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Enzymes and Protein Markers

Intermethod Discordance for α-Fetoprotein Measurements in Fanconi Anemia

Bruno Cassinat,[1*] Diane Darsin,[1] Philippe Guardiola,[2] Marie-Elisabeth Toubert,[1] Jean-Didier Rain,[1] Eliane Gluckman,[2] and Marie-Hélène Schlageter[1]

Background: **The significantly higher serum α-fetoprotein (AFP) in patients with Fanconi anemia (FA) than in non-FA aplastic patients has potential diagnostic utility, but the increase is method-dependent. The aim of this study was to compare five AFP assays on FA and non-FA samples and to investigate possible explanations for FA-specific discrepancies.**

Methods: **Two methods available in our laboratory (Kryptor and IMx) were compared on 59 FA and 27 non-FA patient samples. Kryptor, Immulite, Elecsys, Immuno-I, and Elsa-2 methods were then compared on 14 FA and 14 non-FA patient samples. The AFP glycosylation profile was analyzed by electrophoretic separation in a lectin-containing gel.**

Results: **With all six methods, AFP values were significantly higher in FA than in non-FA patients, but the diagnostic precision and optimal cutoff values varied. Indeed, two methods reached 100% sensitivity and specificity, but in other methods, one or both of these parameters were significantly <100%. Neither heterophilic antibodies nor a specific glycosylation profile was detected in FA samples.**

Conclusions: **AFP results are method-dependent in FA. New methods must be evaluated before use in differential diagnosis of aplastic patients.**

α-Fetoprotein (AFP)[3] is a glycoprotein essentially expressed in the fetal liver. At the moment of birth, the AFP promoter is switched off, and AFP blood concentrations decrease to reach adult values after 2 years of life *(1)*. AFP is widely used as a tumor marker, essentially for yolk sac tumors and hepatocellular carcinoma *(2, 3)*, but it is also useful in screening for neural tube defects *(4)* and Down syndrome *(5, 6)*. Increased serum AFP is also found in liver cirrhosis, viral hepatitis *(7)*, rare cases of hereditary persistence of AFP *(8)*, and in ataxia telangiectasia *(9)*.

Fanconi anemia (FA) is an autosomal recessive disorder characterized by congenital physical abnormalities and by progressive bone marrow aplasia, associated with a high risk of developing either solid tumors or leukemia. The disease is genetically heterogeneous: eight complementation groups have been described *(10)*, and three genes have been cloned *(11–13)*. This genetic heterogeneity is probably the basis for the phenotypic variation observed in FA, which is responsible, in numerous cases, for an absence or a delay in the diagnosis. The delay in diagnosis is deleterious because younger patients have a better clinical outcome after bone marrow transplantation, which is the only satisfactory treatment for FA *(14)*. We recently demonstrated that FA could be easily distinguished from other aplastic syndromes by the circulating AFP concentrations. Indeed, patients with FA had significantly higher AFP concentrations than non-FA aplastic patients *(15)*, and no liver pathology could explain this difference.

Because of the wide use of AFP measurements, a large number of methods are available; all of these methods are immunoassays. Because of differences in the specificity and the labeling of antibodies, immunoassays are subject to intermethod discrepancies, as have been reported for prostate-specific antigen (PSA) *(16)* and troponin *(17)*.

As soon as we detected increased AFP in samples from Fanconi patients, we noticed a discrepancy between two methods available in our laboratory. Although both tests gave significantly different results between FA and non-FA patients, the increase above the upper limit of the reference interval in FA patients was much more evident with the Kryptor assay than with the IMx assay. Because

[1] Nuclear Medicine Department and [2] Hematology:Bone Marrow Transplantation Department, Assistance Publique-Hôpitaux de Paris, Saint-Louis Hospital, 75010 Paris, France.

*Address correspondence to this author at: Service de Médecine Nucléaire, Hôpital Saint-Louis, 1 Avenue Claude Vellefaux, 75010 Paris, France. Fax 33-1-42-49-94-05; e-mail cassinat@chu-stlouis.fr.

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[3] Nonstandard abbreviations: AFP, α-fetoprotein; FA, Fanconi anemia; and PSA, prostate-specific antigen.

AFP measurement could be important for diagnosis of FA, we tested the ability of six different AFP assays to distinguish FA from non-FA aplasia. The methods tested represented 56% of laboratories participating to the European external quality control Oncocheck for AFP measurement. Furthermore, to explain intermethod discrepancies, we checked whether heterophilic antibodies *(18)* or specific AFP isoforms *(19)* could be detected in FA samples.

Materials and Methods

PATIENTS

We tested two distinct cohorts of patients with Fanconi and non-Fanconi aplasia. FA was diagnosed by cytogenetic analysis showing increased chromosomal breaks after incubation with DNA cross-linking agents, and by cell cycle analysis after incubation with alkylating agents. The first cohort consisted of 59 Fanconi patients (30 males and 29 females; median age, 13 years; age range, 1–53 years) and 27 non-Fanconi patients (9 males and 18 females; median age, 26 years; age range, 5–56 years) with acquired aplastic anemia (n = 19), paroxysmal nocturnal hemoglobinuria (n = 2), dyskeratosis congenita (n = 3), Diamond-Blackfan anemia (n = 1), Schwachman syndrome (n = 1), or Glanzmann disease (n = 1).

The second cohort consisted of 14 Fanconi patients (8 males and 6 females; median age, 13 years; age range, 7–30 years) followed in our institution for several years with stable AFP concentrations as measured by the Kryptor assay. We tested sera from 14 control patients (9 males and 5 females; median age, 25 years; age range, 7–49 years) with aplasia of different origins (acquired aplastic anemia, n = 9; dyskeratosis congenita, n = 2; paroxysmal nocturnal hemoglobinuria, n = 1; leukemia, n = 2) at the time of sampling. Both groups of patients had given informed consent for this study.

AFP MEASUREMENTS

Sera were aliquoted and stored at −30 °C until testing. We measured AFP by five nonradioactive automated immunoassays [IMx (Abbott, Rungis, France), Immulite (DPC France, La Garenne-Colombes, France), Immuno-1 (Bayer Diagnostics, Puteaux, France), Elecsys (Roche Diagnostics, Meylan, France), and Kryptor (Brahms France, Sartrouville, France)] and one manual RIA [Elsa-2 (Cis-Bio International, Gif-sur Yvette, France)]. All of these methods used two monoclonal antibodies, except for the Immulite method, which used one monoclonal and one polyclonal antibody. Only the Kryptor is an homogeneous phase assay. The manufacturers' suggested cutoff values (expressed as 95th percentiles) in the control populations differed among assays: 5.8 kilounits/L for the Elecsys, 5.5 kilounits/L for the Immuno-1, 2.6 kilounits/L for the Immulite, 8 kilounits/L for the Kryptor, 5.5 kilounits/L for the IMx, and 8 kilounits/L for the Elsa-2 assay.

TESTING OF HETEROPHILIC ANTIBODIES

To test for heterophilic antibodies, we measured AFP in eight sera before and after incubation in Heterophilic Blocking Tubes (Scantibodies Laboratories Inc.). In keeping with the manufacturer's instructions, just before each AFP assay was performed, we incubated 0.5 mL of serum for 1 h in a tube coated with a specific binder that inactivates heterophilic antibodies.

LECTIN AFFINITY ELECTROPHORESIS

To test the lectin affinity of AFP in our samples, we used the AFP differentiation reagent set L (Wako Chemicals GmbH) according to the manufacturer's instructions. Briefly, 2 μL of serum was electrophoresed on a *Lens culinaris* agglutinin agarose gel and then blotted on a mouse anti-AFP-coated nitrocellulose membrane. After incubation with a second rabbit anti-AFP antibody and then with a peroxidase-labeled anti-rabbit antibody, membrane-bound AFP was detected by a colorimetric reaction. After AFP migration and visualization, distinct fractions (L1, L2, and L3), which are relatively disease-specific, could be identified. We tested four FA samples and three non-FA samples (one ataxia telangiectasia, one testis cancer, and one hepatitis sample), together with a control sample containing L1 and L3 bands (supplied by the manufacturer).

STATISTICAL ANALYSIS

Results of serum AFP measurements in Fanconi and non-Fanconi aplastic patients were compared using the nonparametric Wilcoxon test. In addition, the sensitivity and specificity, which are interpretable regardless of the prevalence of the disease, were calculated for each method.

Results

COMPARISON OF AFP IN FA AND NON-FA APLASTIC PATIENTS

We first compared AFP measurements in 59 Fanconi and 27 non-Fanconi aplastic patients by the IMx and Kryptor methods. The distribution of AFP values was significantly different between FA and non-FA patients ($P = 0.0001$) with both methods (Fig. 1). The Kryptor method was able to detect FA with a sensitivity of 93% and a specificity of 100% (95th percentile = 8 kilounits/L), whereas the IMx method was less discriminant with a specificity of 89% and sensitivity of only 59% (95th percentile = 5.5 kilounits/L). Interestingly, intermethod discrepancies appeared when individual samples were compared. Although in our experience the Kryptor and IMx AFP methods were highly correlated in a population of healthy controls and patients with hepatitis or cancer (n = 75; $r^2 = 0.98$; not shown) as well as in non-FA aplastic patients (n = 27; $r^2 = 0.69$; Fig. 1), it was noteworthy that these methods correlated badly when FA patients were tested (n = 59; $r^2 = 0.45$; Fig. 1).

We then extended the comparison to four additional

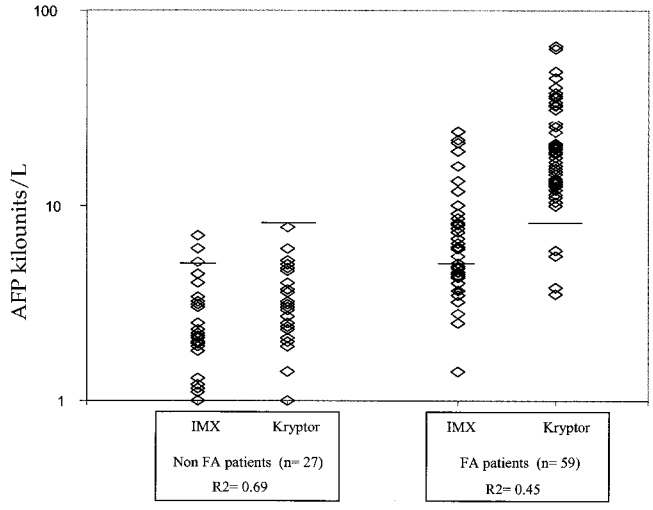

Fig. 1. Results of AFP measurements by the Kryptor and IMx assays on serum samples from Fanconi or non-Fanconi aplastic patients.

Horizontal bars indicate cutoff values expressed as 95th percentile. *R2*, correlation coefficient between IMx and Kryptor values.

methods, using sera from 14 FA patients and 14 non-FA aplastic patients. As shown in Table 1, although AFP concentrations were very similar from one method to another for non-FA samples, important intermethod variations were observed for FA samples. Some samples (e.g., samples 9 and 10) exhibited large discrepancies, most of them giving much higher results by the Kryptor assay than by other methods. Nevertheless, when AFP concentrations were considered with the 95th percentile of each method, four of the five methods tested showed normal values for non-FA aplastic patients and increased values in most FA samples. Only the Kryptor and Immuno-1 methods gave 100% sensitivity and specificity. The Elecsys and Elsa-2 assays had lower sensitivity but good specificity (Fig. 2). Only one method (Immulite) did not discriminate between FA and non-FA samples (specificity, 57%). Although two of five methods had similar sensitivity and specificity, it appeared that interpretation of the Kryptor results was easier because the gap between the highest normal value and the lowest abnormal value was much larger (Fig. 2).

absence of heterophilic interference

AFP values (12.7–101 kilounits/L) changed by <5% when measured by the Kryptor assay before and after treatment of eight FA sera with Heterophilic Blocking Tubes, making this interference unlikely as the source of increased AFP in FA.

analysis of afp lectin affinity

One of the most useful methods to detect different AFP isoforms consists of electrophoresis in a lectin (*L. culinaris* agglutinin) agarose gel *(20)*. As shown in Fig. 3, testis cancer exhibited a strong L2 band, as expected, but FA and ataxia telangiectasia samples exhibited only the L1 fraction.

Table 1. Results of AFP measurements in serum samples from Fanconi or non-Fanconi aplastic patients, using five methods.

	AFP, kilounits/L				
	Kryptor	**Immulite**	**Elecsys**	**Immuno-1**	**Elsa-2**
FA patients					
1	13.5	3.69	6.5	9.3	7
2	17	1.91	4.2	5.7	6.1
3	11.9	3.86	5.4	7.6	5.4
4	19.1	4.22	6.5	8.8	8.8
5	24.1	3.41	8.7	12.2	9.8
6	20.4	5.57	9	11.6	11.1
7	24.6	6.2	7	10.2	7.9
8	14.6	2.4	4.6	6.5	6.5
9	50.5	4.99	9	12.4	18
10	69.9	4	17.2	23.9	33
11	23.1	3.7	5.9	7.6	6.1
12	18.1	4.69	8.1	10.5	11.8
13	23	3.12	4.6	6.2	5.1
14	43.9	5.5	7.4	10.1	8.2
Median	21.7	3.9	6.7	9.7	8
(95% CI)[a]	(15–37)	(3.2–5.3)	(4.8–8.9)	(6.8–12)	(6.1–11.5)
Non-FA patients					
15	3	1.65	1.4	2	2
16	3	2.88	2	3.1	3.2
17	4	3.17	3	4	3.4
18	3	2	2.7	3.8	3.3
19	3	3.44	2.8	4	3.3
20	4.6	2.49	3.5	5.2	3.9
21	1	1.98	1.5	1.6	1.7
22	5	2.32	3.6	5.2	4
23	4.5	4.32	3.7	5.3	4.7
24	4	2.66	3.3	4.4	3.4
25	2	1.84	1.7	2.4	2.2
26	2	1.26	1.4	1.7	2.1
27	1	1.27	1.1	1.5	1.9
28	5.5	4.45	4.6	6.3	4.5
Median	3	2.4	2.7	3.9	3.3
(95% CI)	(2–4.5)	(1.6–3.3)	(1.4–3.5)	(1.8–5.2)	(2.1–3.9)

[a] CI, confidence interval.

Discussion

Intermethod discordance is a frequent phenomenon in immunoassays, as demonstrated recently for carcinoembryonic antigen measurements *(21)*. Indeed, the basis of these assays is the recognition of an immunogen by a pair of specific antibodies. The antigen–antibody reaction is defined by affinity characteristics that are specific to each antibody according to the recognized epitope, but the reaction also depends on the physicochemical composition of the incubation mixture. For example, discrepancies in PSA measurements are known to be dependent on the ability of PSA assays to recognize α_1-antichymotrypsin-

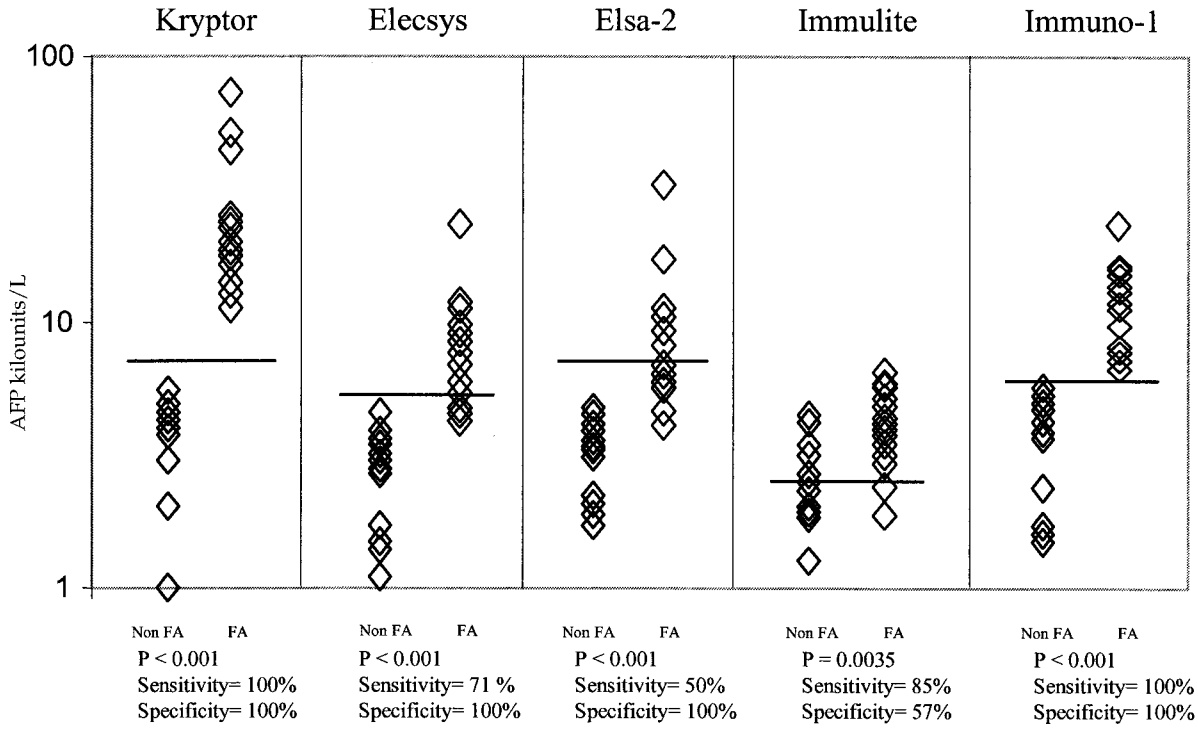

Fig. 2. Comparison of AFP measurements by five AFP assays on serum samples from 14 Fanconi or 14 non-Fanconi aplastic patients.
Horizontal bars indicate cutoff values expressed as 95th percentile.

bound PSA or free PSA *(16)*. Because we recently reported on the usefulness of AFP measurement for FA diagnosis *(15)* and because we observed differences in AFP measurements between two methods available in our laboratory (Kryptor and IMx) in samples from Fanconi patients but not from other patients, we compared four additional methods (three automated and one manual) for their ability to distinguish between samples obtained from FA and non-FA aplastic patients. Our results first confirmed that all methods tested gave significantly higher AFP values for samples from FA compared with samples from non-FA patients. However, our results clearly demonstrated discrepancies in AFP values obtained by the different methods in FA samples, whereas this discrepancy was not noted in samples from non-FA patients.

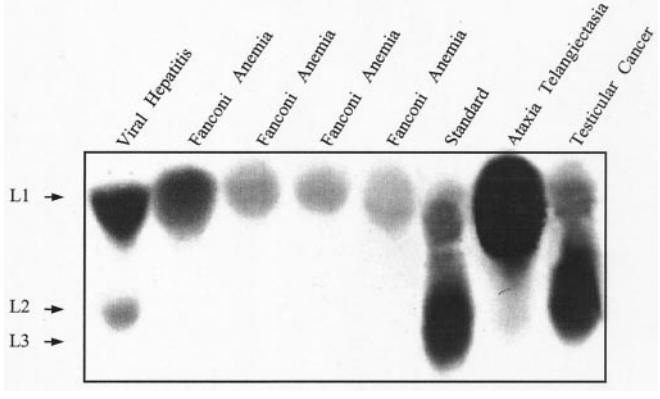

Fig. 3. Lectin affinity gel electrophoresis of samples from different pathologic origins.
The *Standard* contains L1 and L3 fractions.

To explain the observed discrepancies, two hypotheses were tested. The first hypothesis was that discrepancies could occur as a result of heterophilic antibodies in FA sera. This hypothesis might be excluded because AFP values were not modified by treatment of FA samples with Heterophilic Blocking Tubes. The second hypothesis was that discrepancies in AFP measurement might be linked to a difference in AFP recognition by each method. Indeed, to the best of our knowledge, the five tested methods used distinct pairs of antibodies. Thus, it could be hypothesized that in FA patients, posttranslational modifications of AFP could modify the antigenicity of some epitopes, thereby altering AFP recognition by some antibodies but not by others. AFP is known to exhibit specific glycosylation profiles in pathologies such as germ cell tumors or hepatocarcinomas. This particularity has been proposed as a diagnostic test to predict the occurrence of cancer in patients with viral hepatitis or cirrhosis *(19, 22)*. No FA-specific AFP isoform could be shown by electrophoretic separation in a lectin-containing gel. The only isoform detectable was the L1 band, which was found in every hepatitis, cirrhosis, or hepatocarcinoma sample. Interestingly, a similar result was obtained for an ataxia telangiectasia sample. Although this method is the most widely used for the analysis of the AFP glycosylation profile, our results cannot exclude a structural origi-

nality in AFP molecules expressed in FA blood samples that could induce discrepancies in antibody recognition. Indeed, it has been demonstrated that some AFP epitopes are cryptic and could become more antibody-reactive in different situations [reviewed in Ref. *(23)*]. The immunologic heterogeneity of AFP has also been suggested by the separation of several subfractions by immunoaffinity chromatography using distinct monoclonal antibodies *(24)*.

Clinical and biological diagnosis of FA is often long and difficult. A rapid, reproducible, and cost-effective test, such as AFP measurement, could be a useful aid for diagnosis, provided that the method is highly specific, sensitive, and easily interpreted. As demonstrated in our study, AFP methods differed in terms of specificity and sensitivity for FA diagnosis. Furthermore, the Kryptor method seems to be the most suitable for a routine analysis because little or no crossover of AFP values was seen between FA and non-FA patients. On the other hand, the Immulite and IMx methods should probably be avoided for FA diagnosis. Three methods (Elsa-2, Immuno-1, Elecsys) could be used but with careful interpretation. Finally, our results clearly demonstrated that any AFP assay must be validated before its use for FA diagnosis.

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