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Enolase isozymes in seminoma

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Summary. We determined concentrations of α and γ-enolases in normal testis and in seminoma tissues by enzyme immunoassay. Concentrations of α-enolase were 4,170 ± 2,040 ng/mg protein in normal testis (n = 8) and 8,140 ± 4,480 ng/mg protein in seminoma (n = 8). Concentrations of γ-enolase in seminoma (460 ± 571 ng/mg protein) were significantly higher than those of normal testis (59 ± 15 ng/mg protein). Immunohistochemistry showed positive tumor cells for γ-enolase in 6 of 8 seminoma cases (75%). Serum γ-enolase levels were elevated (> 6.0 ng/ml) in 9 of 12 patients (75%) with seminoma: 60% of stage I, and 100% of stages II and III. In 10 patients treated by surgical excision and chemotherapy, serum γ-enolase was significantly reduced after the treatment. These findings indicate that elevated serum γ-enolase is derived from enhanced γ-enolase in seminoma tissues, and that serum γ-enolase could be a useful biomarker for staging and monitoring clinical course in patients with seminoma.

Key words: Seminoma – Enolase – Isozyme – Enzyme immunoassay – Tumor marker

Enolases (EC 4.2.1.11) are glycolytic enzymes catalyzing the reaction pathway between 2-phosphoglycerate and phosphoenolpyruvate, and are widely distributed in mammalian tissues. Their molecules are dimers composed of three immunologically distinct subunits, α, β and γ, and 5 isozymes are known (αα, ββ, γγ, αβ and αγ) with molecular weights of approximately 100,000 [5, 27]. The α subunit of enolase (α-enolase) is widely distributed in various tissues, while the β subunit (β-enolase) is mainly localized in the heart and striated muscle. The γ subunit of enolase (γ-enolase), designated neuron-specific enolase (NSE), is localized at high levels in neuronal cells [28] and neuroendocrine cells, and tumors derived from these cells [30, 36]. We demonstrated that γ-enolase is widely distributed in various tissues and in many types of cells other than just neuronal and neuroendocrine ones by immunohistochemical [8] and enzyme innunoassay methods [15–17]. Several studies showed that serum γ-enolase could be a useful marker for neuroendocrine tumors [10, 26, 33], small cell carcinoma of the lung [3, 7], and renal cell carcinoma [9, 32].

Recent advances in cisplatin-based chemotherapy have lead to improved survival in patients with testicular cancer. Serum markers such as α-fetoprotein (AFP) and human chorionic gonadotropin (HCG) have made important contributions to the diagnosis and management of testicular cancer, particularly, of nonseminomatous germ cell tumors (NSGCT) [12]. But, until now, no useful serum markers for seminoma had been reported. Clinical demand for a marker for seminoma prompted us to study enolase isozymes in seminoma, because of the recent report that the seminoma produces a significant amount of γ-enolase [18, 25].

In this study, to evaluate γ-enolase as a tumor marker for seminoma, we determined the concentrations of both α and γ-enolases in tissues and sera with special reference to tumor stage and clinical course of patients, and immunohistochemically localized α and γ-enolases in normal testis and seminoma tissues.

Materials and methods

Tissue samples

Samples were taken from 8 seminomas, one teratocarcinoma, and one embryonal carcinoma. Histologically normal testicular tissues (n = 8) adjacent to the tumor tissues were also prepared. All samples were obtained from surgical operations and were immediately frozen and kept at -80°C. For enolase assay, about 0.5 gm of each sample was homogenized at 0°C with a glass homogenizer in 10 volumes (volume per weight) of 0.05 M sodium phosphate buffer (pH 7.0) containing 5 mM magnesium sulfate. The homogenate was centrifuged at 4°C at 15,000 *g* for 20 minutes, and the soluble fraction was used for immunoassay of enolase subunits. Protein concentrations in the extracts were measured with Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA), using a dye binding method [1].

Table 1. Enolase isozymes in tissues of normal testis and testicular cancer

	No. of samples	α-enolase (ng/mg protein)	γ-enolase (ng/mg protein)	$\frac{\gamma}{\alpha+\gamma}$ (%)
Normal testis	8	4,170 ± 2,040[a]	59 ± 15[a]	1.7 ± 0.7[a]
Seminoma	8	8,140 ± 4,480	460 ± 571[b]	7.0 ± 8.0
NSGCT	2	2,770, 5,320	49, 64	1.7, 1.2

NSGCT = nonseminomatous germ cell tumor

[a] Values are means ± standard deviation

[b] Significantly higher than normal testis ($p<0.01$), ranging from 62 to 1,660 ng/mg protein

For immunohistochemistry, samples obtained from surgical operations (8 seminomas and 4 normal testicular tissues) were fixed in periodate-lysine-4% paraformaldehyde [24] for 6 hours, washed in phosphate-buffered saline (PBS, pH 7.2) containing increasing concentrations of sucrose, embedded in OCT compound (Tissue-Tek, Naperville, IL), and frozen quickly in dry ice and ethanol.

Serum samples

Serum samples were taken from 12 patients with seminoma (5 of stage I, 2 of stage II, 3 of stage III, and 2 of recurrent disease). Ages ranged from 24 to 57 years with a median of 32. All of the 12 patients underwent radical orchiectomy. Five patients with stage I and one with IIA had retroperitoneal radiation; 6 patients with stages IIB or III, or recurrent disease had chemotherapy. Serum samples both before and after treatment were available for 10 of the 12 patients with seminoma. Serum samples were also taken from 6 patients with NSGCT. All serum samples were kept frozen at -80°C until analysis, and the samples examined were not hemolyzed. When measurements were taken, the serum samples were thawed and 10 μl aliquots were subjected in duplicate to immunoassay of enolase subunits.

Assay of enolase isozymes

Concentrations of enolase isozymes in tissue extracts and serum samples were determined with the enzyme immunoassay system reported previously [17]. The α-enolase assay system consisted of monospecific antibodies to the α subunit of human enolase, and reacted not only with the αα form but also with the αβ and αγ forms of enolase which contain the α subunit in the molecules. The γ-enolase assay system, prepared with monospecific antibodies to the γ subunit of human enolase, reacted with the γγ and αγ forms of enolase. Because αα and γγ forms of enolase were used as the standards for their respective assays, the results were expressed as αα-enolase-equivalent nanograms and γγ-enolase-equivalent nanograms per milliliter serum (ng/ml) or per milligram soluble protein (ng/mg).

To assess γ-enolase, the proportion of γ-enolase to total enolases, that is γ-enolase/(α-enolase + γ-enolase) or [γ/(α + γ)], was calculated. β-enolase was disregarded, because the proportion of β-enolase to total enolases in sera and testicular tissues is small [16].

Serum concentrations of α and γ-enolases in healthy subjects were reported previously [7]. Serum concentrations of α-enolase were 67 ± 24 ng/ml (mean ± standard deviation), ranging from 28 to 135 ng/ml; and of γ-enolase 3.1 ± 0.9 ng/ml, ranging from 1.6 to 5.8 ng/ml, in 100 healthy subjects. The upper limit of normal serum γ-enolase, defined as the mean plus three standard deviations, was 6.0 ng/ml, and so all values greater than 6.0 ng/ml were considered positive.

Immunohistochemistry

α and γ-enolases were localized by the direct peroxidase-labeled antibody method described previously [9]. Briefly, serial cryostat sections were placed on albumin-coated slides and dried at room temperature. The sections were treated with 100% methanol and 0.03% hydrogen peroxide in PBS to inactivate endogeneous peroxidase. They were immersed in nonimmune rabbit serum, washed in PBS, and then reacted with the horseradish peroxidase (HRP)-labeled Fab' fragments of the antiserum. Control sections were treated with the HRP-labeled antibody absorbed with the relevant purified antigens or with HRP-labeled Fab' fragments of nonimmune rabbit γ-globulin. After being washed in PBS, the sections were reacted with 0.025% diaminobenzidine solution containing 10 mM hydrogen peroxide and 10 mM sodium azide, then counterstained with methyl green.

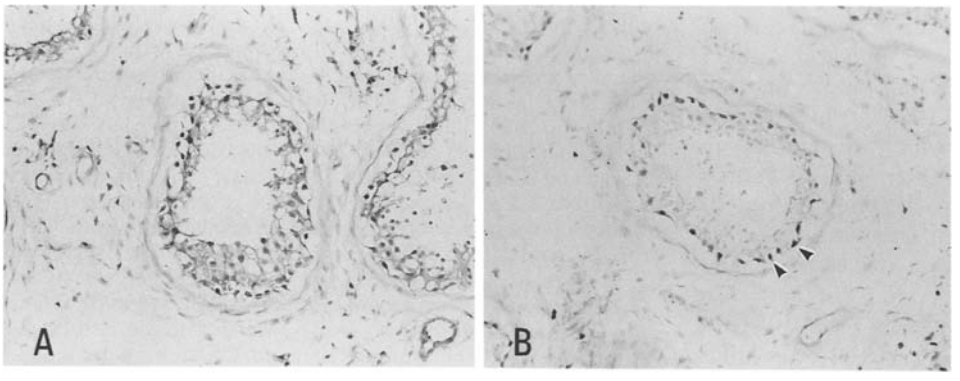

Fig. 1. Localization of α-enolase **A** and γ-enolase **B** in normal testicular tissue (×115). α-enolase was immunostained in spermatogonia, spermatocyte, spermatid and sperm. γ-enolase was localized in spermatogonia (arrowheads)

Table 2. Serum enolase in patients with testicular cancer and in healthy subjects

	No. of samples	α-enolase (ng/ml)	γ-enolase (ng/ml)	$\frac{\gamma}{\alpha+\gamma}$ (%)
Seminoma	12	137 ± 71[a]	10.6 ± 8.8[a]	7.9 ± 4.7
NSGCT	6	82 ± 36	7.4 ± 3.0	9.3 ± 4.8
Healthy subjects	100	67 ± 24	3.1 ± 0.9	4.7 ± 1.4

NSGCT = nonseminomatous germ cell tumor.
[a] Significantly higher than healthy subjects ($p<0.05$)
All values are means ± standard deviation

Staging and statistics

Tumors were staged according to the classification of Caldwell and associates [2]. Data are expressed as mean ± standard deviation. The results were compared by Wilcoxon rank-sum test or signed-rank test.

Results

Concentrations of α and γ-enolases in seminoma tissues

Table 1 shows the concentrations of α and γ-enolases in soluble extracts of the normal testis, seminoma and NSGCT. The mean concentration of α-enolase in seminoma tissues was 2.0 times higher than in normal testicular tissues. The mean level of γ-enolase in seminoma was 7.8 times higher than in normal testis with statistical significance ($p < 0.01$). Seven of 8 cases (88%) had elevated tissue levels (>110 ng/mg protein) of γ-enolase. Consequently, the mean $\gamma/(\alpha + \gamma)$ value of seminoma tissues was 7.0%, and that of normal testis tissues was 1.7%. Two cases of NSGCT (one teratocarcinoma and one embryonal carcinoma) had 49 and 64 ng/mg protein of γ-enolase, respectively, as low as in normal testis.

Immunohistochemical localization of α and γ-enolases in normal testis and seminoma tissues

Among germ cells of normal testis, α-enolase was positive in spermatogonia, spermatocyte, spermatid and sperm (Fig. 1A), whereas, γ-enolase was confined to spermatogonia (Fig. 1B).

In seminoma, α-enolase was positive in the cytoplasm, and occasionally nucleus of tumor cells in all 8 cases (Fig. 2A). γ-enolase was immunostained in 6 of 8 cases (75%). γ-enolase was also localized in the cytoplasm, and occasionally nucleus of tumor cells (Fig. 2B). Staining intensity and number of positive tumor cells for γ-enolase correlated well with tissue concentrations of γ-enolase in the 7 cases in which both immunohistochemical and quantitative studies were performed.

Serum levels of γ-enolase in patients with seminoma

Serum γ-enolase in 12 patients with seminoma ranged from 3.8 to 35.9 ng/ml with a mean of 10.6 ± 8.8 ng/ml (Table 2, Fig. 3). Serum γ-enolase in seminoma was significantly higher than in healthy subjects (3.1 ± 0.9 ng/ml, $p<0.05$). Serum levels of α-enolase in patients with seminoma were also higher than in healthy subjects ($p<0.05$). The mean $\gamma/(\alpha+\gamma)$ in patients with seminoma (7.9%) was higher than that in healthy subjects (4.7%, not significant). Serum γ-enolase and $\gamma/(\alpha+\gamma)$ value in patients with NSGCT were elevated as in patients with seminoma (Table 2). No significant association was found between serum levels and tissue concentrations of γ-enolase in the 7 patients with seminoma.

Overall, serum levels of γ-enolase were elevated (>6.0 ng/ml) in 9 of 12 patients with seminoma (75% positive), and in 4 of 6 (67%) with NSGCT (Fig. 3). Figure 4 shows the distribution of serum γ-enolases in patients with

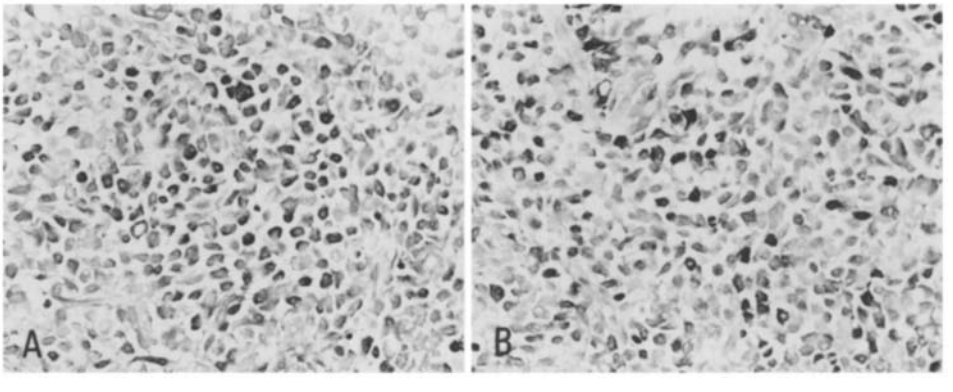

Fig. 2. Localization of α-enolase **A** and γ-enolase **B** in seminoma tissue (×170). Both α and γ-enolases were positively stained in cytoplasm and occasionally nucleus of tumor cells

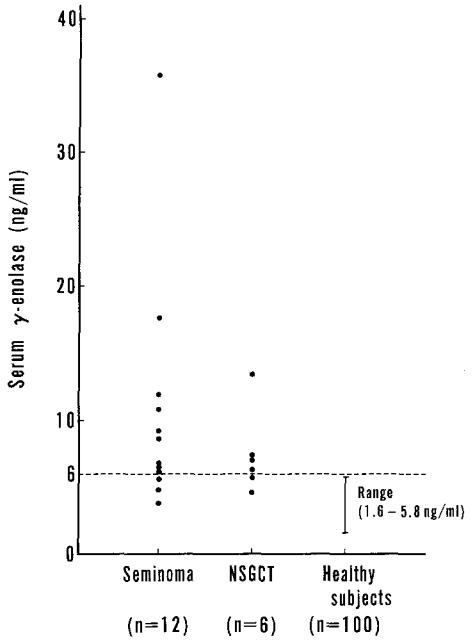

Fig. 3. Serum levels of γ-enolase in patients with seminoma and nonseminomatous germ cell tumors (NSGCT). In healthy subjects, serum level of γ-enolase was 3.1 ± 0.9 ng/ml (mean ± standard deviation), ranging from 1.6 to 5.8 ng/ml. The upper limit of normal serum γ-enolase, defined as the mean plus 3 standard deviations, was 6.0 ng/ml (dashed line)

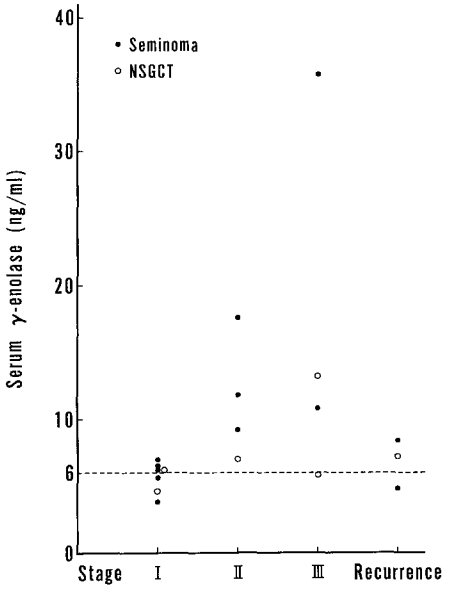

Fig. 4. Serum levels of γ-enolase in patients with testicular cancer of stages I to III, and recurrent disease. Twelve patients with seminoma and 6 with nonseminomatous germ cell tumors (NSGCT)

seminoma and NSGCT of stages I to III, and of recurrent disease. In seminoma, the positive rates were 60% (3/5) of patients with stage I, 100% (2/2) of state II, 100% (3/3) of stage III, and 50% (1/2) of patients with recurrent disease. In 10 patients with seminoma treated by surgical excision and chemotherapy, which induced complete

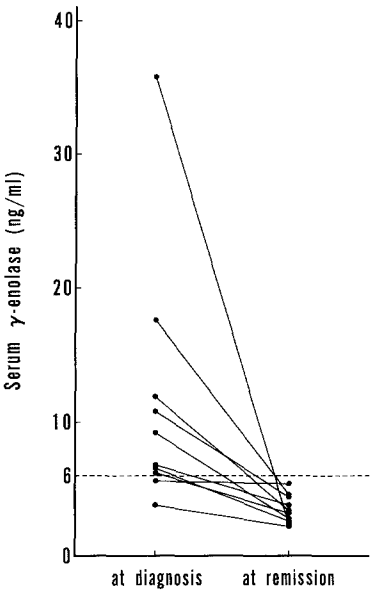

Fig. 5. Serum γ-enolase levels at diagnosis and at remission in patients with seminoma. Serum γ-enolase levels were significantly reduced after treatment ($p<0.01$)

remission, serum γ-enolase (preoperative levels of 11.4 ± 9.5 ng/ml) was significantly reduced after treatment (3.4 ± 1.1 ng/ml, $p<0.01$, Fig. 5).

Discussion

Recent advances in chemotherapy have lead to improved survival of patients with testicular cancer. Serum markers are useful for staging, evaluating efficacy of chemotherapy, monitoring recurrence, and deciding surgical intervention following chemotherapy. In particular, AFP and HCG are useful markers for NSGCT. On the other hand, only about 8–20% of patients with seminoma had elevated HCG [11–13]; no patients with pure seminoma had elevated serum AFP [11]. New serum markers for seminoma have been investigated, including lactate dehydrogenase [22, 29, 34] and its isozymes [4, 23], placental alkaline phosphatase [6, 13, 14, 20], γ-glutamyl transpeptidase [13], hydroxybutyric dehydrogenase [6, 22], and pregnancy specific β_1 glycoprotein [19, 31]. Lange and Winfield [21] suggested that placental alkaline phosphatase is a useful tumor marker and that the other tumor markers listed above have not demonstrated sufficient sensivity or specificity for routine use. Several reports [6, 13, 14, 20] showed that 40–57% of patients with active seminoma had elevated serum levels of placental alkaline phosphatase. Further study is needed about whether γ-enolase provides additional information to placental alkaline phosphatase.

Several investigators reported that γ-enolase is a useful serum marker for neuroendocrine tumors [10, 26, 33],

small cell carcinoma of the lung [3, 7] and renal cell carcinoma [9, 32]. Therefore, neither γ-enolase nor other serum markers is specific for testicular cancer. Of note, however, was the finding that benign diseases or disorders in liver or kidney other than hemolytic ones do not affect serum levels of γ-enolase.

Recently Kuzmits et al. [18] reported that serum γ-enolase is useful for patients with seminoma. Both they [18] and Niehans et al. [25] immunohistochemically localized γ-enolase in seminoma tissues. We here determined the tissue concentrations of both α and γ-enolases, and the value of $\gamma/(\alpha+\gamma)$, and clarified the reason for the elevated serum γ-enolase in patients with seminoma.

In tumor cells, glycolytic activities of glycolytic enzymes are enhanced, accompanied by increased anaerobic glycolysis [35]. We reported that both γ-enolase levels and $\gamma/(\alpha+\gamma)$ values are elevated in tissues of neuroendocrine tumors [10] and small cell carcinoma of the lung (SCCL) [7]. The $\gamma/(\alpha+\gamma)$ of seminoma was 7.0%: lower than that of neuroblastoma (33%) and small cell carcinoma of the lung (16%), but higher than in nonneuroendocrine tumors such as Wilms' tumor (3.3%), rhabdomyosarcoma (2.5%), and non-small cell carcinoma of the lung (2.5%). These findings suggest that enhanced γ-enolase in seminoma tissues is due not only to the increased glycolysis, but also to the preferential production of γ-enolase as found in neuroendocrine tumors and SCCL. Several previous investigations [7, 9, 32] suggested that serum γ-enolase is derived from tumor cell destruction, not from adjacent normal tissues compressed or invaded by tumors.

The present immunohistochemical study showed tumor cells positive for γ-enolase in 75% of seminoma cases, corresponding to the 82% shown by Niehans et al. [25]. The frequency of immunohistochemically positive tumor cells for γ-enolase varied from case to case and from site to site in the tumors examined. Such immunohistochemical heterogeneity among cases and within each tumor might explain the wide variation in tissue concentrations of γ-enolase determined by enzyme immunoassay. No significant correlation was found between the frequency of immunohistochemically positive cells for γ-enolase or the tissue concentration of γ-enolase and the serum levels of patients. Serum γ-enolase appeared to be associated with tumor burden and stage. Further study of larger series will be needed to determine factors affecting serum γ-enolase in patients with seminoma.

In conclusion, the present study showed that elevated serum γ-enolase is derived from preferentially enhanced γ-enolase in seminoma tissues, and that serum γ-enolase could be a useful marker for staging and monitoring treatment in patients with seminoma.

References

1. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248
2. Caldwell WL, Kademian MT, Frias Z, Davis TE (1979) The management of testicular seminomas, 1979. Cancer 45:1768
3. Carney DN, Marangos PJ, Ihde DC, Bunn PA Jr, Cohen MH, Minna JD, Gazdar AF (1982) Serum neuron-specific enolase: a marker for disease extent and response to therapy of small-cell lung cancer. Lancet I:583
4. von Eyben FE, Blaabjerg O, Petersen PH, Hørder M, Nielsen HV, Andersen SK, Parlev E (1987) Lactate dehydrogenase isoenzyme 1 in testis cancer. Lancet II:1035
5. Fletcher L, Rider CC, Taylor CB (1976) Enolase isozymes: III. chromatographic and immunological characteristics of rat brain enolases. Biochim Biophys Acta 452:245
6. Ford TF, Butcher DN, Samuell CT, Masters JRW, Parkinson MC, Oliver RTD (1985) Serum and tissue tumour markers in seminomas. Br J Urol 57:750
7. Fujita K, Haimoto H, Imaizumi M, Abe T, Kato K (1987) Evaluation of γ-enolase as a tumor marker for lung cancer. Cancer 60:362
8. Haimoto H, Takahashi Y, Koshikawa T, Nagura H, Kato K (1985) Immunohistochemical localization of γ-enolase in normal human tissues other than nervous and neuroendocrine tissues. Lab Invest 52:257
9. Haimoto H, Takashi M, Koshikawa T, Asai J, Kato K (1986) Enolase isozymes in renal tubules and renal cell carcinoma. Am J Pathol 124:488
10. Ishiguro Y, Kato K, Ito T, Nagaya M (1983) Determination of three enolase isozymes and S-100 protein in various tumors in children. Cancer Res 43:6080
11. Javadpour N, McIntire KR, Waldmann TA (1978) Human chorionic gonadotropin (HCG) and alpha-fetoprotein (AFP) in sera and tumor cells of patients with testicular seminoma: a prospective study. Cancer 42:2768
12. Javadpour N (1980) The role of biologic tumor markers in testicular cancer. Cancer 45:1755
13. Javadpour N (1983) Multiple biochemical tumor markers in seminoma: a double-blind study. Cancer 52:887
14. Jeppsson A, Wahren B, Stigbrand T, Edsmyr F, Andersson L (1983) A clinical evaluation of serum placental alkaline phosphatase in seminoma patients. Br J Urol 55:73
15. Kato K, Ishiguro Y, Suzuki F, Ito A, Semba R (1982) Distribution of nervous system-specific forms of enolase in peripheral tissues. Brain Res 237:441
16. Kato K, Ishiguro Y, Ariyoshi Y (1983) Enolase isozymes as disease markers: Distribution of three enolase subunits (α, β, and γ) in various human tissues. Dis Mark 1:213
17. Kato K, Asai R, Shimizu A, Suzuki F, Ariyoshi Y (1983) Immunoassay of three enolase isozymes in human serum and in blood cells. Clin Chim Acta 127:353
18. Kuzmits R, Schernthaner G, Krisch K (1987) Serum neuron-specific enolase: a marker for response to therapy in seminoma. Cancer 60:1017
19. Lange PH, Bremner RD, Horne CHW, Vessella RL, Fraley EE (1980) Is SP-1 a marker for testicular cancer? Urology 15:251
20. Lange PH, Millan JL, Stigbrand T, Vessella RL, Ruoslahti E, Fishman WH (1982) Placental alkaline phosphatase as a tumor marker for seminoma. Cancer Res 42:3244
21. Lange PH, Winfield HN (1987) Biological markers in urologic cancer. Cancer 60:464
22. Lippert MC, Javadpour N (1981) Lactic dehydrogenase in the monitoring and prognosis of testicular cancer. Cancer 48:2274
23. Liu F, Fritsch HA, Trujillo JM, Samuels ML (1982) Serum lactate dehydrogenase isozyme 1 in patients with advanced testicular cancer. Am J Clin Pathol 78:178
24. McLean IM, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative: A new fixation for immunoelectron microscopy. J Histochem Cytochem 22:1077
25. Niehans GA, Manivel JC, Copland GT, Scheithauer BW, Wick MR (1988) Immunohistochemistry of germ cell and trophoblastic neoplasms. Cancer 62:1113
26. Prinz RA, Marangos PJ (1982) Use of neuron-specific enolase as a serum marker for neuroendocrine neoplasms. Surgery 92:887

27. Rider CC, Taylor CB (1974) Enolase isoenzymes in rat tissues: Eletrophoretic chromatographic, immunological and kinetic properties. Biochim Biophys Acta 365:285
28. Rider CC, Taylor CB (1975) Evidence for a new form of enolase in rat brain. Biochem Biophys Res Commun 66:814
29. Robertson AG, Read G (1982) The value of lactate dehydrogenase as a nonspecific tumour marker for seminoma of the testis. Br J Cancer 46:994
30. Schmechel D, Marangos PJ, Brightman M (1978) Neuron-specific enolase is a molecular marker for peripheral and central neuroendocrine cells. Nature 276:834
31. Steffens J, Friedmann W, Nagel R (1985) Immunohistochemical and radioimmunological determination of β-HCG and pregnancy-specific β_1-protein in seminomas. Urol Int 40:72
32. Takashi M, Haimoto H, Tanaka J, Murase T, Kato K (1989) Evaluation of γ-enolase as a tumor marker for renal cell carcinoma. J Urol 141:830
33. Tapia FJ, Polak JM, Barbosa AJA, Bloom SR, Marangos PJ, Dermody C, Pearse AGE (1981) Neuron-specific enolase is produced by neuroendocrine tumours. Lancet I:808
34. Taylor RE, Duncan W, Horn DB (1986) Lactate dehydrogenase as a marker for testicular germ-cell tumours. Eur J Cancer Clin Oncol 22:647
35. Warburg O, Minami S (1923) Versuche am überlebenden Carcinomgewebe. Klin Wochenschr 2:776
36. Wharton J, Polak JM, Cole GA, Marangos PJ and Pearse AGE (1981) Neuron-specific enolase as an immunocytochemical marker for the diffuse neuroendocrine system in human fetal lung. J Histochem Cytochem 29:1359

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