncommon tumors of subjects with familial multiple endocrine neoplasia type 1.
J. Clin. Endocrinol. Metab. 1997, *82:* 1416-1420.

19. Zhuang Z., Ezzat S.Z., Vortmeyer A.O., Weil R., Oldfield E.H., Park W-S.S., Huang S., Agarwal S.K., Guru S.C., Manickam P., Debelenko L.V., Kester M.B., Olufemi S.-E., Heppner C., Crabtree J.S., Burns A.L., Spiegel A.M., Marx S.J., Chandrasekharappa S.C., Collins F.S., Emmert-Buck M.R., Liotta L.A., Asa S.L., Lubensky I.A.
Mutations of the MEN1 tumor suppressor gene in pituitary tumors.
Cancer Res. 1997, *57:* 5446-5451.

20. Prezant T.R., Levine J., Melmed S.
Molecular characterization of the MEN1 tumor suppressor gene in sporadic pituitary tumors.
J. Clin. Endocrinol. Metab. 1998, *83:* 1338-1391.

21. Poncin J., Stevenaert A., Beckers A.
Somatic MEN1 gene mutation does not contribute significantly to sporadic pituitary tumorigenesis.
Eur. J. Endocrinol. 1999, *140:* 573-576.

22. Larsson C., Calender A., Grimmond S., Giraud S., Hayward N.K., Teh F., Farnebo F.
Molecular tools for presymptomatic testing in multiple endocrine neoplasia type 1.
J. Intern. Med. 1995, *238:* 239-244.

23. Chandrasekharappa S.C., Guru S.C., Manickam P., Olufemi S-E., Collins F.S., Emmert-Buck M.R., Debelenko L.V., Zhuang Z., Lubensky I.A., Liotta L.A., Crabtree J.S., Wang Y., Roe B.A., Weisemann J., Boguski M.S., Agarwal S.K., Kester M.B., Kim J.S. Heppner C., Dong Q., Spiegel A.M., Burns A.L., Marx S.J.
Positional cloning of the gene for multiple endocrine neoplasia-type 1.
Science 1997, *276:* 404-407.

24. Agarwal S., Kester M.B., Debelenko L.V., Heppner C., Emmert-Buck M., Skarulis M.C., Doppman J.L., Kim Y.S., Lubensky I.A., Zhuang Z., Green J.S., Guru S.C., Manickam P., Olufemi S.-E., Liotta L.A., Chandrasekharappa S.C., Collins F.S., Spiegel A.M., Burns A.L., Marx S.J.
Germline mutations of the MEN1 gene in familial *MEN1* and related states.
Hum. Mol. Genet. 1997, 6: 1169-1175.

25. Tanaka C., Kimura T., Yang P., Moritani M., Yamaoka T., Yamada S., Sano T., Yoshimoto K., Itakura M.
Analysis of loss of heterozygosity on chromosome 11 and infrequent inactivation of the MEN1 gene in sporadic pituitary adenomas.
J. Clin. Endocrinol. Metab. 1998, *83:* 2631-2634.

26. Herman J.G., Latif F., Weng Y., Lerman M.I., Zbar B., Liu S., Samid D.S., Gnarra J.R., Linehan W.M.
Silencing of the VHL tumor-suppressor gene by DNA metylation in renal carcinoma.
Proc. Natl. Acad. Sci. USA 1994, *91:* 9700-9704.

27. Merlo A., Herman J.G., Mao L., Lee D.J., Gabrielson E., Burger P.C., Baylin S.B., Sidransky D.
5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers.
Nat. Med. 1995, *1:* 686-692.

28. Asa S.L., Somers K., Ezzat S.
The MEN1 gene is rarely downregulated in pituitary adenomas.
J. Clin. Endocrinol. Metab. 1998, *83:* 3210-3212.