

MASS SCREENING OF LIVER CANCER BY DETERMINATION OF ALPHA-FETOPROTEIN IN DRIED
BLOOD SPOTS ON FILTER PAPER

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Primary hepatocellular carcinoma (PHC) ranks the highest among the cancer mortalities of male ($25.7/10^5$) and the second highest among the cancer mortalities of female ($7.9/10^5$) in Taiwan (1,2). Surgical resection is the only favorable treatment for PHC at the present time (3). But up-to-date, only 2-9% symptomatic PHC patients are surgically resectable in Taiwan (4,5). Early detection may increase the resection rate and improve long-term survival of patients with PHC.

Determination of serum alpha-fetoprotein (AFP) and/or ultrasound examination have been used for early detection of PHC in individuals known to be at high-risk (6-9). Ultrasound examination is technically difficult and requires an experienced ultrasonographer as well as high-resolution machines, therefore may not be suitable as a first-line screening method for a mass PHC screening program. Since more than 70% of the patients with PHC showed an elevated AFP value, assay of AFP was suggested to be used as the first-line screening test for early detection of this cancer in a large population (10). A mass survey of PHC by screening of serum AFP levels using a radiorocket electrophoresis has been reported (11). However, the techniques used for AFP assay in the above-mentioned studies were less than satisfactory for a mass screening program because of the difficulty of collecting, storing and transporting serum samples and the complexity and (or) insensitivity of the method. Therefore, a method for measuring AFP in dried blood samples spotted on filter paper using a

sensitive and simple two-site enzyme immunoassay was developed by us for mass screening of PHC (12). The dried blood spot technique, a method widely used in neonatal screening for congenital metabolic diseases, was employed in our assay system to simplify the sample collection and transportation processes. We herein reported the application of this technique for mass screening of PHC in Taiwan.

MATERIAL AND METHODS

Samples

Blood samples collected by venepuncture or skin puncture were spotted onto the special filter paper (Toyo Kagaku Sangyo Co., Japan), which is used for neonatal screening, and air-dried. These spotted specimens on filter paper were sealed in a zip-lock plastic bag and sent by mail to the central laboratory (Clinical Biochemistry Research Laboratory, Dept. of Medical Research, Veterans General Hospital, Taipei), where the specimens were kept at 4°C. The stability of AFP in dried blood spots is very good (12, 13).

Determination of AFP in Dried Blood Spots

AFP in dried blood spots were measured by a method described previously (12) with minor modifications. In brief, a 4.7 mm dried blood disc (equivalent to about 6 μ l of whole blood) obtained from standards or test samples together with 300 μ l of buffer (25 g/L bovine serum albumin, 0.1 mol/L Tris, and 0.1 mol/L NaCl, pH 7.5) containing anti-AFP antibody conjugated with alkaline phosphatase were added to each plastic tube (12 x 75 mm). A polystyrene bead coated with monoclonal anti-AFP antibody was then added and the mixtures were gently shaken at room temperature overnight. Beads were washed 4 times using deionized water and transferred to new tubes. Then, 300 μ l of p-nitrophenyl phosphate (2.69 mmol/L) in 1.04 mol/L diethanolamine (pH 10.0) was added to the washed beads. After incubation at 37°C for half an hour, 1 ml of 0.25 mol/L NaOH was added to stop the reaction. Absorbance was read at 405 nm using a spectrophotometer

(Stasar III, Gilford Instrument, Oberlin, Ohio). The sensitivity of AFP assay by this method is 5 $\mu\text{g}/\text{L}$, and AFP assay by our method has a good correlation with conventional radioimmunoassay (Serono Diagnostic SA, Conisions/VD, Switzerland) (12).

Other Tests and Methods

Ultrasound examination (Aloka SSD 256 with 3.5 MHZ transducer, Aloka Co., Ltd., Tokyo) of the liver was performed at Veterans General Hospital, Taipei, for cases with positive AFP ($>20 \mu\text{g}/\text{L}$ plasma) screening result. For the cases receiving ultrasound examination, serum samples were collected for the following tests. Hepatitis B surface antigen (HBsAg) was detected by a commercial kit (Clinical Assay, Connaught Laboratories, Willowdale, Ontario, Canada). The serum proteins, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), total bilirubin, alkaline phosphatase, and γ -glutamyl transferase were measured by an autoanalyzer (SMAC-II, Technicon Instruments Corporation, Tarry Town, NY).

RESULTS AND DISCUSSION

Community-Based Programs

Luh-Guu Township. A community-based pilot project for screening of liver cancer using dried blood samples was carried out in Luh-Guu Township, a rural area in Central Taiwan (Fig. 1), during late 1985 and early 1986 (5,14,15). Dried blood samples were collected from 1894 men over 40 years of age, which represented an effective collection rate of 82%. There were 20 cases (20/1894, 1%) with initial screening AFP levels greater than 20 $\mu\text{g}/\text{L}$ of plasma. The range of elevation of AFP concentrations was 21.2 to 535.7 $\mu\text{g}/\text{L}$. Of the 19 cases who received ultrasound examinations, space-occupying lesions were detected in three by initial recall (Table I), and the fourth was discovered at the 5-month follow-up. The remaining 15 persons except one suffered from other types of hepatobiliary tract disease (e.g. alcoholic liver disease, chronic hepatitis B, active cirrhosis) with AFP ranged from 23.9 to 79.7 $\mu\text{g}/\text{L}$ (5).

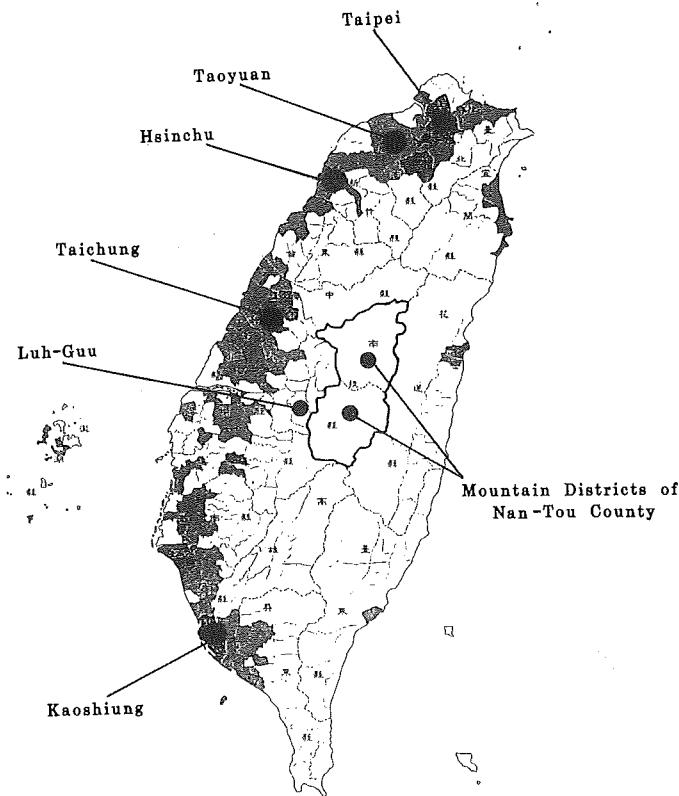


Fig. 1. Geographical distribution of the liver cancer mass screening projects in Taiwan. Shaded area represents the district with higher population density.

Although all 4 PHC cases were serum HBsAg positive, their biochemical data were not characteristic for cirrhosis or PHC (5). Computerized tomography and celiac arteriography also strongly favored the diagnosis of PHC in these four cases. In contrast to the low resection rate (5/57, 8.8%) of tumors among symptomatic PHC patients who were hospitalized during the period of this survey, these four cases were all subjected to successful removal of their tumors. The surgical specimens were all proved to be PHC histologically (5).

TABLE I

PHC DETECTED BY DRIED BLOOD SPOT AFP MASS SCREENING IN THE LUH-GUU TOWNSHIP IN TAIWAN

Case No.	Sex	Age (yrs)	AFP (μg/ml)	HBsAg	Tumor (cm)
L1	M	47	535.7	+	4.5
L2	M	68	43.9	+	2.5
L3	M	60	21.2	+	2
L4	M	70	29.2 1795*	+	1.5

* AFP level 5 months after initial screening.

One of our patients (Case No. L4) was a chronic hepatitis B carrier who was thought to have alcoholic liver disease after initial screening, but proved to have a small PHC at 5-month follow-up. Thus, patients discovered by mildly elevated serum AFP levels should be closely followed up by regular AFP testing and ultrasound examinations, even if they initially seemed to have a non-malignant disease.

Mountain districts of Nan-Tou County. During February - March, 1987, another community-based screening project was carried out in the mountain districts of Nan-Tou County (Fig. 1). 4,084 dried blood samples were collected from men (53%) and non-pregnant women (47%) over 30 years of age. There were approximate 8,000 people age over 30 years old in that area at that time. The aborigine people lived in the mountain districts in Taiwan are known to have high alcohol intake. There were 48 cases (Male:28, Female:20) found with AFP level greater than 20 μg/L. The AFP screening positivity rate was around 1.2% which is similar to that found in the Luh-Guu project. 44 of the 48 positive cases were successfully recalled for ultrasound examination and biochemical liver function tests. Two symptomatic cases suspected with PHC, one male and one female (both AFP >500 μg/L), expired before the follow-up examination.

PHC were detected in 5 men, but only one of them had small tumor and was successfully resected (Table II). One pregnancy and 14 other types of hepatobiliary disease were also found in the recalled cases. There were no specific finding in the remaining 24 recalled cases.

A year later, during March - June, 1988, from the 904 cases found with positive AFP results and/or with positive serum HBsAg marker during the initial screening in the mountain districts of Nan-Tou County, 591 cases (Male:338, Female:253) were successfully recalled for follow-up dried blood spot AFP test and ultrasound examination. There were 15 cases (Male:11, Female: 4) found with AFP level greater than 20 $\mu\text{g}/\text{L}$. The AFP positivity rate (2.6%) of the follow-up test was about twice of that of initial screening. One male case (Case No. N6) with AFP level at 89.8 $\mu\text{g}/\text{L}$ was detected with small PHC ($\sim 2\text{cm}$) (Table II). The tumor was then successfully resected. In the remaining 14 AFP positive cases,

TABLE II

PHC DETECTED BY DRIED BLOOD SPOT AFP MASS SCREENING IN THE MOUNTAIN DISTRICTS OF NAN-TOU COUNTY IN TAIWAN

Case No.	Sex	Age (yrs)	AFP ($\mu\text{g}/\text{L}$)	HBsAg	Tumor (cm)
N1	M	61	2997	+	>5
N2	M	69	>450	+	3 (both lobes)
N3	M	51	32.7	+	$\sim 2^{**}$
N4	M	46	>500	+	>5 (symptomatic)
N5	M	52	30.5	-	multi-nodules
N6	M	61	<10 89.8*	+	$\sim 2^{**}$

* AFP level one year after initial screening.

** Surgically resected.

two pregnancies and 6 with other hepatobiliary disease were confirmed and the other 6 had no specific finding. But among the cases with negative AFP ($<10 \mu\text{g}/\text{L}$) follow-up result, a patient of PHC (4cm, multi-nodules) was detected by

ultrasound examination.

Although the initial AFP screening of the PHC case (Case No. N6) detected by the follow-up test was negative, his serum HBsAg was positive. HBsAg carriers are considered to be a high-risk population for the development of PHC (16,17). Therefore, besides the cases with elevated AFP, the HBsAg carriers should also be closely monitored. However, as demonstrated by the HBsAg negative PHC case (Case No. N5) detected in this mass survey, the screening of liver cancer by AFP should not be limited to the HBsAg carriers only.

Non-Community-Based Program

After the demonstration of the practicability and acceptability of the dried blood spot AFP mass screening method by the community-based pilot project, a larger non-community-based mass screening pilot program (18) was carried out in 5 metropolitan areas in Taiwan. Between August, 1986 and June, 1987, 39,500 samples (58% male, 42% female) were collected from government and company employee during the anti-cancer education seminars provided by the Cancer Society of the Republic of China in the 5 metropolitan areas, namely Taipei, Taoyuan, Hsinchu, Taichung and Kaoshiung (Fig. 1). The subjects were apparently healthy and not pregnant. Most of them were between 20 and 60 years old. 111 cases had AFP levels higher than 20 µg/L. The positivity rate (0.3%) was much lower than that of the community-based programs in the rural areas. The difference of the positivity rate seems not caused by the age difference between the screened populations. The different age groups had similar AFP positivity rate (0.2 - 0.5%) in the metropolitan areas (Table III). Among the 111 AFP positive cases, ten of them were found to be pregnant. 87 of the remaining 101 cases received ultrasound examination. Four cases (Male:3, Female:1) with small PHC and a case with ovarian cancer were detected (Table IV).

All of the 5 cases found with cancer had their tumors removed successfully.

TABLE III

AGE DISTRIBUTION AND THE AFP POSITIVE RATE OF THE MASS SCREENING PROGRAM IN THE METROPOLITAN AREAS IN TAIWAN

Age (yrs)	Male			Female			Total		
	Cases	Positive	(%)	Cases	Positive	(%)	Cases	Positive	(%)
≤20	556	3	0.5	572	1	0.2	1131	4	0.4
21-24	934	3	0.3	1877	9	0.5	2816	12	0.4
25-29	4020	13	0.3	3919	16	0.4	7951	29	0.4
30-34	5427	14	0.3	3501	7	0.2	8936	21	0.2
35-39	4778	11	0.2	3018	1	0.03	7801	12	0.2
40-44	2443	6	0.2	1557	3	0.2	4005	9	0.2
45-49	1618	6	0.4	917	2	0.2	2540	8	0.3
50-54	983	3	0.3	523	4	0.8	1510	7	0.5
55-59	1049	5	0.5	271	0	0	1323	5	0.4
≥60	1179	4	0.3	251	0	0	1434	4	0.3
Total	23016	68	0.3	16430	43	0.3	39500	111	0.3

* AFP level higher than 20 µg/L was considered as positive.

They are living well at the present time (Sept. 1988; 19-22 months after resection). 54 cases were found with other hepatobiliary disease and 89% of them had positive serum HBsAg marker, which was much higher than the expected HBsAg carrier rate (15-20%) in the general population in Taiwan (16,17).

Since the AFP positivity rate was relatively low in the metropolitan screening program, the cases with borderline AFP screening results were rechecked. 132 second dried blood spot samples were collected from 141 cases who had an AFP level between 10-20 µg/L on the first screening test. Two cases were found to be pregnant. 22 of the rest of the follow-up cases still had an

TABLE IV

TUMORS DETECTED BY DRIED BLOOD SPOT AFP MASS SCREENING IN THE METROPOLITAN AREAS IN TAIWAN

Case No.	Sex	Age (yrs)	AFP (μg/L)	HBsAg	Tumor (cm)
M1	F	37	316	+	PHC 2.0
M2	M	32	499	+	PHC 2.0
M3	M	56	41	+	PHC 0.4
M4	M	26	>800	+	PHC 4.0
M5	F	24	583	-	Ovarian 15 Cancer

AFP lever greater than 10 μg/L and were recalled for ultrasound examination.

But no tumor was detected. 16 of them were found to have non-malignant hepatobiliary disease, and most of them were serum HBsAg positive. The cases with positive AFP results and/or with positive serum HBsAg marker in this program should be closely followed up by AFP determination and ultrasound examination. The results of this mass screening program in the metropolitan areas in Taiwan is summarized in Fig. 2.

The serum AFP level has been reported to be elevated in other cancers besides PHC, especially in the germ cell tumors (19). As shown by the ovarian cancer detected in our AFP screening program, other cancers as well as PHC should be ruled out in the asymptomatic cases with positive AFP screening result. Previous studies (6-9) and our follow-up project in the mountain districts of Nan-Tou County showed that some patients (10-20%) with PHC may have normal serum AFP level and would not be detected by measurement of AFP alone. Therefore, such persons who are already known to be at high risk for the development of PHC, e.g. chronic HBsAg carriers or patients with cirrhosis,

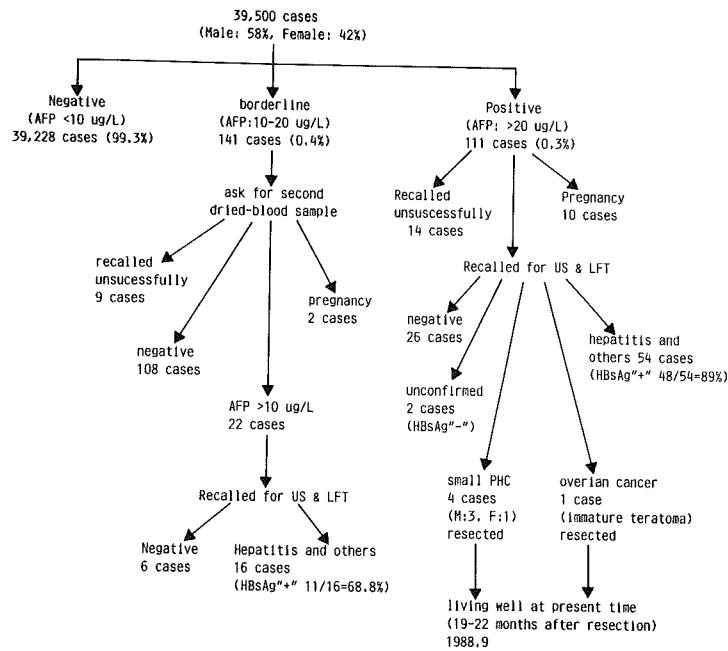


Fig. 2. Results of dried blood spot AFP mass screening program in the metropolitan areas in Taiwan. US: Ultrasound examination, LFT: Liver function tests.

should be monitored closely by both ultrasound examination and AFP testing. However, in the population at large, assay for AFP remains the first-line screening test for PHC (10,11,20). Our results indicate that this simple, sensitive and convenient method for collecting samples and determination of AFP may be used as the primary test in the mass screening program for the early detection of PHC, especially in rural areas, and as a follow-up test to monitor the patients at high risk for the development of PHC. However, from the cost-effective point of view, the optimal selection of the population to be screened and the optimal duration between the follow-up tests for the high-risk group remain to be established.

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