Immunohistochemical Study of the Expression of Human Chorionic Gonadotropin-β in Oral Squamous Cell Carcinoma

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BACKGROUND. Human chorionic gonadotropin (hCG) is a glycoprotein hormone comprised of two dissimilar subunits (α and β) and normally is synthesized by trophoblastic tissue. Although hCG expression has been identified in a variety of neoplastic tissues, to the authors' knowledge no investigation has centered on tumors of oral origin.

METHODS. Oral squamous cell carcinomas (OSCC) were studied in comparison with oral fibromas for the presence of hCGβ using the avidin-biotin-peroxidase complex immunohistochemical technique.

RESULTS. hCGβ immunoreactivity was identified in 29 of 45 OSCC (64%). The positively staining cells in each tumor specimen were few (range, 0.5-5%) and were scattered throughout the tumor. When tumors were classified according to grade, it was found that hCGβ staining was positive in 5 of 15 well differentiated OSCC (33%), in 12 of 15 moderately differentiated OSCC (80%), and in 12 of 15 moderately to poorly differentiated OSCC (80%). hCGβ immunoreactivity could not be demonstrated in any of the oral fibromas.

CONCLUSIONS. The presence of hCGβ positive tumor cells appears potentially to reflect a malignant behavior of OSCC. ***Cancer*** **1999;85:757–62.**
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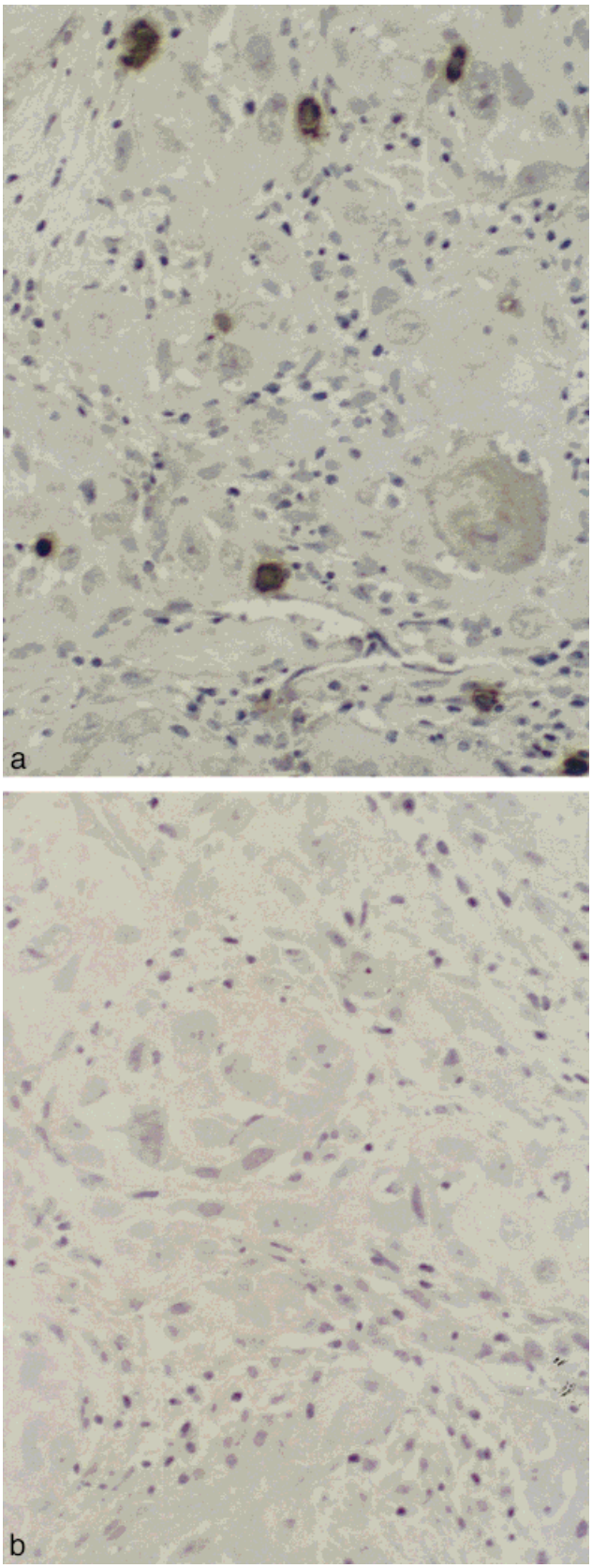
Human chorionic gonadotropin (hCG) was discovered by Hirose[1] and Ascheim and Zondeck[2] 78 and 70 years ago, respectively. It is a glycoprotein hormone comprised of noncovalently linked α and β subunits.[3-5] Although the α subunit is shared by other glycoprotein hormones such as luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone, the β subunits are unique for each hormone.[6-8] hCG is secreted by placental syncytiotrophoblasts and in elevated amounts by neoplastic cells in tumors of trophoblastic origin.[9,10] hCG also can be secreted by various nontrophoblastic neoplasms.[11] Measurement of hCG in serum has been the basis of pregnancy tests and as a marker for many trophoblastic and nontrophoblastic tumors.[10] Of fundamental importance is the fact that normal or benign cells do not express hCGβ. Thus, hCGβ can be a marker of malignant transformation, and hCGβ assays can serve as indicators of tumor progression.[12]

The current study assessed the expression of hCGβ in oral squamous cell carcinoma (OSCC), using avidin-biotin-horseradish peroxidase complex (ABC) immunohistochemistry.



MATERIALS AND METHODS

Tissue Samples

Forty-five formalin fixed and paraffin embedded biopsy specimens from clinically diagnosed and microscopically confirmed OSCC were used. They included 15 well differentiated, 15 moderately differentiated, 14 moderately to poorly differentiated, and 1 poorly differentiated OSCC. In addition, 15 formalin fixed, paraffin embedded biopsy specimens of oral fibromas were used as controls. Four-micron thick sequential sections were cut from each specimen. One slide was stained with hematoxylin and eosin. Another slide was subjected to the immunohistochemical procedure for detection of hCGβ. The third slide served as a negative control with the primary antibody omitted.

Sections of a formalin fixed, paraffin embedded normal placenta were used as positive control tissue for a titration assay to determine the optimal dilution of anti-hCGβ antibody and the proper incubation period that permitted specific antigen detection with minimal background staining.

Reagents

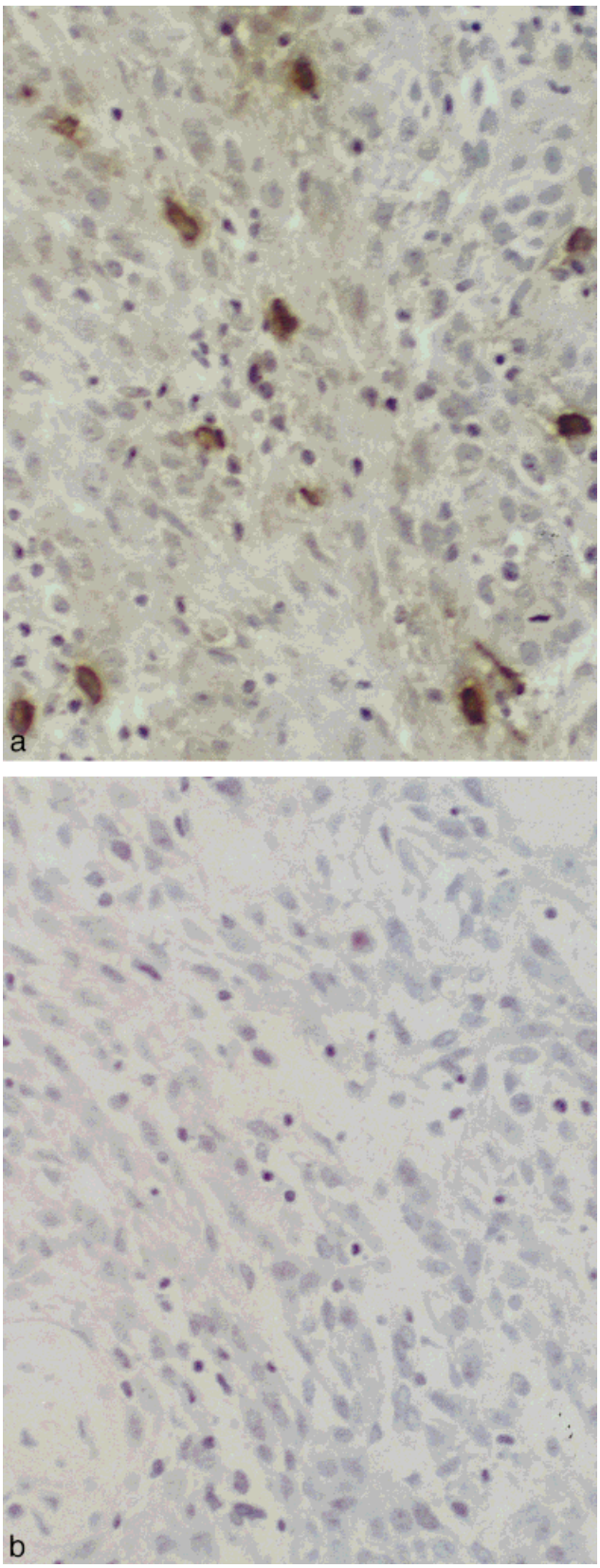
Reagents were the primary antibody (mouse anti-hCGβ), secondary antibody (biotin-goat antimouse immunoglobulin [Ig] G), peroxidase-avidin complex, diaminobenzidine tetrahydrochloride/nickel-cobalt kit (DAB-Black Kit), Peroxo-Block (specific inhibitor of endogenous peroxidase activity), CAS Block (nonimmune immunoglobulin), pepsin, phosphate-buffered saline (PBS) (pH 7.4), PBS with Tween 20, hematoxylin, and Histomount.

All the reagents were obtained from Zymed Laboratories (San Francisco, CA) except for the PBS with Tween 20, which was obtained from Sigma Laboratories (St. Louis, MO). All the reagents were stored at 4 °C except for hematoxylin and Histomount, which were stored at room temperature.

Immunostaining Procedure

The ABC immunohistochemical technique was a modification of that described by Yakeishi et al.[5] After deparaffinization, the sections were treated with Peroxo-Block. Pepsin digestion at 37 °C was conducted

FIGURE 1. (a) Human chorionic gonadotropin (hCG) immunoreactivity in a moderately poorly differentiated oral squamous cell carcinoma (OSCC) treated with an immunohistochemical procedure that included the primary antibody. Black immunostaining is present (immunostain for β-hCG, $\times$100). (b) hCG immunoreactivity in the same moderately poorly differentiated OSCC treated with an immunohistochemical procedure without the primary antibody. No immunostaining is present (immunostain for β-hCG, $\times$100).



for antigen retrieval and CAS Block was applied to reduce nonspecific background staining. The primary antibody was applied for 20 hours at 4 °C, followed by biotinylated secondary antibody (goat antimouse monoclonal antibody at 1:200 dilution) for 10 minutes at room temperature. Then, avidin-conjugated peroxidase (1:200 dilution) was applied for 10 minutes at room temperature. Chromogen, diaminobenzidine tetrahydrochloride/nickel-cobalt, substrate system was applied and sections were counterstained with hematoxylin, dehydrated, and mounted in histomount.

Negative control sections were subjected to the same staining procedures in deletion of the primary antibody. The sections were examined microscopically for the presence and proportion of positively stained tumor cells at ×100 magnification. Any section that had one or more stained cells was classified as positive.

Statistical Analysis

The different grades of OSCC and oral fibromas were compared blindly for differences in staining using Fisher's exact test. A Bonferroni adjustment was made to the *P* values to adjust for multiple tests.

RESULTS

hCGβ immunoreactive cells were found in 29 of the 45 specimens of OSCC (64%). The black staining ranged from moderate to high intensity. It involved single cells or small clusters of cells scattered throughout the tumor (Figs. 1a and 2a). The staining was specific because it was not detected in negative control sections in which the primary antibody to hCGβ was not applied (Figs. 1b and 2b). hCGβ staining was positive in 5 of 15 well differentiated OSCC (33%), in 12 of 15 moderately differentiated OSCC (80%), and in 12 of 15 moderately poorly to poorly differentiated OSCC (80%).

The percentage of positively staining cells in the tumor specimens was small (range, 0.5-5%). The one poorly differentiated OSCC specimen showed positive staining. The oral fibroma specimens consistently were negative. In addition, normal tissues surround-

FIGURE 2. (a) Human chorionic gonadotropin (hCG) immunoreactivity in a moderately differentiated oral squamous cell carcinoma (OSCC) treated with an immunohistochemical procedure that included the primary antibody. Black immunostaining is present (immunostain for β-hCG, ×100). (b) hCG immunoreactivity in the same moderately differentiated OSCC treated with an immunohistochemical procedure without the primary antibody. No immunostaining is present (immunostain for β-hCG, ×100).

TABLE 1
Summary of Immunohistochemical Studies Localizing hCG in Carcinomas

Source, year	No.	Tumor type	Method	% positive staining	Control
McManus et al., 1976[14]	9	Renal	IMPX	89	NIS
Horne et al., 1976[24]	50	Breast	IMPX	60	NIS, AA
Nishiyama et al., 1980[25]	35	Lung	IMPX	20	NIS
Nishiyama et al., 1980[25]	16	Breast	IMPX	6	NIS
Bellet et al., 1980[26]	53	Breast	IMPX	4	NIS, AA
Wilson et al., 1981[27]	61	Lung	PAP	84	NIS
Heyderman et al., 1982[28]	40	Lung	IMPX	5	AA
Wachner et al., 1984[29]	129	Breast	IMPX	12	NIS, AA
Kimura et al., 1985[30]	72	Lung	PAP	57	NIS
Lee et al., 1985[18]	233	Breast	ABC	15	NIS, AA
Burg-Kurland et al., 1986[21]	33	Esophageal	PAP	33	AA
Kuida et al., 1988[17]	11	Lung	ABC	36	NIS, AA
Kuida et al., 1988[17]	10	Breast	ABC	10	NIS, AA
Kuida et al., 1988[17]	10	Renal	ABC	0	NIS, AA
Yamagushi et al., 1989[15]	388	Colorectal	PAP	22	NIS
Yakeishi et al., 1990[5]	92	Gastric	ABC	53	NIS
Current study, 1997	45	Oral	ABC	64	NIS

hCG: human chorionic gonadotropin; IMPX: indirect immunoperoxidase; NIS: nonimmune serum; AA: absorbed antiserum; PAP: peroxidase-antiperoxidase; ABC: avidin-biotin-horseradish peroxidase complex.

ing the tumors consistently were negative. The immunostaining was localized in the cytoplasm of cancer cells. The nuclei consistently were negative and occasionally a discrete band of staining near the surface of the malignant cells was noticeable. In general, it appeared that the most anaplastic or poorly differentiated cells tended to be positive whereas well differentiated cells were negative.

Statistical analysis showed that the difference in staining between moderately poorly differentiated OSCC and oral fibroma and between moderately differentiated OSCC and oral fibroma was significant ($P = 0.001$). However, the difference in staining between well differentiated OSCC and oral fibroma was not significant ($P = 0.227$). The statistical analysis also revealed that the difference in staining between moderately to poorly differentiated OSCC and well differentiated OSCC, as well as between moderately differentiated OSCC and well differentiated OSCC, was approaching statistical significance. ($P = 0.141$)

DISCUSSION

Elevated serum levels of hCG occur in patients with trophoblastic tumors and in some patients with malignancies of the lung, liver, gastrointestinal tract, adrenal cortex, and urogenital tract.[13,14] McManus et al.,[14] using frozen tissue sections and immunohistochemistry, found that hCG was detectable in 89% of tumor specimens. Subsequent immunohistochemical studies showed a marked variation in the frequency of hCG detection in tumor specimens (Table 1). In the current study, 64% of OSCC showed hCGβ immunoreactivity, a frequency less than that reported by McManus et al.,[14] but higher than the results of the majority of other studies.

In the current study, tissues with a few immunoreactive cells in the tumor were evaluated as positive and these positive cells were observed more often in moderately and moderately to poorly differentiated OSCC. The same tendency also was observed by other investigators.[15] This observation suggests that the presence of hCG in tumor defines the aggressiveness of the tumors in which it is found, or in other words, hCG positive tumors could indicate a higher grade of malignancy. Although the mechanism of hCG production in nontrophoblastic tumors is unknown, our results support the differentiation theory in which it is believed that hCG production could be an event in the course of dedifferentiation or arrested differentiation of the malignant cells.[16]

Based on the results of many studies, it is possible that the majority nontrophoblastic tumors synthesize small amounts of hCG but that the immunohistochemical techniques may not be sensitive enough to detect the marker, especially in formalin fixed, paraffin embedded tumor tissue.[17] Finally, there also could be some differences in the production of hCG among different types of nontrophoblastic tumors; therefore, more studies are needed to investigate hCG immunoreactivity in OSCC and other tumors.

Some of the previous studies indicate that the percentage of positively stained tumor cells in each specimen was small.[17,18] This supports the findings of the current study. The low frequency of cancer cells

stained by hCG can be explained by tumor cell heterogeneity. Even though some tumors have a multicellular origin, the majority of tumors are monoclonal.[19] Nonetheless, tumors are comprised of different cell populations with diverse morphologic, genetic, immunologic, and biologic characteristics.[20] Epigenetic as well as genetic phenomena govern the development of tumor cell heterogeneity. Cancer cells genetically are unstable, and this instability increases with tumor progression and results in the generation of subpopulations of cells with increasingly malignant potential.[20] This may explain why in the current study in a single tumor there was only a small percentage of tumor cells that expressed hCGβ immunoreactivity. The hCGβ immunoreactive tumor cells may be more aggressive and have higher metastatic potential.

The current study indicates that hCG is produced by the majority of OSCC lesions. The results also suggest that hCGβ expression could indicate a higher grade of malignancy. hCGβ appears to be a tumor-associated marker because it was not expressed by normal or uninvolved oral epithelium. Thus, hCGβ could be useful in cytologic studies for the identification of OSCC cells. hCGβ may contribute to a means of subclassifying OSCC based on the frequency of the presence of the marker.[21] There is evidence indicating that certain tumor markers are associated with the biologic and clinical behavior of the tumors.[22,23] Therefore, immunohistochemical classification of OSCC could provide more prognostic information than classification based exclusively on morphology.[21]

REFERENCES

1. Hirose T. Exogenous stimulation of corpus luteum formation in the rabbit: influence of extracts of human placenta, decidua, fetus, hydatid mole, and corpus luteum on the rabbit gonad. *J Jpn Gynecol Soc* 1920;16:1055.
2. Ascheim, Zondek B. Die Schwangerschaftsdiagnose aus dem harn durch Nachweis des Hypophysenvorderlappen-hormone. 2. Practishe und theoretiche Ergebnisse aus den harnuntersuchungen. *Klin Wochenschr* 1928;7:1453-7. In: O'Connor J, Birken S, Lustbader JW, Krichevsky A, Chen Y, Canfield RE. Recent advances in chemistry and immunochemistry of human chorionic gonadotropin: impact on clinical measurements. *Endocr Rev* 1994;15:650-83.
3. Nagelberg SB, Marmorstein B, Khazaeli MB, Rosen SW. Isolated ectopic production of the free beta subunit of chorionic gonadotropin by an epidermoid carcinoma of unknown primary site. *Cancer* 1985;55:1924-30.
4. Cowley G, Smith JA, Ellison M, Gusterson B. Production of β-human chorionic gonadotropin by human squamous cell carcinoma cell lines. *Int J Cancer* 1985;35:575-9.
5. Yakeishi Y, Mori M, Enjoji M. Distribution of β-human chorionic gonadotropin-positive cells in noncancerous gastric mucosa and in malignant gastric tumors. *Cancer* 1990;66: 695-701.
6. Hattori M, Fusake M, Yoshimi H, Matsakura S, Imura H. Ectopic production of human chorionic gonadotropin in malignant tumors. *Cancer* 1978;42:2328-33.
7. Rosen SW, Weinstraub BD, Vaitukitis JL, Sussman HH, Hershman JM, Muggia FM. Placental proteins and their subunits as tumor markers. *Ann Intern Med* 1975;82:71-83.
8. Cosgrove DE, Campain JA, Cox GS. Chorionic gonadotropin synthesis by human tumor cell lines: examination of subunit accumulation, steady-state levels of mRNA, and gene structure. *Biochim Biophys Acta* 1989;1007:44-54.
9. Marcillac I, Troalen F, Bidart J-M, Ghillani P, Ribrag V, Escadier B, et al. Free human chorionic gonadotropin β subunit in gonadal and nongonadal neoplasms. *Cancer Res* 1992;52,3901-7.
10. O'Connor JF, Birken S, Lustbader JW, Krichevsky A, Chen Y, Canfield RE. Recent advances in the chemistry and immunochemistry of human chorionic gonadotropin: impact on clinical measurement. *Endocr Rev* 1994;15:650-83.
11. Gailani C, Chu TM, Nussbaum A, Ostrander M, Christoff N. Human chorionic gonadotrophins (hCG) in non trophoblastic neoplasms: assessment of abnormalities of hCG and CEA in bronchogenic and digestive neoplasms. *Cancer* 1976;38:1684-6.
12. Regelson W. Have we found the "Definitive cancer biomarker"? The diagnosis and therapeutic implications of human chorionic gonadotropin-beta expression as a key to malignancy. *Cancer* 1995;76:1299-301.
13. Braustein GD, Vaitukitis JL, Carbone PP, Ross GT. Ectopic production of human chorionic gonadotropin by neoplasms. *Ann Intern Med* 1973;78:39-45.
14. McManus LM, Maughton MA, Martinez-Hernandez A. Human chorionic gonadotropin in human neoplastic cells. *Cancer Res* 1976;36:3476-81.
15. Yamagushi A, Ishida T, Nishimura G, Kumaki T, Katoh M, Kosaka T, et al. Human chorionic gonadotropin in colorectal cancer and its relationship to prognosis. *Br J Cancer* 1989; 60:382-4.
16. Uriel J. Retrodifferentiation and fetal patterns of gene expression in cancer. *Adv Cancer Res* 1979;29:127-74.
17. Kuida CA, Braustain GD, Shintaku P, Said JW. Human chorionic gonadotropin expression in lung, breast, and renal carcinomas. *Arch Pathol Lab Med* 1988;112:282-5.
18. Lee AK, Rosen PP, DeLellis RA, Saigo PE, Gangi MD, Groshen S, et al. Tumor marker expression in breast carcinomas and relationship to prognosis: an immunohistochemical study. *Am J Clin Pathol* 1985;84:687-96.
19. Fialkow PJ. Clonal origin of human tumors. *Biochim Biophys Acta* 1976;458:283-321.
20. Drews RE, Schnipper LE. Development of tumor heterogeneity. In: Moosa AR, Schimpff SC, Robson MC, editors. Comprehensive textbook of oncology. 2nd edition. Baltimore: Williams and Wilkins, 1991:123-30.
21. Burg-Kurland CL, Purnell DM, Combs JW, Hillman EA, Harris CC, Trump BF. Immunocytochemical evaluation of human esophageal neoplasms and preneoplastic lesions for β-chorionic gonadotropin, placental lactogen, carcinoembryonic antigen, and nonspecific cross-reacting antigen. *Cancer Res* 1986;46:2936-43.
22. Higashiyama M, Kodama K, Yokouchi H, Takami K, Adashi M, Taki T, et al. KA11/CD82 expression in nonsmall lung carcinoma is a novel, favorable prognostic factor. *Cancer* 1998;83:466-74.
23. Pizer ES, Lax SF, Kuhajda FP, Pasternack GR, Kurman RJ. Fatty acid synthase expression in endometrial carcinoma correlation with cell proliferation and hormone receptors. *Cancer* 1998;83:528-37.

24. Horne CHW, Reid IN, Milne GD. Prognosis significance of inappropriate production of pregnancy proteins by breast cancer. *Lancet* 1976;2:279-82.
25. Nishiyama T, Stolbach LL, Rule AH. Expression of oncodevelopmental markers (Regan isozyme, β-hCG, CEA) in tumor tissues and uninvolved bronchial mucosa: an immunohistochemical study. *Acta Histochem Cytochem* 1980;13:245-53.
26. Bellet D, Arrang JM, Contesso G, Caillaud JM, Bohuon C. Localization of the β subunit of human chorionic gonadotropin in various tumors. *Eur J Cancer* 1980;16:433-9.
27. Wilson TS, McDowell EM, McIntire KR, Trump BF. Elaboration of human chorionic gonadotropin by lung tumors. *Arch Pathol Lab Med* 1988;112:282-5.
28. Heyderman E, Chapman DV, Richardson TC. Biological markers in lung cancer: an immunocytochemical approach. *Cancer Detect Prev* 1982;5:429-49.
29. Wachner R, Wittekind C, Kleist SV. Immunohistological localization of β-hCG in breast carcinomas. *Eur J Cancer Clin Oncol* 1984;20:679-84.
30. Kimura N, Ghandur-Mnaymen L. An immunohistochemical study of keratin, carcinoembryonic antigen, human chorionic gonadotropin and alpha-fetoprotein in lung cancer. *Tohoku J Exp Med* 1985;145:23-38.