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An Improved Sensitive Assay Method for the Heterogeneity of α -Foetoprotein: Possible Application for Early Differential Diagnosis

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Summary: A combination of lectin-affinity electrophoresis and antibody-affinity blotting was used for the qualitative determination of molecular species of α -foetoprotein. Concanavalin A and erythrophytohaemagglutinin were used as lectins. This method was able to quickly discriminate between the molecular species of α -foetoprotein of recurrent ovarian yolk sac tumour and those of non-malignant liver diseases and cord sera at term, using only 3 μ l of sera containing 200 μ g/l of α -foetoprotein. The results indicate that this assay method might be useful for the differential diagnosis of recurrent yolk sac tumour from drug- or blood transfusion-induced liver diseases and for the monitoring of the serum α -foetoprotein level of patients with yolk sac tumour producing less than 200 μ g/l of α -foetoprotein.

Introduction

α -Foetoprotein is classified a typical tumour marker for hepatocellular carcinoma and malignant germ cell tumours with yolk sac elements (1–3). In ovarian tumours with yolk sac elements, the elevated serum α -foetoprotein level in patients shows a good correlation with the clinical status of malignancy in more than 75% cases; in addition, an increase in serum α -foetoprotein after a transient decrease due to surgical resection of the tumour is a sign of recurrence (3–6). In the follow-up study after the surgical resection of yolk sac tumours, it is important to determine whether the slight increase of serum α -foetoprotein around

300 μ g/l might be due to the recurrence of the tumour or regeneration of the host hepatocytes, after treatment with hepatotoxic anticancer agents or by blood transfusion (3, 7, 8).

It has been reported that α -foetoprotein produced by the yolk sac, foetal liver or regenerating hepatocytes with non-malignant liver diseases can be differentiated by different reactivities against concanavalin A or erythrophytohaemagglutinin (9–11). This heterogeneity of α -foetoprotein with respect to lectin affinity is ascribed to differences in the carbohydrate moiety (12).

Recently a highly sensitive technique for the detection of small amounts of α -fetoprotein was reported by *Taketa et al.* (10, 13). In the present work we applied this rapid and sensitive method to the clinical differential diagnosis of the early stages of recurrent yolk sac tumour from non-malignant liver diseases.

Materials and Methods

Sera from patients

Frozen samples of sera were obtained from patients with yolk sac tumour without recurrent disease but with hepatic disorders (2 cases) and with pathologically confirmed recurrent disease (10 cases, 7 of these with chronic liver diseases indicated by a considerable elevation of serum α -fetoprotein from 150 to 500 $\mu\text{g/l}$). Samples were also collected from patients with non-malignant liver diseases including liver cirrhosis (30 cases) and from cord sera at term (30 cases).

Determination of serum α -fetoprotein level

Serum α -fetoprotein levels were examined using radioimmunoassay (RIA) (14) and *Mancini's* method (15) with a Japanese α -fetoprotein standard (Nippon Bio-Test Lab., Japan) (13) as a reference.

Specific antibody to α -fetoprotein

Specific antiserum against human α -fetoprotein was produced in a horse by weekly subcutaneous injections of 1 mg of purified α -fetoprotein (16) emulsified in *Freund's* complete adjuvant. Specific antibody against α -fetoprotein was purified by affinity chromatography on BrCN-activated Sepharose 4B (Pharmacia, Sweden) coupled to human α -fetoprotein (14).

Isolation of molecular species of α -fetoprotein

α -Fetoprotein was fractionated by concanavalin A — or erythrophytohaemagglutinin — affinity agarose gel electrophoresis followed by blotting as described previously (10, 13). Briefly, antibody-coated nitrocellulose papers were prepared by incubating with 100 mg/l affinity-purified horse antibody to human α -fetoprotein, then by fixing with glutaraldehyde vapour, neutralizing with NaBH_4 , followed by washing with Tris-buffered saline (20 mmol/l Tris HCl, pH 7.5, 500 mmol/l NaCl, Tris-buffered saline). The nitrocellulose was blocked with 5 g/l Tween 20 in Tris-buffered saline for 30 min, then washed with Tris-buffered saline.

Electrophoresis was carried out on 10 g/l agarose gel plates (Litex HSA, Litex, Denmark) in barbital/barbital-Na buffer, pH 8.6 containing 1 g/l concanavalin A (Sigma, USA) or 0.5 g/l erythrophytohaemagglutinin (Sigma, USA) or without lectins. Three microlitres of patients' sera or reference α -fetoprotein at various concentrations (diluted with the gel buffer or α -fetoprotein-free normal human sera) was applied to the plate and electrophoresis was performed at 4 °C. α -Fetoprotein-free sera were prepared by passing sera from healthy subjects through an anti-human α -fetoprotein antibody-affinity column. After lectin-affinity electrophoresis, α -fetoprotein was transferred, by capillary blotting, to nitrocellulose coated with antibody to α -fetoprotein. The nitrocellulose was then washed twice in 0.5 g/l Tween 20 in Tris-buffered saline, then treated with rabbit anti-human α -fetoprotein IgG (DAKO, Denmark), followed by treatment with swine antibody against rabbit IgG

labelled with horseradish peroxidase. Colour was developed by incubating the nitrocellulose with 3,3'-diaminobenzidine tetrahydrochloride (Polyscience, USA) and H_2O_2 in Tris-buffered saline.

Chemicals

All chemicals were used of reagent grade.

Results and Discussion

Post-operative anticancer chemotherapy with a combination of vincristine, actinomycin D and cyclophosphamide has been employed in patients with recurrent yolk sac tumours. Approximately 70% of patients treated in this way experienced liver injury, due at least in part to the side effects of the therapy. In these cases serum α -fetoprotein levels generally tend to increase. A method to determine at an early stage whether the increase in serum α -fetoprotein is due to liver injury or the recurrence of yolk sac tumour (tab. 1) would therefore be clinically advantageous.

The antibody-affinity blotting method showed sensitive results with a detection limit for α -fetoprotein of less than 3 μl of a solution containing 200 $\mu\text{g/l}$. In the qualitative analysis, α -fetoprotein bands could be identified in a sample containing less than 100 $\mu\text{g/l}$ (300 pg) of α -fetoprotein (fig. 1). By this sensitive method, all the yolk sac tumour α -fetoprotein from patients with recurrent yolk sac tumour (10/10) was separated into two bands of concanavalin A-reactive minor and concanavalin A-non-reactive major α -fetoprotein (fig. 1 a, fig. 2). In contrast, all the α -fetoprotein obtained from cord sera (30/30) and from the sera of patients with non-malignant liver diseases (30/30) showed a single concanavalin A-reactive band (figs. 2, 4). The same result was also observed in the sera of clinically tumour-free patients with liver injury (data not shown). When erythrophytohaemagglutinin was used as the lectin, all the yolk sac tumour α -fetoprotein from the patients with recurrent yolk sac tumours (10/10) was fractionated into 3 bands, i.e. strong, weak and non-reactive bands. However, erythrophytohaemagglutinin affinity electrophoresis separated normal α -fetoprotein into one or two minor reactive bands and a major non-reactive band (fig. 1 b, fig. 3). One case (case 8) indicated co-elevation of yolk sac tumour α -fetoprotein and normal α -fetoprotein (tab. 1). In this case, α -fetoprotein was separated into concanavalin A-reactive and concanavalin A-non-reactive bands with the same colour intensity (fig. 4). The patient had clinically been diagnosed as recurrent yolk sac tumour with chronic liver damage, so that the result described above coincided well with the clinical diagnosis. The interre-



Fig. 1. Sensitivity and typical patterns of antibody-affinity blotting technique for the detection of α -fetoprotein separated by agarose gel electrophoresis with concanavalin A (A), erythrophytohaemagglutinin (B). Serum from patient with recurrent yolk sac tumour was serially diluted with α -fetoprotein-free serum (A, B) and 3 μ l of each sample (1. 100 μ g/l, 2. 200 μ g/l, 3. 500 μ g/l, 4. 1000 μ g/l) was applied. Electrophoresis was run until bromophenol blue migrated 5.0 cm from the origin.



Fig. 2. Typical α -fetoprotein (AFP) bands separated by concanavalin A-affinity electrophoresis. Serum from a patient with liver cirrhosis (liver α -fetoprotein, 245 μ g/l, b) and from recurrent yolk sac tumour (yolk sac α -fetoprotein; case 9, a; case 4, c) were used. Other conditions of assay, see figure 1.

lation between the types of α -fetoprotein bands and the pathophysiological status of increased α -fetoprotein production is schematically summarized in figure 5.

Heterogeneous reactivity of α -fetoprotein against lectins has been detected by lectin-affinity-crossed immunoelectrophoresis (9, 11, 17) as well as the combination of lectin-affinity mini-column chromatography with the radioimmunoassay of α -fetoprotein (18, 19). However, the sensitivity of the former method for α -fetoprotein is around 2 mg/l, unless radiolabeled antibody to α -fetoprotein is available. This means that the method described in this paper may have 20–50 times greater sensitivity than that of the former method.

Tab. 1. Clinical data of patients

Patient	Disease	Blood transfusion (ml) ¹⁾	Liver function			
			Aspartate aminotransferase ²⁾	Alanine aminotransferase ²⁾	γ -Glutamyl transferase ²⁾	α -Foetoprotein ²⁾
1	recurrent ³⁾	800	50	75	180	350
2	recurrent	600	15	8	42	214
3	recurrent	1500	18	11	40	190
4	recurrent	2000	120	95	150	240
5	recurrent	800	54	50	120	217
6	recurrent	—	35	25	49	150
7	recurrent	—	80	43	60	188
8	recurrent	2000	148	95	110	480
9	recurrent	2400	77	65	69	280
10	recurrent	800	54	35	80	297
11	no evidence ⁴⁾	3000	196	130	120	292
12	no evidence	2200	210	147	98	386

¹⁾ Blood transfusion was performed either at operation or during post-operative adjuvant chemotherapy.

²⁾ Aspartate aminotransferase (reference range 5–40 Karmen units);

Alanine aminotransferase (reference range 0–35 Karmen units);

γ -Glutamyl transpeptidase (reference range 0–40 U/l) were measured by autoanalyser.

α -Foetoprotein (reference range 0–2 μ g/l) was determined with RIA.

³⁾ Clinically and pathologically confirmed recurrent disease.

⁴⁾ Clinically no evidence of disease.

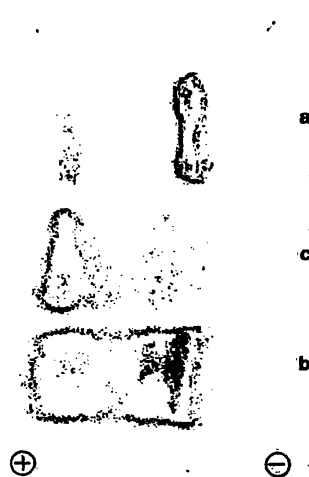


Fig. 3. Typical α -fetoprotein (AFP) bands separated by erythrophytohaemagglutinin-affinity electrophoresis. Sera from two recurrent yolk sac tumour (yolk sac α -fetoprotein, case 2, a and case 9, b) and cord serum at term (liver α -fetoprotein, 190 μ g/l, c) were used. Other conditions of assay, see figure 1.

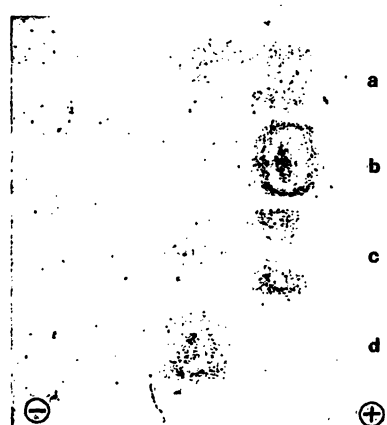


Fig. 4. Analytical α -fetoprotein (AFP) bands separated by concanavalin A-affinity electrophoresis. Sera from patients with recurrent yolk sac tumour (case 5, a; case 10, b), with recurrent yolk sac tumour with hepatic injury (case 8, c), and with liver cirrhosis (liver α -fetoprotein, 250 μ g/l, d) were used. In lane c, liver α -fetoprotein with the same mobility as lane d was separated from yolk sac α -fetoprotein and these two bands indicated the same intensity of colour development suggesting the co-elevation of both types of α -fetoprotein.

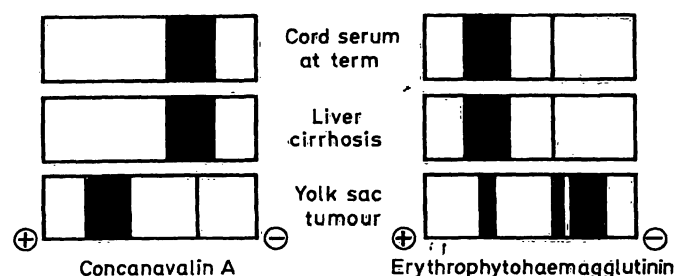


Fig. 5. Schematic representation of α -fetoprotein bands separated by affinity-electrophoresis with concanavalin A and erythrophytohaemagglutinin.

As shown by Govindarajan et al. (19), concanavalin A-reactive and non-reactive α -fetoprotein fractions in the sera of patients with liver- and germ cell-tumours can be quantitatively determined in 200 μ l of serum at α -fetoprotein concentrations as low as 58 μ g/l. This means that the quantitation of bound and non-bound fractions of α -fetoprotein by the latter method is clearly superior to the qualitative analysis presented here. But in this latter method it should be noted that serum samples must be appropriately diluted before passing through the concanavalin A column, to avoid incorrect results due to the presence of other glycoproteins with mannose-type carbohydrate moieties.

Thus, the main advantage of the antibody-affinity-blotting method is the fact that very low volumes of serum (3 μ l) are sufficient for analysis. In comparison with established techniques, the method seems to have a significantly higher sensitivity. Qualitative analysis was based on the identification of the typical reactive patterns of α -fetoprotein bands with lectins for α -fetoprotein concentrations as low as 100 μ g/l. This novel method avoids the troublesome manipulation of serum samples, and it is potentially useful for the rapid and early differential diagnosis of recurrent yolk sac tumour and non-malignant liver diseases.

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