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Use of Serum Gamma-enolase and Aldolase A in Combination as Markers for Renal Cell Carcinoma

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To clarify whether measurement of serum γ-enolase and aldolase A in combination is useful for diagnosis and prediction of prognosis in cases of renal cell carcinoma (RCC), levels of both markers were evaluated by enzyme immunoassay in 132 patients with RCC. Serum γ-enolase was elevated in 53 of the cases (40%) whereas serum aldolase A was elevated in 45 (34%). At least one of the two markers was elevated in 54% of the patients (71/132), this value being significantly higher than the positive rates for either γ-enolase (40%) or aldolase A (34%) evaluated singly. Expression of the two markers assessed in combination became more positive with stage progression, values being 37% in stage I, 59% in stage II, 72% in stage III, and 74% in stage IV. In contrast, patients with benign urological diseases demonstrated positive rates for γ-enolase and aldolase A as low as 3% and 6%, respectively. Increase in serum γ-enolase was correlated with stage, tumor size, and histological grade, whereas elevated levels of serum aldolase A were associated only with advancing stage. In 15 patients with recurrent diseases, 11 (73%) had elevated levels of γ-enolase and 5 (33%) had elevated levels of aldolase A, indicating that γ-enolase is the more sensitive of the two for detection of recurrence. Patients with elevated levels of both γ-enolase and aldolase A had less favorable survival than those expressing no or only one of the markers, indicating that simultaneous measurement of the two markers provides information directly relevant to prognosis in cases of RCC.

Key words: Enolase — Aldolase — Isozyme — Renal cell carcinoma — Biomarker

Enolase (EC 4.2.1.11), a glycolytic enzyme catalyzing the reaction between 2-phosphoglycerate and phosphoenolpyruvate, consists of dimers of 3 immunologically distinct subunits, α, β, and γ. Five isozymes (αα, ββ, γγ, αβ, and αγ) are known, each with a molecular weight of about 100,000.[1,2] The γ subunit of enolase (γ-enolase), though designated neuron-specific enolase (NSE), is distributed widely in various tissues and in many types of cells other than neuronal and neuroendocrine cells.[3,4] Having investigated tissue concentrations and immunohistochemical localization of enolase isozymes in renal cell carcinoma (RCC[4]), we first reported that serum γ-enolase was elevated in patients with RCC.[5,6]

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), another glycolytic enzyme with a molecular weight of about 160,000, has a tetrameric form with three immunologically distinct subunits: A, B, and C.[7,8] The A subunit (aldolase A), which is the dominant fetal form, is present in large amounts in muscles, and in smaller amounts in the kidney. The B subunit (aldolase B) is found predominantly in the liver and kidney, whereas the C subunit (aldolase C) is localized mainly in the brain. Aldolase A has attracted attention as a potential biomarker for malignant tumors, including hepatocellular carcinomas,[9] and recently we immunochemically localized this enzyme in renal tissues and RCCs, showing it to be enhanced during renal carcinogenesis.[10] Furthermore, we determined serum levels of aldolase A in RCC patients, demonstrating elevation in many cases.[10,11]

New biomarkers for RCC are required because those presently available do not have sufficiently high sensitivity or specificity. Since no comparative evaluation of γ-enolase and aldolase A as serum biomarkers for clinical application has been performed, the present study of their relation to diagnosis and prognosis in RCC patients was conducted.

MATERIALS AND METHODS

Serum samples Serum samples of 132 patients, 107 men and 25 women, with RCC were preoperatively obtained by venipuncture. Serum samples were stored at either −80°C or −20°C until analysis. Serum samples from 100 patients with benign urological diseases were also obtained for determining levels of γ-enolase. Samples exhibiting hemolysis were not used.

Age of the patients with RCC ranged from 23 to 88 years, with a mean age of 59.8±11.3 (SD) years. Of the

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[4] Abbreviations: RCC, renal cell carcinoma; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; S100-α, the α subunit of S100 protein; CK-B, the B subunit of creatine kinase.

132 patients, 63 (48%) had stage I, 17 (13%) had stage II, 18 (14%) had stage III, and 34 (26%) had stage IV tumors according to Robson's staging system.[12] Tumor size varied from 2 to 22 cm in diameter with a mean value of 7.5±4.2 (SD) cm.

Immunoassay methods for γ-enolase and aldolase A isozyme The enzyme γ-enolase was assayed by the sandwich-type enzyme immunoassay system reported previously.[3] In brief, the serum samples were incubated with polystyrene balls bearing immobilized monospecific antibodies to the human γ-enolase antigen, and then the balls were incubated with the same antibodies labeled with β-D-galactosidase from *Escherichia coli*. The galactosidase activity bound to the balls was assayed with 4-methylumbelliferyl-β-D-galactoside as a substrate. The assay was confirmed to be highly sensitive, and the minimum detection limit of γ-enolase was 1 pg per assay tube. Aldolase A isozyme was assayed by an immunoassay method similar to that for γ-enolase as described previously.[13] The assay was also highly sensitive, and the minimum detection limit of aldolase A was 10 pg per assay tube. The immunoassays of γ-enolase and aldolase A were performed with purified human γγ enolase and aldolase A_4 as standards, respectively, and the results were expressed as nanograms of γγ enolase equivalent or homomeric aldolase A_4 per milliliter of serum.

Human γγ enolase was purified from the brain by using a chromatofocusing column, as previously reported.[14] The final preparation was found to be virtually homogeneous on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE).[14] Aldolase A_4 was purified from human pectoral muscles, as described by Penhoet *et al.*[15] with some modifications.[13] The final preparation had a specific activity of about 15 U on the basis of mg protein, and showed a single band on SDS-PAGE.[13]

Statistical analysis Quantitative data were expressed as mean±standard deviation (SD) values and compared using Wilcoxon's rank-sum test. The results for frequencies were compared by means of the chi-square test or the direct probability exact test. Average serum level of γ-enolase in healthy adults (n=100) was measured to be 3.1±0.9 ng/ml with a range of 1.6 to 5.8. Any value higher than 6.0 ng/ml, the mean plus 3 SDs was tentatively defined as abnormal.[6] Serum level of aldolase A of healthy adults (n=100) was estimated to be 198±51 ng/ml. Any value higher than 300 ng/ml, which was the mean plus 2 SDs, was tentatively defined as abnormal.[11]

Cumulative survival curves were depicted by Kaplan-Meier's method,[16] the generalized Wilcoxon test[17] being used for assessment of the statistical significance of differences in survival.

RESULTS

Serum levels of γ-enolase and aldolase A in patients with RCC Serum levels of γ-enolase in 132 patients with RCC ranged from 1.5 to 60.4 ng/ml with a mean value of 7.4±7.7 (SD) ng/ml. Elevation (more than 6.0 ng/ml) was observed in 53 of the 132 patients (40% positive rate). Serum levels of aldolase A in 132 patients with RCC ranged from 64 to 1710 ng/ml with a mean value of 317±272 ng/ml, and elevation (more than 300 ng/ml) was found in 45 cases (34% positive rate). Table I summarizes concentration values and positive rates for γ-enolase and aldolase A in different RCC stages.

Evaluation of γ-enolase and aldolase A in combination revealed at least one of the two markers to be elevated in 71 of the 132 patients (54% positive rate), this figure being significantly higher than those for γ-enolase ($P<0.05$) and aldolase A ($P<0.01$) evaluated singly.

Table I. Levels and Positive Rates for Serum γ-Enolase and Aldolase A in 132 Patients with Renal Cell Carcinoma

	No. of samples	Serum γ-enolase		Serum aldolase A		Positive rate using the two markers in combination[a]
		Mean±SD	Positive rate	Mean±SD	Positive rate	
Renal cell ca.	132	7.4±7.7	40% (53/132)	317±272	34% (45/132)	54% (71/132)
Stage I	(63)	5.5±6.3	24% (15/63)	245±147	22% (14/63)	37% (23/63)
Stage II	(17)	5.8±3.4	35% (6/17)	283±197	35% (6/17)	59% (10/17)
Stage III	(18)	9.3±7.1[b]	56% (10/18)[d]	405±326[d]	50% (9/18)[d]	72% (13/18)[d]
Stage IV	(34)	10.5±10.3[c]	65% (22/34)[e]	424±390[d]	47% (16/34)[d]	74% (25/34)[b]
Healthy subjects	100	3.1±0.9[f]	—	198±51[g]	—	—

a) Proportion of patients with elevated levels of at least one of the two biomarkers.
b) Significantly higher than stage I ($P<0.01$). *c*) Significantly higher than stage I ($P<0.001$) or II ($P<0.05$).
d) Significantly higher than stage I ($P<0.05$). *e*) Significantly higher than stage I ($P<0.001$).
f) Significantly different from stage I ($P<0.01$), II ($P<0.01$), III ($P<0.01$), or IV ($P<0.001$).
g) Significantly different from stage I ($P<0.05$), III ($P<0.05$), or IV ($P<0.01$).

Table II. Levels and Positive Rates for Serum γ-Enolase and Aldolase A in Patients with Renal Cell Carcinoma Relative to Tumor Size, Cell Type, and Histological Grade

	No. of samples	Serum γ-enolase		Serum aldolase A	
		Mean±SD	Positive rate	Mean±SD	Positive rate
Tumor size					
≤5 cm	46	5.5±4.0	30% (14/46)	310±297	28% (13/46)
5–10 cm	62	7.3±7.3[a)]	37% (23/62)	291±183	35% (22/62)
>10 cm	24	11.0±11.9[b)]	67% (16/24)[c)]	401±387	42% (10/24)
Cell type					
clear cell	95	6.6±6.3	37% (35/95)	280±228	28% (27/95)
granular cell	17	8.3±6.3	47% (8/17)	494±445	53% (9/17)
mixed	11	6.7±3.9	55% (6/11)	291±195	36% (4/11)
Histological grade					
grade 1	30	5.5±3.4	30% (9/30)	310±205	37% (11/30)
grade 2	82	7.1±6.9	39% (32/82)	301±291	28% (23/82)
grade 3	12	8.0±5.0	67% (8/12)[d)]	372±302	50% (6/12)
Normal subjects	100	3.1±0.9[e)]	—	198±51[f)]	—

a) Significantly higher than in tumors of 5 cm or less ($P<0.05$).
b) Significantly higher than in tumors of ≤ 5 cm ($P<0.01$) or 5–10 cm ($P<0.05$).
c) Significantly higher than in tumors of ≤ 5 cm ($P<0.01$).
d) Significantly higher than in grade 1 ($P<0.05$).
e) Significantly different from each subgroup of tumor size, cell type, or histological grade ($P<0.05$ to $P<0.001$).
f) Significantly different from each subgroup of tumor size, cell type, or histological grade ($P<0.05$ to $P<0.001$) except mixed type and grade 3 tumors.

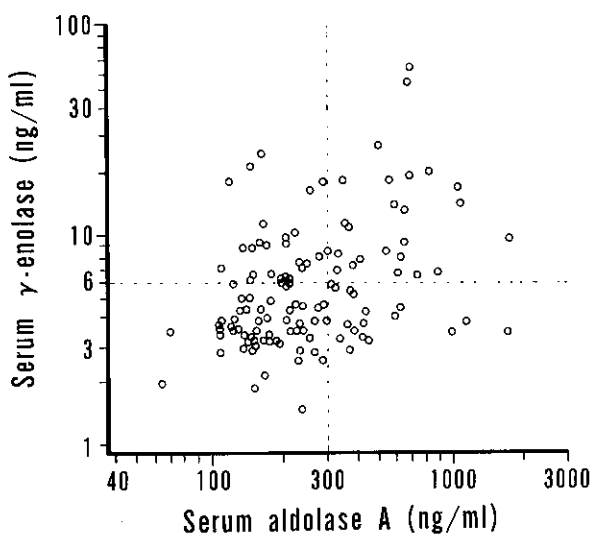

Fig. 1. Comparison of γ-enolase and aldolase A levels in sera of patients with renal cell carcinoma. The correlation coefficient is 0.31 (n=132, $P<0.01$).

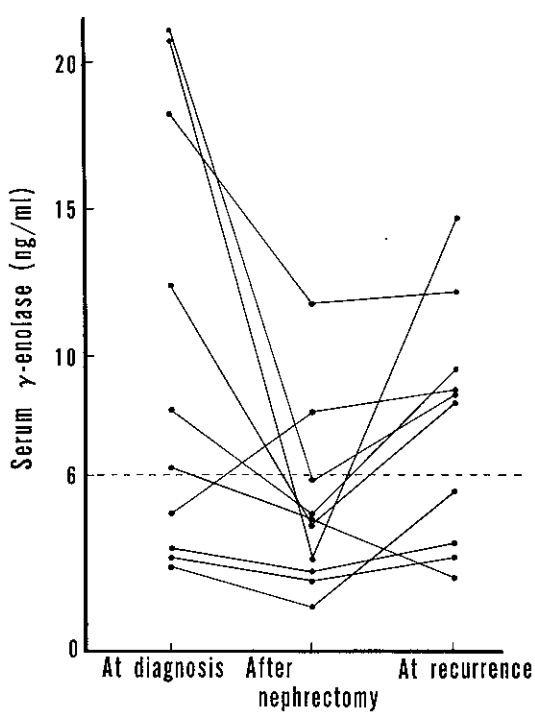

Fig. 2. Changes in serum γ-enolase levels during the clinical courses of 10 patients with renal cell carcinoma. Upper limit of normal serum γ-enolase was 6.0 ng/ml (broken line).

Simultaneous evaluation of the two markers resulted in positive rates that increased with advancing stage, the value being 37% in stage I, 59% in stage II, 72% in stage III, and 74% in stage IV (Table I, right side).

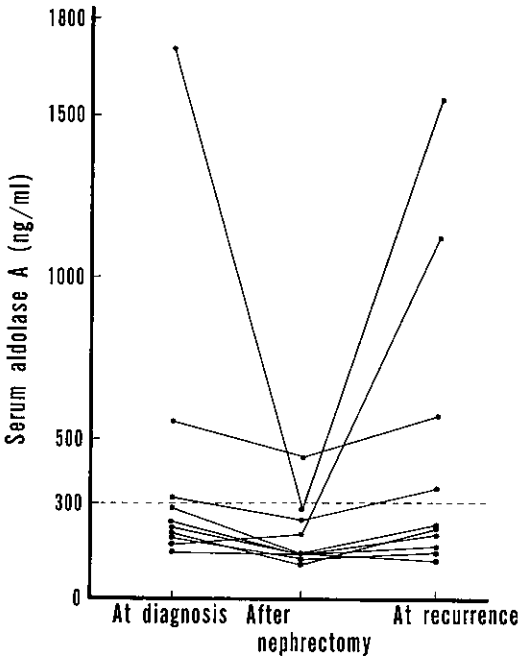

Fig. 3. Changes in serum aldolase A levels during the clinical courses of 10 patients with renal cell carcinoma. Upper limit of normal serum aldolase A was 300 ng/ml (broken line).

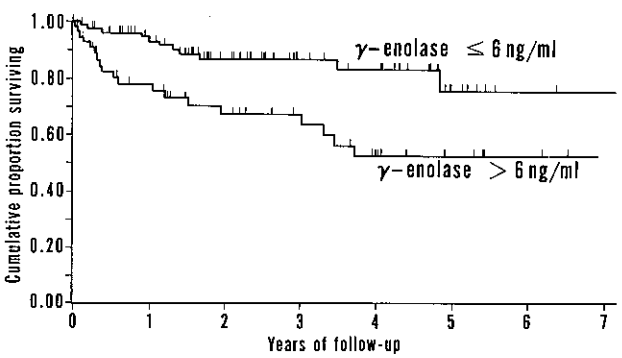

Fig. 4. Cumulative survival curves for patients with and without elevated levels of γ-enolase.

Table II shows the positive rates and levels of γ-enolase and aldolase A relative to tumor size, grade, and cell type. Serum levels of γ-enolase were significantly increased with stage, tumor size and histological grade, while serum levels of aldolase A only demonstrated a clear correlation with stage.

In 100 patients with benign urological diseases the positive rate for γ-enolase was 3%. The positive rate of

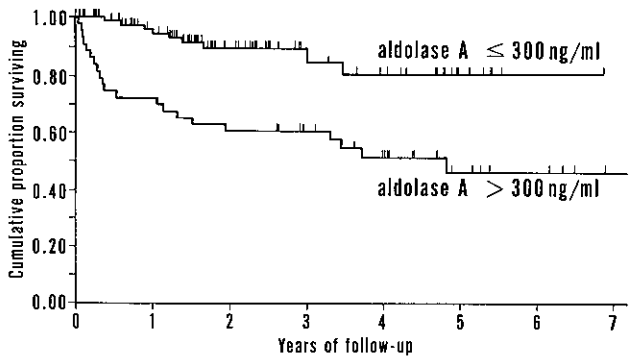

Fig. 5. Cumulative survival curves for patients with and without elevated levels of aldolase A.

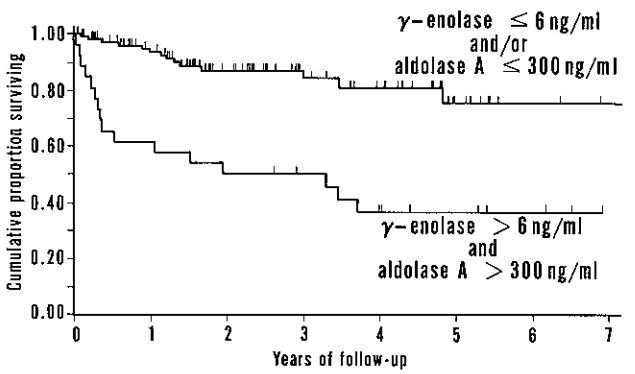

Fig. 6. Cumulative survival curves for patients with elevated levels of both γ-enolase and aldolase A as compared with those demonstrating no or only one elevated marker.

aldolase A was earlier found to be 6% for benign urological diseases.[11]

Comparison of γ-enolase and aldolase A levels in sera of patients with RCC Figure 1 illustrates the positive correlation between γ-enolase and aldolase A levels in sera of RCC patients (Pearson's correlation coefficient, $r=0.31$, $n=132$, $P<0.01$).

Clinical course changes in serum levels of γ-enolase and aldolase A In 15 patients with recurrent disease the positive rates of γ-enolase and aldolase A were 73% (11/15) and 33% (5/15), respectively, the former demonstrating a tendency to be higher than the latter ($P=0.07$). Of the 15 patients, 12 (80%) had elevated levels of at least one of the two markers.

Figures 2 and 3 illustrate changes in serum levels of γ-enolase and aldolase A during the clinical courses of 10 patients from whom samples were serially taken. The markers changed consistently with clinical course. Of the

10 patients with recurrent disease 6 (60%) had elevated levels of γ-enolase and 4 (40%) had elevated levels of aldolase A. At least one of the two markers were elevated in 7 of the 10 patients (70%); both of them were elevated in 3 (30%).

Prognostic significance of γ-enolase and aldolase A

Figure 4 shows survival curves for patients with (>6 ng/ml) and without (≤6 ng/ml) elevated levels of γ-enolase, the former group demonstrating a poorer survival ($P=0.002$). Figure 5 similarly shows survival curves for patients with (>300 ng/ml) and without (≤300 ng/ml) elevated levels of aldolase A. Increase was again associated with a less favorable survival ($P<0.0001$). Figure 6 shows survival curves for patients with elevated levels of both γ-enolase and aldolase A and for those with no or only one elevated marker. The former group had a poorer survival than the latter ($P<0.0001$), and the difference between patients with a favorable prognosis and those with an unfavorable one became more evident when the two markers were considered.

DISCUSSION

To date no reliable biomarkers for diagnosis, monitoring of clinical course, and prediction of survival of patients with RCC have been reported. Several acute phase reactant proteins have been investigated, including C-reactive protein,[18] haptoglobin,[19] fibrinogen,[20] and β_2-microglobulin.[21] However, these proteins all suffer from the disadvantage of lack of specificity. In recent years Baumann *et al.*[22] have proposed serum phosphohexose isomerase as a potential biomarker for RCC but did not describe changes of the enzyme levels during the clinical course or the predictive significance for prognosis. Hershman *et al.*[23] evaluated serum CA-50 antigen, defined by a monoclonal antibody binding to an epitope present on two different carbohydrate structures, sialosyl-Lewis[a] and sialosyllactotetraose, and reported 7 of 15 patients with RCC (47%) to demonstrate elevated levels. In our own search for useful biomarkers for RCC we have evaluated γ-enolase,[6] aldolase A,[11] the α subunit of S100 protein (S100-α),[24] and the B subunit of creatine kinase (CK-B)[25] in sera of RCC patients. Among them, we chose γ-enolase and aldolase A as most suitable for the present comparative study because the positive rates for γ-enolase (40%) and aldolase A (34%) in RCC cases were higher than those for S100-α (29%) and CK-B (24%), and because the positive rates of γ-enolase (3%) and aldolase A (6%) for patients with benign urological diseases were lower than those of S100-α (12%) and CK-B (11%).[24, 25]

The fact that γ-enolase and aldolase A are glycolytic enzymes is of interest since anaerobic glycolysis is generally enhanced in neoplastic tissues.[26] In addition, γ-enolase and aldolase A are considered to be isozymes indicating oncofetal reversion during renal carcinogenesis.[5, 10] There have been reports that serum γ-enolase was elevated in patients with neuroendocrine tumors, lung cancer, and testicular seminoma[6, 27] and that serum aldolase A was enhanced in patients with hepatocellular carcinoma.[9] These findings indicate that γ-enolase and aldolase A are not specific for RCC. In this study simultaneous measurement of γ-enolase and aldolase A resulted in an improved positive rate of 54% as compared with rates of 40% and 34% obtained for γ-enolase and aldolase A measured singly. Thus, this approach allowed increased sensitivity.

Furthermore, concentrations of γ-enolase and aldolase A changed in parallel with the clinical course of patients with RCC, this being particularly clear for γ-enolase. Our finding also indicated that γ-enolase might be more useful for detecting recurrent diseases than aldolase A although the number of patients available for this analysis was relatively small. The present study further revealed that patients with elevated levels of γ-enolase and aldolase A had a poor survival and demonstrated that simultaneous measurement of the two markers in combination allowed particularly clear differentiation of two groups of patients with favorable and unfavorable prognoses.

Our experience indicates that potential new serum markers must have the following characteristics: (1) proteins or other substances which are produced by tumor cells and are markedly enhanced during renal carcinogenesis, while being found at only very low concentrations in normal tissues including the kidney, typical examples being oncofetal proteins or isozymes; (2) small-molecular-weight substances of less than 100,000 daltons, which should have easy entry into the blood stream; and (3) substances having a short half-life, for example, within 24 h as found in the case of γ-enolase.[6] Further study is required to find optimal serum markers with the above characteristics. However, in conclusion, the present study indicates that simultaneous measurement of γ-enolase and aldolase A in combination may be valuable for monitoring the clinical course of RCC patients and predicting patient survival.

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