# Human Kallikrein 10 Expression in Surgically Removed Human Pituitary Corticotroph Adenomas: An Immunohistochemical Study

*Ashley Di Meo, BSc,*†‡ Fabio Rotondo, BSc,* Kalman Kovacs, MD, PhD,*† Michael D. Cusimano, MD, PhD,§ Luis V. Syro, MD,‖ Antonio Di Ieva, MD, PhD,§ Eleftheros P. Diamandis, MD, PhD,‡ and George M. Yousef, MD, PhD*†*

**Abstract:** Human kallikrein 10 (hk10), a secreted serine protease, was reported to function as a tumor suppressor. hK10 immunoexpression has been demonstrated in lactrotrophs and corticotrophs of the nontumorous human adenohypophysis. In the present study, for the first time we report hK10 immunoexpression in various surgically removed corticotroph adenoma subtypes. Specimens were fixed in formalin and embedded in paraffin. Immunostaining was performed using the streptavidin-biotin-peroxidase complex method with an hK10-specific rabbit polyclonal antibody. Results showed that the endocrinologically active adrenocorticotropic hormone (ACTH)-producing pituitary tumors and the silent subtypes were immunopositve for hK10. Intensity of staining varied between the different subtypes. Intensity was lowest in the silent subtypes (silent corticotroph subtypes 1 and 2) compared with nontumorous human adenohypophysial corticotrophs, whereas the endocrinologically active subtypes (ACTH-secreting adenomas, corticotroph carcinomas, Crooke cell adenomas, Crooke cell carcinomas), showed the highest hK10 immunoexpression. Immunopositivity in the nuclei of the ACTH-secreting adenomas and carcinomas, as well as dual cytoplasmic and nuclear localization of hK10 in some of the secreting tumor types was an intriguing finding. Immunoexpression of hK10 in the ACTH-secreting tumors as well as in the Crooke cell tumors was significantly increased when compared with the nonfunctioning tumors and in the corticotrophs of nontumorous pituitaries.

**Key Words:** ACTH, human kallkrein 10, immunohistochemistry, neoplasms, pituitary adenoma, prognostic marker

(*Appl Immunohistochem Mol Morphol* 2014;00:000–000)

The human tissue kallikrein family is encoded by 15 genes located in tandem on chromosomal locus 19q13.3-4.[1,2] Various members of the human tissue kallikrein family have been reported to play an essential role in tumor invasion and metastasis.[3,4] Human kallikrein 10 (hK10), also known as normal epithelial cell-specific 1 (NES1) protein, is a secreted serine protease belonging to the kallikrein family of tissue proteases. Unlike other members of the kallikrein family, hK10 has been shown to have a putative role as a tumor suppressor due to its ability to inhibit tumor formation in nude mice.[1,5] The expression of hK10 was demonstrated in various human tissues including breast, ovary, prostate, glandular epithelium, and cells within the central and peripheral nervous system.[1] Furthermore, hK10 has been shown to be aberrantly expressed in prostate, breast, and ovarian cancers. In ovarian cancer cell lines, hK10 expression was upregulated whereas in prostate and breast cancer cell lines, it was downregulated.[3,6]

Pituitary corticotroph cell adenomas are adrenocorticotropic hormone (ACTH)-producing tumors often associated with Cushing disease. Crooke cell adenomas are a rare histologic variant of corticotroph adenomas that account for $<1\%$ of pituitary tumors.[7,8] In some cases, this variant seems to be glycocorticoid hormone dependent and occurs in the corticotrophs of nontumorous pituitary of patients with elevated serum cortisol levels.[9] Crooke cell adenomas present as aggressive macroadenomas that invade the sphenoid and cavernous sinuses leading to visual disturbances. As a result of invasion into adjacent tissues, Crooke cell adenomas are usually difficult to operate and have a high recurrence rate.[7,9] Cushing's disease is a consequence of ACTH-secreting pituitary adenomas and is associated with a prolonged period of elevated blood cortisol levels.

Received for publication March 24, 2014; accepted April 29, 2014.

From the *Department of Laboratory Medicine, Keenan Research Centre for Biomedical Science, Li Ka Shing Knowledge Institute, St. Michael's Hospital; †Department of Laboratory Medicine and Pathobiology; §Department of Neurosurgery, St. Michael's Hospital, University of Toronto; ‡Departments of Pathology and Laboratory Medicine, Division of Clinical Biochemistry, Mount Sinai Hospital and Clinical Biochemistry, University Health Network, Toronto, ON, Canada; and ‖Department of Neurosurgery, Hospital Pablo Tobon Uribe and Clinica Medellin, Medellin, Colombia.

Supported by grants from the Canadian Institute of Health Research, Kidney Foundation of Canada, the Kidney Cancer Research Network of Canada, and Prostate Cancer Canada.

The authors declare no conflict of interest.

Reprints: Fabio Rotondo, BSc, Department of Laboratory Medicine, Division of Pathology, Keenan Research Centre for Biomedical Science, Li Ka Shing Knowledge Institute, St. Michael's Hospital, 30 Bond Street, Toronto, ON, Canada M5B 1W8 (e-mail: rotondof@smh.ca).

Other variants of corticotroph adenomas include silent subtype 1 and 2 tumors. The clinically silent corticotroph adenomas account for 5% to 6% of all nonfunctioning pituitary adenomas. Despite ACTH immmunoreactivity, these adenomas display no clinical and biochemical evidence of Cushing's disease. Silent subtype 1 adenomas are indistinguishable from clinically functioning corticotroph adenomas by histologic and ultrastructural analysis.[10] Silent subtype 2 adenomas are usually sparsely granulated, their secretory granules are smaller, and are not spherical. Both subtypes 1 and 2 are aggressive macroadenomas associated with high persistence and recurrence rate.[11] Pituitary carcinomas, according to the World Health Organization classification,[12] can be diagnosed only when distant cerebrospinal and/or systemic metastatases are documented.

The aim of the present study was to determine the clinical value of hK10 as a prognostic biomarker for ACTH-producing pituitary tumors. We found that hK10 immunoexpression is elevated in ACTH-secreting tumor cells versus normal tissues. We also noted that hK10 immunoreactivity was significantly lower in the silent corticotroph adenomas suggesting that the histologic expression of hK10 may distinguish nonfunctioning corticotroph adenomas from functioning corticotroph adenomas.

## MATERIALS AND METHODS

In this study, we examined 58 cases of surgically removed corticotroph adenomas. Specimens included 16 ACTH-secreting adenomas associated with Cushing disease, 6 ACTH-secreting carcinomas, 10 Crooke cell adenomas, 6 Crooke cell carcinomas, and 20 clinically nonfunctioning adenomas (10 silent subtype 1, 10 silent subtype 2 adenomas). Only the primary tumors (carcinomas) were studied and the metastases were not investigated. In addition, 10 nontumorous autopsy obtained pituitaries removed from patients with no endocrine diseases were also collected and used as controls.

Specimens were formalin-fixed, dehydrated in graded ethanol, and paraffin-embedded. Sections of 4-μm thickness were stained with hematoxylin-eosin, periodic acid-Schiff, and the Gordon-Sweet silver method for the demonstration of reticulin fibers. Immunohistochemical analysis using antisera directed toward all adenohypophysial hormones, as well as, the α-chain of the glycoprotein hormones (α-subunit), and Ki-67 using the MIB-1 antibody. Antibody sources and specifications were previously reported.[13] In addition, all tumors were fixed in 2.5% glutaraldehyde, osmicated, routinely processed, and investigated by transmission electron microscopy. Immunohistochemical and ultrastructural investigation was used to accurately classify the tumors and assess their apparent endocrine activity. Tumors were classified according to the criteria defined by the World Health Organization.[12]

Immunohistochemical staining of hK10 was achieved by the streptavidin-biotin-peroxidase complex protocol using an hK10-specific rabbit polyclonal antibody supplied by E.P. Diamandis (dilution 1:150), LSAB+ Kit (DAKO, Carpenteria, CA). Tissues were exposed to primary antibody overnight at 4°C. Formalin-fixed, paraffin-embedded normal pituitary served as a positive control, whereas substitution of hK10 antibody with phosphate-buffered saline served as a negative control.

Immunohistochemical findings were assessed semiquantitatavely by 3 of the authors (A.D.M., F.R., K.K.) using a combined score resulting from multiplying the intensity of staining and frequency of immunoreactive cells, as described by Nakagawa et al.[14] Staining intensity was 4-tier, including 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Frequency of immunoreactive cells was ranked in a 4-tier including 1 ( < 10%), 2 (10% to 50%), 3 (51% to 90%), and 4 (> 90%). Scores for hK10 staining fell into 2 groups, negative (0.1 to 1.9) and positive (2 to 12).

## RESULTS

Using the polyclonal hK10 antibody, immunoreactivity was mainly localized in the cytoplasm of all pituitaries studied. hK10 immunoexpression was also noted in the nuclei of ACTH-secreting pituitary adenomas with Cushing disease, ACTH-producing carcinomas, Crooke cell adenomas, and Crooke cell carcinomas. Staining in the cystoplasm and nuclei varied both within the individual cases and within the various tumor types. Not all immunopositive cells contained nuclear staining.

In all the pituitary adenomas studied, hK10 immunopositivity was highest in the endocrinologically active pituitary tumors (9.0 to 5.4), compared with nontumorous (3.0) (Fig. 1) and silent adenomas (1.9 to 1.5) (Fig. 2) where reactivity was less intense and showed a more diffuse pattern of staining localized only in the cytoplasm. Both ACTH-producing corticotroph and Crooke cell carcinomas showed extensive cytoplasmic immunoexpression as well as nuclear immunopositivity in

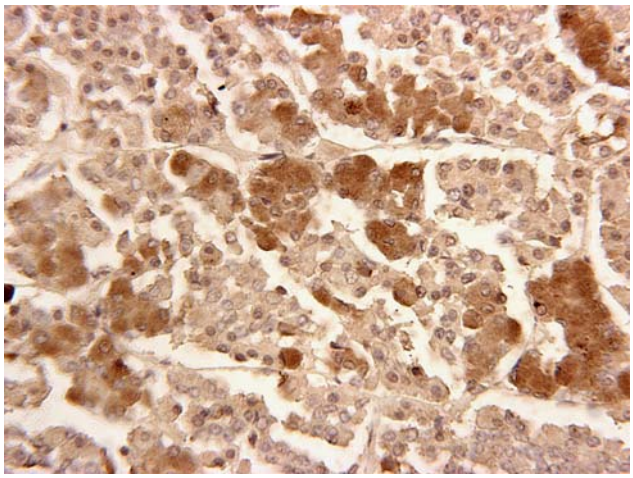

**FIGURE 1.** Diffuse, strong cytoplasmic immunopositivity of human kallikrein 10 in the corticotroph cells of the nontumorous pituitary. Few adenohypophysial cells show mild nuclear positivity. Original magnification: ×250.

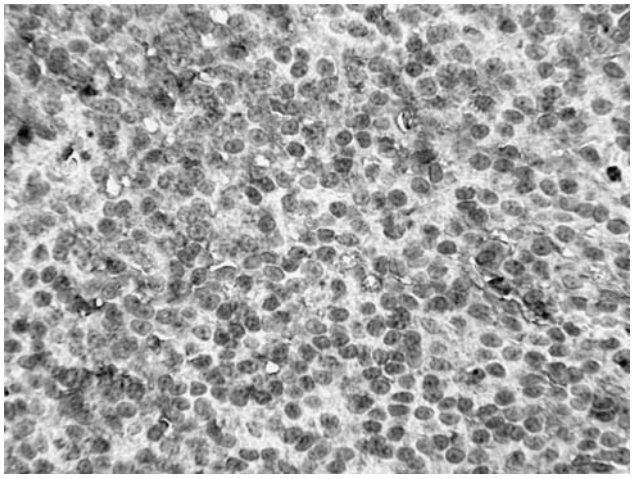

**FIGURE 2.** Weak cytoplasmic immunopositivity in a few cells of silent 1 corticotroph adenoma of the pituitary. Original magnification: ×400.

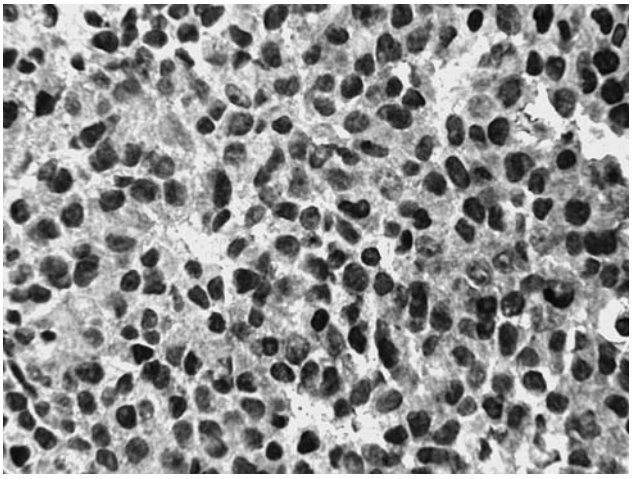

**FIGURE 4.** Corticotroph carcinoma showing strong cytoplasmic and nuclear immunopositivity for human kallikrein 10. Original magnification: ×400.

the majority of the cells (Figs. 3, 4). Nuclear immunoexpression was not seen in all of the cells that demonstrated cytoplasmic positivity. In regards to the hK10 immunoexpression between carcinomas and adenomas of both the ACTH-secreting and Crooke cell tumors, the carcinomas showed a significant increase hK10 immunoexpression in both the cytoplasm and nuclear regions.

The nontumorous pituitaries showed immunopositivity for hK10, achieving a score (3.0) and displaying a diffuse pattern with many stronger staining cells randomly distributed compared with the widespread immunostaining seen in the endocrinologically active tumors (Fig. 4). Double immunostaining was not performed. Further studies are needed to assess the cell types immunopositive for hK10 in the nontumorous adenohypophysis. Scores for hK10 immunostaining are summarized in Table 1.

Replacement of the primary antibody with phosphate-buffered saline abolished immunopositivity in all tissues confirming the specificity of the staining.

No statistically significant correlation was found between hK10 immunopositivity and sex, age, tumor size, or recurrence rate. Correlation between hK10 staining and Ki-67, a nuclear antigen marker for cellular proliferation showed that hK10 immunoexpression was noticeably higher in cases that also had higher Ki-67 labeling index. The pituitary carcinomas which all had Ki-67 values of $>10$ demonstrated the highest hK10 immunoexpression. The results suggest that the location and abundance of hK10 in carcinoma cells may play a role in their initation and/or progression.

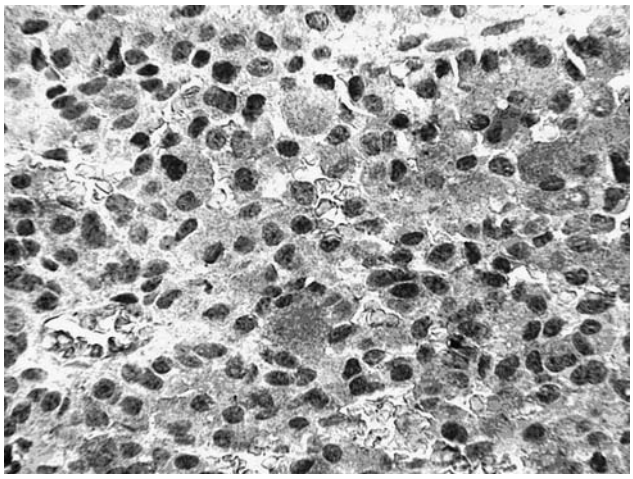

**FIGURE 3.** Corticotroph adenoma accompanied by Cushing disease. The majority of cells show both cytoplasmic and nuclear immunopositivity for human kallikrein 10. Original magnification: ×400.

## DISCUSSION

The human tissue kallikrein family has been reported to play an essential role in tumor progression, invasion, and metastasis.[3] hK10 has been shown to have a putative role as a tumor suppressor and it was found to be abundantly expressed in breast cancer cell line, 76N-MEC, but is downregulated in the radiation transformed equivalent N76-R30.[15] Furthermore, hK10 immunoexpression was shown to be higher in GH-producing pituitary adenomas treated with octreotide, a long-acting somatostatin analog, compared with untreated tumors (Rotondo et al, unpublished data). The finding that hK10 inhibited tumor formation in nude mice further supported the role of hK10 as a tumor suppressor.[5] Luo et al[16] reported hK10 expression to be significantly increased in ovarian carcinoma versus normal ovarian tissue suggesting that hK10 may have the ability to promote tumor cell migration through the breakdown of extracellular barriers. Here, we also report an increase in hK10 immunoexpression in functioning ACTH-secreting corticotroph adenomas and a decrease in silent cortico-

**TABLE 1.** Immunoexpression of Human Kallikrein 10 in Corticotroph Tumors and Nontumorous Pituitaries

| Tissue Type | No. Cases | M:F Ratio | Mean Age | Age Range | Human Kallikrein 10 Immunoexpression (Intensity×Frequency) |
|---|---|---|---|---|---|
| Pituitary tumors | | | | | |
| Crooke cell carcinoma | 6 | 3:3 | 47 | 24-72 | 9.0 |
| ACTH carcinoma | 6 | 3:3 | 50 | 31-70 | 8.7 |
| ACTH adenoma with Cushing | 16 | 6:10 | 51 | 32-67 | 5.9 |
| Crooke cell adenoma | 10 | 4:6 | 59 | 44-79 | 5.4 |
| Silent 1 adenoma | 10 | 6:4 | 52 | 29-80 | 1.9 |
| Silent 2 adenoma | 10 | 5:5 | 55 | 45-63 | 1.5 |
| Nontumorous | 10 | 5:5 | 60 | 49-79 | 3.0 |

ACTH indicates adrenocorticotropic hormone.

troph adenomas. The evidence suggests that hK10 may have a role in tumor suppression or progression and that it may be cancer specific.

We report for the first time the nuclear localization of hK10 in ACTH-secreting pituitary adenomas, Crooke cell adenomas, and Crooke cell carcinomas. This is contrary to what has previously been reported as immunohistochemical analysis demonstrated that hK10 localization is mainly cytoplasmic.[1] The observed nuclear localization of hK10 is similar to what other groups have documented for hK4. It was noted that hK4 is predominately localized in the nucleus by immunofluorescence and cell fractionation studies.[17] It has also been demonstrated that hK4 plays a role in the promotion of cell proliferation, tumor aggressiveness, chemotherapeutic drug resistance, metastasis, and epithelial to mesechymal transition.[18] As a result, we suggest that the nuclear localization of hK10 may alter hK10 function and contribute to tumor progression in a similar manner observed with hK4.

Previous reports have shown that hK4 is predominantly localized in the nucleus because it lacks sequences in exon 1 responsible for encoding a signal peptide which targets the protein for secretion.[17] Similarly, the subset of hK10 with nuclear localization may result from a loss or aberrant function of the signal peptide caused by mutations in the *KLK10* gene.

Human tissue kallikrein expression is regulated by steroid hormones.[1] Prostate-specific antigen, a member of the kallikrein protease family and a well-known prostate cancer biomarker, has been reported to be regulated by estrogens, androgens, glucocorticoids, and progestins.[19–23] hK2 and hK3 responded to androgens in prostate cancer cell lines, whereas hK6 and hK10 have been documented to respond to estrogens in breast cancer cell lines.[3] In addition, hK10 has been shown to be upregulated primarily by estrogens as well as androgens and progestins in the breast cancer cell line BT-474.[1] It appears that cortisol plays an essential role in the upregulation of hK10 in Cushing syndrome patients presenting with hypercortisolemia. This would explain the reduced immunoexpression of hK10 in silent corticotroph adenomas as these patients present with normal serum cortisol level.

Cushing disease is a result of an ACTH-secreting pituitary adenoma that causes an increase in serum cortisol levels.[7,9] The observed expression of human tissue kallikreins in the anterior pituitary by Petraki et al[1] supported the suggestion that kallikreins are involved in the processing of precursors to peptide hormones such as ACTH. Thus, increased hK10 expression may be involved in the excessive secretion of ACTH by functioning corticotroph adenomas.

Some kallikreins have demonstrated diagnostic and prognostic potential in various cancer types. hK3, also known as prostate-specific antigen, is the most well-known kallikrein, a widely used biomarker for prostate cancer.[21] Recently, other kallikreins have been found to have potential use as biomarkers in various diseases including cancer. hK2 has been suggested to have diagnostic potential for prostate cancer.[6] Darson et al[24] provided immunohistochemical evidence that hK2 may be a valuable biomarker capable to identifying more aggressive prostate tumors. hK4, hK5, and hK8 have been examined as potential prognostic biomarkers for ovarian cancer and hK10 was found to have diagnostic and prognostic potential in late-stage ovarian cancer.[16] Here for the first time we demonstrate the diagnostic potential of hK10 in a specific subset of pituitary tumors. hK10 intensity was high in functioning ACTH-secreting adenoma subtypes and low in nonfunctioning silent corticotroph subtypes 1 and 2 adenomas. This may suggest that hK10 has a potential prognostic value in distinguishing functioning ACTH-secreting corticotroph adenomas from silent corticotroph adenomas which has significant implications for patient management and treatment options.

Jin et al[25] found that galactin-3, a β-galactoside-binding protein, is associated with functioning ACTH tumors and is infrequently expressed in silent ACTH adenomas. Thodou et al[26] also demonstrated that galactin-3 is highly expressed in functioning corticotroph adenomas of the pituitary gland, whereas silent adenomas display very focal to null expression of galactin-3. These results suggest that both hK10 and galactin-3 can help distinguish functioning from silent adenomas of the pituitary.

The aim of our study was to determine the clinical value of hK10 as a prognostic biomarker for corticotroph tumors. In this study, we found that hK10 immunoexpression is elevated in ACTH-secreting tumor

cells versus normal tissues. We also found that hK10 immunoreactivity was significantly lower in the silent corticotroph adenomas suggesting that the histologic expression of hK10 may distinguish functioning corticotroph adenomas from nonfunctioning corticotroph adenomas.

## ACKNOWLEDGMENTS

*Authors are grateful to the Jarislowsky and Lloyd Carr-Harris Foundations for their generous support and to Drs BW. Scheithauer and S. Yamada for sharing their valuable tissue (carcinoma cases) for this project.*

## REFERENCES

1. Petraki CD, Karavana VN, Luo LY, et al. Human kallikrein 10 expression in normal tissues by immunohistochemistry. *J Histochem Cytochem*. 2002;50:1247–1261.
2. Yousef GM, White NM, Michael IP, et al. Identification of new splice variants and differential expression of the human kallikrein 10 gene, a candidate cancer biomarker. *Tumour Biol*. 2005;26:227–235.
3. Pepin D, Shao ZQ, Huppe G, et al. Kallikreins 5, 6 and 10 differentially alter pathophysiology and overall survival in an ovarian cancer xenograft model. *PLoS One*. 2011;6:1–12.
4. Borgono CA, Diamandis EP. The emerging roles of human tissue kallikreins in cancer. *Nat Rev Cancer*. 2004;4:876–890.
5. Sidiropoulos M, Pampalakis G, Sotiropoulou G, et al. Down-regulation of human kallikrein 10 (KLK10/NES1) by CpG island hypermethylation in breast, ovarian and prostate cancers. *Tumour Biol*. 2005;26:324–336.
6. Petraki CD, Gregorakis AK, Papanastasiou PA, et al. Immunohistochemical localization of human kallikreins 6, 10 and 13 in benign and malignant prostatic tissues. *Prost Cancer Prostat Dis*. 2003;6:223–227.
7. Rotondo F, Cusimano M, Scheithauer BW, et al. Atypical, invasive, recurring Crooke cell adenoma of the pituitary. *Hormones (Athens)*. 2012;11:94–100.
8. Saeger W, Ludecke DK, Buchfelder M, et al. Pathohistological classification of pituitary tumors: 10 years of experience with the German Pituitary Tumor Registry. *Eur J Endocrinol*. 2007;156:203–216.
9. George DH, Scheithauer BW, Kovacs K, et al. Crooke's cell adenoma of the pituitary: an aggressive variant of corticotroph adenoma. *Am J Surg Pathol*. 2003;27:1330–1336.
10. Alahmadi H, Lee D, Wilson JR, et al. Clinical features of silent corticotroph adenomas. *Acta Neurochir (Wien)*. 2012;154:1493–1498.
11. Scheithauer BW, Jaap AJ, Horvath E, et al. Clinically silent corticotroph tumors of the pituitary gland. *Neurosurgery*. 2000;47:723–730.
12. Kovacs K, Scheithauer BW, Horvath E, et al. The World Health Organization classification of adenohypophysial neoplasms. A proposed five-tier scheme. *Cancer*. 1996;78:502–510.
13. Kovacs K, Lloyd R, Horvath E, et al. Silent somatotroph adenomas of the human pituitary. A morphologic study of three cases including immunocytochemistry, electron microscopy, in vitro examination, and in situ hybridization. *Am J Pathol*. 1989;134:345–353.
14. Nakagawa SA, Lopes A, Lopes de Carvalho A, et al. Nitric oxide synthases, cyclooxygenase-2, nitrotyrosine, and angiogenesis in chondrosarcoma and their relation to prognosis. *J Bone Joint Surg Am*. 2010;92:1738–1746.
15. Shvartsman HS, Lu KH, Lee J, et al. Overexpression of kallikrein 10 in epithelial ovarian carcinomas. *Gynecol Oncol*. 2003;90:44–50.
16. Luo LY, Katsaros D, Scorilas A, et al. Prognostic value of human kallikrein 10 expression in epithelial ovarian carcinoma. *Clin Cancer Res*. 2001;7:2372–2379.
17. Xi Z, Klokk TI, Korkmaz K, et al. Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer. *Cancer Res*. 2004;64:2365–2370.
18. Zhang SQ, Cai B, Liu L, et al. Kallikrein 4 overexpression in endometrial carcinoma and upregulation by estrogen via mitogen-activated protein kinase signal pathway. *Int J Gynecol Cancer*. 2009;19:1377–1383.
19. Diamandis EP, Yousef GM, Luo LY, et al. The new human kallikrein gene family: implications in carcinogenesis. *Trends Endocrinol Metab*. 2000;11:54–60. 200.
20. Yousef GM, Diamandis EP. The new human tissue kallikrein gene family: structure, function, and association to disease. *Endocr Rev*. 2001;22:184–204.
21. Libe R, Barbetta L, Dall'Asta C, et al. Evaluation of serum prostate-specific antigen concentrations in women with Cushing's disease. *Horm Metab Res*. 2003;35:330–332.
22. Luke MC, Coffey DS. Human androgen receptor binding to the androgen response element of prostate specific antigen. *J Androl*. 1994;15:41–51.
23. Slagter MH, Gooren LJ, de Ronde W, et al. Serum and urine tissue kallikrein concentrations in male-to-female transsexuals treated with antiandrogens and estrogens. *Clin Chem*. 2006;52:1356–1365.
24. Darson MF, Pacelli A, Roche P, et al. Human glandular kallikrein 2 expression in prostate adenocarcinoma and lymph node metastases. *Urology*. 1999;53:939–944.
25. Jin L, Riss D, Ruebel K, et al. Galectin-3 expression in functioning and silent ACTH-producing adenomas. *Endocr Pathol*. 2005;16:107–114.
26. Thodou E, Argyrakos T, Kontogeorgos G. Galectin-3 as a marker distinguishing functioning from silent corticotroph adenomas. *Hormones (Athens)*. 2007;6:227–232.