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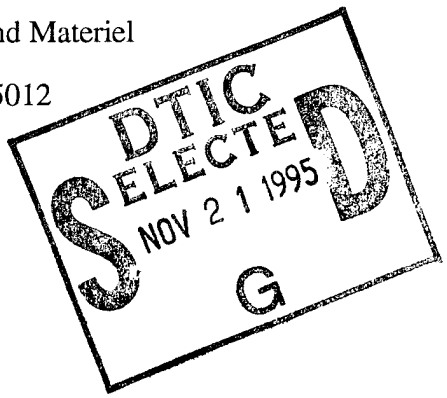

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Zhihong Lu, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Alabama at Birmingham
Birmingham, Alabama 35294-2010

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13. ABSTRACT *(Maximum 200 words)*

5-Fluorouracil (5-FU) is one of the most used chemotherapeutic agents in the treatment of breast cancer. Dihydropyrimidine dehydrogenase (DPD) is the key enzyme in 5-FU catabolism. The major purpose of the project is to characterize the distribution pattern of DPD activity in breast cancer patients, to establish the relationship between DPD activity and 5-FU-associated toxicity, and to improve 5-FU-based chemotherapy in breast cancer patients by monitoring DPD activity. Results from the present study with 336 breast cancer patients demonstrated that DPD activity follows a normal distribution as seen in normal population. However, the mean DPD activity in breast cancer patients was significantly lower than that observed with the general population. Results from the present study with 138 human liver samples demonstrated that liver DPD activity follows a normal distribution, and slight differences among race, gender, and ages were observed. Using a polyclonal antibody against human DPD, we have shown the correlation between DPD activity and the amount of DPD protein, providing the insight of DPD deficiency and the basis for the future clinical use of DPD antibody to quantify DPD activity in cancer patients.

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INTRODUCTION

I. BACKGROUND

Since its introduction into clinical use more than 30 years ago, 5-fluorouracil (5-FU) continues to be one of the most widely used anticancer drugs (1-2), ranking in the top three anticancer drugs prescribed in the U.S. (3). It is used today, either alone or more typically in combination with other drugs, in the treatment of a number of different malignancies, particularly three of the most frequently occurring cancers: carcinomas of the breast, colon, and skin (4). In general, 5-FU is relatively well tolerated at standard doses with the primary toxic effects occurring in the gastrointestinal mucosa and bone marrow (4,5). Neurologic toxicity presenting as cerebellar ataxia and somnolence occurs much less frequently (<5%), but may be dose-related (5). Like many other antineoplastic drugs, 5-FU has a relatively narrow therapeutic index, such that toxicity is likely to increase as the dose (concentration) is escalated.

The biochemical mechanism responsible for most of the toxicities observed with 5-FU is thought to require anabolism of 5-FU to form specific 5-FU nucleotides, which can then act at several sites, in particular, inhibition of thymidylate synthase, but also through incorporation into RNA and DNA (1-2). Until recently, catabolism was not thought to have a major role in 5-FU toxicity. It has been shown in preclinical studies that inhibition of pyrimidine catabolism can indirectly cause increased exposure to 5-FU which in turn can then be anabolized resulting in increased toxicity (2). Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting step in pyrimidine catabolism (6). This enzyme,which is essentially an irreversible step,converts 5-FU to dihydrofluorouracil which is inactive. Thus this enzymatic step is important in regulation of 5-FU metabolism,determining the availability of 5-FU for anabolism and further determining 5-FU therapeutic effects and/or toxicity.

Over the past decade, there have been several clinical studies which have further emphasized the importance of pyrimidine catabolism and the DPD step in particular as a major determinant of 5-FU toxicity. Attempts to increase the antitumor effectiveness of 5-FU through "biochemical modulation" of its anabolism with concomitant administration of thymidine (leading to release of thymine), led to unexpected life-threatening toxicity (7). The biochemical mechanism responsible for this drug interaction was subsequently shown to be competition of thymine and 5-FU for DPD (8). Since 5-FU and thymine have been reported to have similar affinities for DPD (6,9), it is not surprising that 5-FU degradation was inhibited, most likely resulting in altered 5-FU pharmacokinetics and pharmacodynamics, and in turn increased toxicity. Pharmacokinetic studies of patients receiving 5-FU by continuous infusion demonstrated that plasma 5-FU levels varied throughout the day with a circadian pattern (10). Subsequent study examining patients receiving 5-FU by continuous infusion demonstrated that plasma 5-FU levels had a circadian variation that varied inversely with circadian variation in DPD from peripheral blood mononuclear (PBM) cells, suggesting that the plasma 5-FU levels were regulated by DPD (11). The relationship between DPD levels and 5-FU pharmacokinetics was also demonstrated by Fleming et al (12) in patients receiving 5-FU by 5-day continuous infusion.

The importance of DPD in 5-FU pharmacokinetics and toxicity perhaps is best illustrated in patients with DPD deficient (13-18). Following 5-FU-based chemotherapy, these patients developed profound toxicity and eventually died. More importantly, in our earlier studies (14-17), ten of the 11 patients were women with either breast or colorectal cancer. In view of this striking sex difference in patient studies from our laboratory (14-17) and others (13), as well as the recently suggested sex-related difference in 5-FU clearance (18), it is important to determine if there is sex

difference in DPD activity and in 5-FU toxicity by a large scale prospective study. Recently, in an NIH/NCI funded study, we determined the characteristics of DPD activity and established a baseline for this enzyme in general population (17).

There are an increasing number of genetic polymorphisms recognized for drug metabolizing enzymes that may produce not only altered drug metabolism but also increased drug toxicity. Pharmacogenetic syndromes have now been described for many different types of drugs including cancer chemotherapy drugs (19). While decreased drug metabolism may result in altered pharmacokinetics and pharmacodynamics and hence increased toxicity with various classes of drugs, it can be particularly striking with cancer chemotherapy drugs since the therapeutic index is typically narrow. In the present project, we have been studying the pharmacogenetic syndrome of DPD deficiency that may not be rare, potentially accounting for many of the cases of severe 5-FU toxicity, including death, seen in the clinic. The presence of genetic polymorphism in the population due to the presence of unknown molecular alteration(s) in DPD suggests that if DPD can be assessed prior to therapy severe toxicity may be avoided in the future.Although the mechanisms are unclear, the frequency of DPD deficiency in women and breast cancer patients appears to be higher than that in general population.

II. PURPOSE AND SIGNIFICANCE OF THE PROJECT

The long term objective of this project is to improve 5-FU chemotherapy in cancer patients through a better understanding of the genetic polymorphism of DPD and its role in determining 5-FU toxicity. Knowledge acquired from this project should be useful in the future to predict which patients may be susceptible to severe 5-FU toxicity, permitting modification of drug dose before chemotherapy. Specific objectives include:

1.) Determine in a prospective study the relationship between DPD activity and 5-FU toxicity.

Hypothesis 1- *-DPD has a major role in determining 5-FU toxicity with decreased enzyme activity being associated with greater toxicity. DPD activity in breast cancer patients may be used in predicting 5-FU toxicity and permitting modification of drug dose of 5-FU in chemotherapy of breast cancer.*

2.) Determine biochemical properties of DPD from peripheral blood mononuclear cells (PBM-DPD) of normal and deficient individuals.

Hypothesis 2-- *The mechanisms responsible for DPD deficiency may be related to DNA, RNA and protein levels. Comparison of DPD from deficient patients with DPD from normal individuals should provide insight into the mechanism of genetic polymorphism of DPD.*

As noted earlier, 5-FU continues to be one of the most widely used anticancer drugs (1-2), ranking in the top three anticancer drugs prescribed in the U.S. (3). It is one of the major chemotherapeutic agents in the treatment of breast cancer(4). However, the mechanisms responsible for 5-FU toxicity are not completely understood. Although toxicity from 5-FU is generally manageable, severe and life threatening toxicity does occur. As shown above,DPD is the initial rate limiting enzyme in 5-FU catabolism and hence can ultimately regulate the amount of 5-FU available for anabolism. Furthermore, we have shown that there is evidence for variation in enzyme in the population, and that certain individuals who experience severe toxicity after 5-FU

treatment are deficient in DPD activity. Preliminary studies indicate that DPD activity in breast cancer patients is lower than the general population. In addition, most DPD deficient patients identified by us and others were women. Furthermore, the frequency of DPD deficiency in breast cancer patients appears to be much higher than in the general population. Thus it appears highly desirable to determine in a prospective study the relationship between DPD activity and toxicity in patients receiving 5-FU, and to determine the mechanisms responsible for DPD deficiency.

METHOD AND MATERIALS

Chemicals and Radiochemical

5-FU, BSA, NADPH, and Histopaque were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]-5-FU (25 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). The purity of unlabeled and labeled 5-FU was confirmed by HPLC (20) to be greater than 99%. Acrylamide and pre-stained molecular weight markers were purchased from Bio-Rad (Richmond, CA). Alkaline phosphatase-labeled goat anti-rabbit antibody, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt were obtained from Southern Biotechnology (Birmingham, AL). All other solvents and reagents were purchased in the highest grade available.

The major buffer (buffer A) used in both the enzyme preparation and DPD assay contained 35 mM potassium phosphate, pH 7.4, 2.5 mM magnesium chloride, and 10 mM 2-mercaptoethanol. Since it is light-sensitive and unstable with long-term storage, NADPH, the critical cofactor in the enzyme reaction was freshly prepared.

Determination of PBM-DPD Activity in Breast Cancer Patients

Cancer Patients. In collaboration with the Oncology Clinic in the University of Alabama at Birmingham, breast cancer patients who were seen in the clinic and treated or to be treated with 5-FU (in combination with other agents) were assayed for DPD activity. Consent was obtained from each patient. Clinical data were also collected for further analysis.

Blood Collection and Isolation of PBM Cells. Blood samples (25 ml) were drawn from a peripheral vein into heparinized tubes and then loaded onto a centrifuge tube containing 15 ml Histopaque. After centrifugation at 500 x *g* for 30 min at 25°C, the PBM cell fraction was carefully removed and washed 3 times with PBS. Contaminating red blood cells were hypotonically lysed. The resulting PBM cells were used in the subsequent enzyme assay.

Preparation of PBM cytosol. Fresh PBM cells were suspended in buffer A then placed in an ice bath and lysed by sonication (3 times 10 sec with 30 sec interval between sonication). After centrifugation at 14, 000 x *g* for 15 min at 4°C, the supernatant was removed and used in the subsequent enzyme assay. Using the method of Bradford (21), the amount of protein in the sample was determined prior to enzyme assay in order to add appropriate amount protein into the reaction mixture.

Enzyme Assay DPD activity was determined by radioassay, measuring the catabolites of 5-FU formed by reversed-phase HPLC (17, 20). The reaction mixture contained 200 μM NADPH, 20 μM [^{3}H]-5-FU, buffer A, and enzyme solution (250-1000 μg total protein) in a final

volume of 1 ml. The mixture was incubated at 37°C and 175 µl of the reaction sample was taken out at various times (5, 10, 20, 30, 60 min) and mixed with the same volume of ice-cold ethanol to stop the reaction. The mixture was then kept in a freezer (-20°C) for 30 min and subsequently filtered through a 0.2 µm Acro filter (Gelman Sciences, Ann Arbor, MI) prior to HPLC analysis.

Reversed-Phase HPLC Analysis Separation of 5-FU and its catabolites was performed by reversed-phase HPLC using a Hewlett-Packard 1050 HPLC system equipped with a spectrometric detector and chromatographic terminal (HP 3396 Series II Integrator). Two Hypersil 5 µm columns (Jones Chromatography, Littleton, CO) were used in tandem as the stationary phase. The columns were eluted at a flow rate of 1.0 ml/min with the mobile phase containing 1.5 mM potassium phosphate, pH 8.0, with 5 mM tetrabutylammonium hydrogen sulfate (17,20). Fractions (1 ml) were collected into 7-ml scintillation vials, using a Redifrac fraction collector (Pharmacia LKB Biotechnology, Piscataway, NJ) and were mixed with 5.5 ml scintillation solvent. The radioactivity in each fraction was quantitated by liquid scintillation spectrometry (Beckman LS 6000). Under these conditions, typical retention times for dihydrofluorouracil and 5-FU were 9 and 21 min, respectively.

Determination of Liver DPD Activity in General Population

Liver Tissues from Donors. Human livers were obtained from the National Disease Research Interchange (NDRI), Philadelphia, PA, and the University of Alabama Comprehensive Cancer Center Tissue Procurement Shared Facility. The protocol used in this study was approved by both the university Institutional Review Board and NDRI. In the present study, 138 human liver samples (83 males and 55 females) from donors with normal liver function were collected. The 138 human liver samples were free of liver disease. These liver samples were collected in a 2-year period. Liver samples were quickly frozen and kept in -70°C freezer until use. No significant systemic difference in DPD activity was found for samples obtained at various times over a 2-year period.

Sample Preparation. The slowly-thawed liver tissues were washed with ice-cold physiologic saline (0.9% NaCl), weighed, minced, and homogenized in 4 volumes of buffer A. The resulting homogenate was centrifuged at 100,000 x *g* for 60 min at 4 °C. The cytosolic fraction (supernatant) was removed and used in the subsequent analyses. DPD activity was determined immediately following the supernatant preparation. Prior to enzyme assay, the amount of protein in each sample was determined by the method of Bradford (21) in order to add the appropriate amount of protein to the enzyme reaction. The experimental conditions for enzyme reaction and HPLC analysis were the same as described above.

Calculation of DPD Activity

For each sample, 5 determinations were run at various incubation times. After HPLC analysis, the amount of 5-FU catabolites at each time point was quantitated. The data was plotted using products formed (y) versus time (x) to calculate the slope of the reaction (products formed/min) by linear regression analysis. The slope was then divided by the amount of protein added to obtain the final result (DPD activity expressed as nmol/min/mg protein). For samples from cancer patients with DPD deficiency or liver samples with very low DPD activity, at least two separate assays were performed.

As demonstrated in our previous studies (9, 17), the radioassay was sensitive and accurate in determination of DPD activity in peripheral blood mononuclear cells and in liver samples. Using

the assay conditions described above, the variations of inter- and intra-assay were found to be less than 5%. For samples with extremely low enzyme activity, at least two separate assays were performed to verify the results.

Immunoblot Analysis (Western Blot)

The primary antibody used in the study was the purified rabbit polyclonal antibody generated against human liver DPD (9). A 7% SDS-PAGE was performed using the freshly prepared 100,000 x *g* liver supernatant. SDS-PAGE was carried out in a 1.0 mm thick, 7% (w/v) polyacrylamide gel containing 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. Samples were mixed with an equal volume of sample buffer (0.0625 M Tris-HCl, pH 6.8; 10% glycerol; 0.2% SDS (w/v); 80 mM 2-mercaptoethanol) and then boiled for 5 min. Electrophoresis was performed at a constant current of 30 mA for 45 min at 25°C. The proteins were transferred from the gel to a nitrocellulose filter following the method described previously by Towbin *et al.* (22). Following incubation overnight at 4°C with the primary antibody (diluted 1:2000) in a 120 mM borate-saline solution containing 1% (w/v) BSA, pH 8.5, the nitrocellulose filter was washed with borate-saline containing 0.5% (v/v) Tween 20 and then incubated with a secondary, alkaline phosphatase-labeled goat anti-rabbit antibody. The location of immunoreactive proteins on the nitrocellulose filter was detected in a 0.1 M sodium carbonate buffer (100 ml), pH 9.5, containing 30 mg nitro blue tetrazolium (added as a 1 ml solution dissolved in 70% dimethylformamide) and 15 mg 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (added as a 1 ml solution dissolved in 100% dimethylformamide).

Statistical Analysis

Mean DPD activity (and S.D. or S.E.) was calculated for each group by age, gender, and race. The differences of DPD activity among the groups by gender, age, and race were analyzed by ANOVA. To determine the distribution pattern in the general population, probability testing was used.

RESULTS

Population Distribution of PBM-DPD Activity in Breast Cancer Patients

Using freshly prepared PBM cell samples, DPD activities of 336 breast cancer patients were determined. The population characteristics of this study are summarized in Table 1 (page 15). Distribution of PBM-DPD activity in this population is shown in Fig 1 (page 18). Statistical analysis by probability testing indicated that human PBM-DPD activity follows a normal distribution. In order to examine potential differences in age and race, further statistical analyses were carried out. The mean PBM-DPD activities in each group by age and race are also shown in Table 1 (page 15).

Statistical analyses indicated that mean PBM-DPD activity in breast cancer patients was significantly lower than that observed in female controls (17) ($p<0.005$, Table 1, page 15). In breast cancer patients, mean PBM-DPD activity was slightly higher in Caucasians compared to African Americans. Analysis of different age groups indicated that PBM-DPD activities in individuals age of 40's and above were slightly higher than that observed with age of 30's (Table 1, page 15; Fig 2, page 19). However, these differences were not statistically significant. Further

examination of affect of race and age on PBM activity by cross analysis indicated that these differences were not statistically significant (Fig. 3, page 20).

Further examination of affect of disease, race, and age on PBM-DPD activity by ANOVA indicated that only disease difference (cancer vs. normal subjects) was statistically significant after adjustments for race and age.

Population Distribution of Liver DPD Activity in "Normal" Donors

Using frozen liver samples, DPD activities of 138 donor livers collected consecutively over the preceding 24 months were determined. The population characteristics of this study are summarized in Table 2 (page 16). Distribution of human liver DPD activity is shown in Fig 4 (page 21). Statistical analysis by probability testing indicated that human liver dihydropyrimidine dehydrogenase activity follows a normal distribution (Fig 5, page 22). In order to examine potential differences in gender, age and race, further statistical analyses were carried out. The mean liver DPD activities in each group by gender, age, race are shown in Table 2 (page 16). Results from ANOVA analyses are shown in Table 3 (page 17). Mean liver DPD activity in females was significantly higher than that observed in males ($p<0.005$). Although liver DPD activity was higher in Caucasians compared to African Americans, the difference was not statistically significant. Analysis of different age groups indicated that liver DPD activities in individuals age 30-40 yrs and 40-50 yrs were slightly higher than other groups (Table 2, page 16), particularly in females (Fig 6, page 23). However, these differences were not statistically significant. Further examination of affect of gender, race, and age on liver DPD activity by ANOVA indicated that only gender difference was statistically significant after adjustments for race and age (Table 3, page 17).

Correlation between Enzyme Activity and the Amount of DPD Protein in the Liver

Using the affinity-purified polyclonal antibody against human DPD, Western blot analysis demonstrated that DPD protein was significantly decreased in liver cytosol from the two samples with extremely low enzyme activity compared with samples with normal activity. Representative Western blot analyses were shown in Fig 7A, B, C (page 24). As illustrated in Fig. 7B, where an equal amount of protein was loaded onto the gel, the DPD protein band for two liver samples had a very low density. The correlation analysis between DPD activity and the density of DPD protein band on western blot analysis demonstrated a relationship between DPD activity and the amount of DPD protein in the liver (Fig 8, page 25).

DISCUSSION

5-FU is a major chemotherapeutic agent used in the treatment of breast cancer. DPD has an important role in 5-FU catabolism with regulation of the availability of 5-FU for anabolism, potentially determining the resultant anticancer efficacy and/or toxicity of 5-FU(1-3). However, the clinical value of determination of DPD activity has not been widely appreciated until recently. This is due to limited knowledge of "normal" DPD activity in the general population, the relationship between peripheral blood mononuclear cells DPD and liver DPD, as well as the unavailability of a sensitive and reproducible assay. The major purpose of the present project is to characterize the distribution pattern of PBM-DPD activity in breast cancer patients, to establish the relationship between DPD activity and 5-FU-associated toxicity, and to improve 5-FU-based chemotherapy in breast cancer patients by monitoring DPD activity.

Results from the present study demonstrated that PBM DPD activity in breast cancer patients generally follows a normal distribution as seen in normal population (17). However, the major finding of the present study is that mean PBM DPD activity in breast cancer patients was significantly lower than that observed with the general population. This difference (cancer vs. normal subjects) was still statistically significant after adjustments for race and age. The mechanisms responsible for the difference are not clear, and further study is needed.

Results from the present study demonstrated that liver DPD activity generally follows a normal distribution. Slight differences among race, gender, and ages were observed. Gender difference in liver DPD activity was shown to be statistically significant. Of note, 4 liver samples had very low enzyme activity (<0.05 nmol/min/mg protein) and 3 liver samples had very high enzyme activity (>0.85 nmol/min/mg protein). Although the importance of extremely low DPD activity has been shown in cancer patients with deficiency of this enzyme, the significance of very high enzyme activity has not been clear but may have a role in the poor response to 5-FU treatment due to increased catabolism of the drug.

A number of drug metabolizing enzymes have genetic polymorphisms (19). To evaluate the distribution pattern of DPD activity and determine if a genetic polymorphism for DPD exists, several studies have now been undertaken in normal subjects and cancer patient populations (11,12,17,18,23,24). Most of the previous studies utilized a small population without balance in the number of subjects in each subgroup by gender, age, and race. A recent study in our laboratory with 124 subjects (45% males and 55% females) demonstrated a normal distribution of PBM-DPD activity, with an approximate 4-fold difference in enzyme activity (17). No significant differences in the enzyme activity were observed related to gender, age, or race in the general population (17). In a study of cancer patients, Milano *et al.* (18) reported a possible influence of gender on 5-FU clearance and suggested that it may be related to variations in DPD activity. However, more recent reports from the same laboratory showed no significant gender difference in DPD activity (12,25). In the present study, we reported preliminary results of a cohort study of DPD activity in breast cancer patients. PBM-DPD activity was determined in a larger population (336 female breast cancer patients). The distribution pattern of PBM activity was similar to the general population (17). However, the mean enzyme activity was significantly lower than that observed in the general population. Prospective studies of the relationship between dihydropyrimidine dehydrogenase activity and 5-fluorouracil-associated toxicity and/or response are continuing.

Although the liver is thought to be the major site of 5-FU metabolism (26), the population distribution of liver DPD activity has not been known. In a previous study (26), a 288-fold variation in human liver DPD activity was observed, presumably due to variation in the quality of liver tissue preparation. In the present study, human liver DPD activity was for the first time demonstrated to have a normal distribution, while the mean enzyme activity of females was observed to be slightly higher than that of males.

In a continuing effort to study DPD in this laboratory, we have generated a specific polyclonal antibody against human DPD. Using this antibody, both decreased liver DPD activity and decreased protein corresponding to this enzyme were observed in cancer patients identified to be deficient of this enzyme (17). Studies have now shown the usefulness of the polyclonal antibody in quantitating DPD activity in various tissues including liver, peripheral blood mononuclear cells, and fibroblasts (27). Using a larger sample size in the present study, we further established the relationship between DPD activity and the amount of DPD protein, providing the insight of DPD deficiency and the basis for the future clinical use of DPD antibody to quantify DPD activity in cancer patients.

CONCLUSIONS

Thus far, studies in the present project have demonstrated that: 1) Dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells in the breast cancer population generally follows a normal distribution with slight differences in age and race. Significantly decreased DPD activity was observed in breast cancer patients compared to normal population; and 2) Liver dihydropyrimidine dehydrogenase activity in the general population generally follows a normal distribution with slight differences in gender, age and race. Significantly decreased DPD activity was associated with decreased enzyme protein.

Further studies are needed to determine the frequency of DPD deficiency in the cancer patient population, prospectively determine the relationship between DPD activity and 5-FU effectiveness and/or toxicity, the relationship between DPD activities in peripheral blood mononuclear cells and the liver, and the molecular basis for DPD deficiency.

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Table 1. Comparison of PBM-DPD Activity (nmol/min/mg protein) between Breast Cancer Patients and Health Volunteers

Group		Breast Cancer Patients		Health Volunteers (see ref. 17)	
		n	Mean ± S.E.	n	Mean ± S.E.
Total		336	0.264 ± 0.006	68	0.443 ± 0.016
	African American	46	0.241 ± 0.018	28	0.460 ± 0.026
	Caucasian	290	0.270 ± 0.006	40	0.431 ± 0.020
Age (yr)	30-	32	0.231 ± 0.016		0.456 ± 0.021
	40-	99	0.264 ± 0.011		0.431 ± 0.030
	50-	108	0.274 ± 0.010		0.373 ± 0.031
	60-	56	0.283 ± 0.015		
	70-	41	0.265 ± 0.018		

Table 2. Liver Dihydropyrimidine Dehydrogenase (DPD) Activity in Healthy Donors

Group		n	Liver DPD Activity (nmol/min/mg protein)			p Value
			Mean ± S.E.	Highest	Lowest	
Total		138	0.369 ± 0.015	0.894	0.049	
Women		55	0.421 ± 0.024	0.894	0.059	0.005
	African American	10	0.389 ± 0.052			
	Caucasian	45	0.428 ± 0.028			
Men		83	0.335 ± 0.018	0.743	0.049	
	African American	24	0.309 ± 0.033			
	Caucasian	59	0.346 ± 0.021			
African American		34	0.333 ± 0.028	0.689	0.116	0.157
Caucasian		104	0.382 ± 0.017	0.894	0.049	
Age (yr)	20-	12	0.335 ± 0.048	0.667	0.059	0.150
	30-	20	0.426 ± 0.046	0.859	0.049	
	40-	29	0.399 ± 0.026	0.635	0.136	
	50-	24	0.374 ± 0.041	0.894	0.116	
	60-	28	0.299 ± 0.031	0.744	0.071	
	70-	25	0.382 ± 0.032	0.743	0.167	

Table 3. Statistical (ANOVA) Analysis of Liver Dihydropyrimidine Dehydrogenase (DPD) Activity in Healthy Donors

Factors	Group	F Value	p Value	Conclusion
Gender	2	8.33	0.005	Liver DPD activity was significantly higher in women than that in men.
Race	2	2.02	0.157	No significant difference.
Age	6	1.65	0.150	No significant difference.
Gender and Race	2 X 2			Cross analysis by race and gender indicated that liver DPD activity is not race-dependent while gender difference remains.
Gender		7.46	0.007	
Race		2.12	0.148	
Gender and Age	2 X 6			Cross analysis by age and gender indicated that DPD activity is not age-dependent while gender difference remains.
Gender		7.36	0.008	
Age		1.73	0.133	
Race and Age	2 X 6			Cross analysis by age and race DPD activity is neither age- nor race-dependent.
Race		1.96	0.164	
Age		1.67	0.147	
Gender, Race, and Age	2 X 2 X 6			Cross analysis by race and gender indicated that DPD activity is not race-dependent while gender difference remains.
Gender		6.51	0.011	
Race		2.04	0.156	
Age		1.74	0.131	

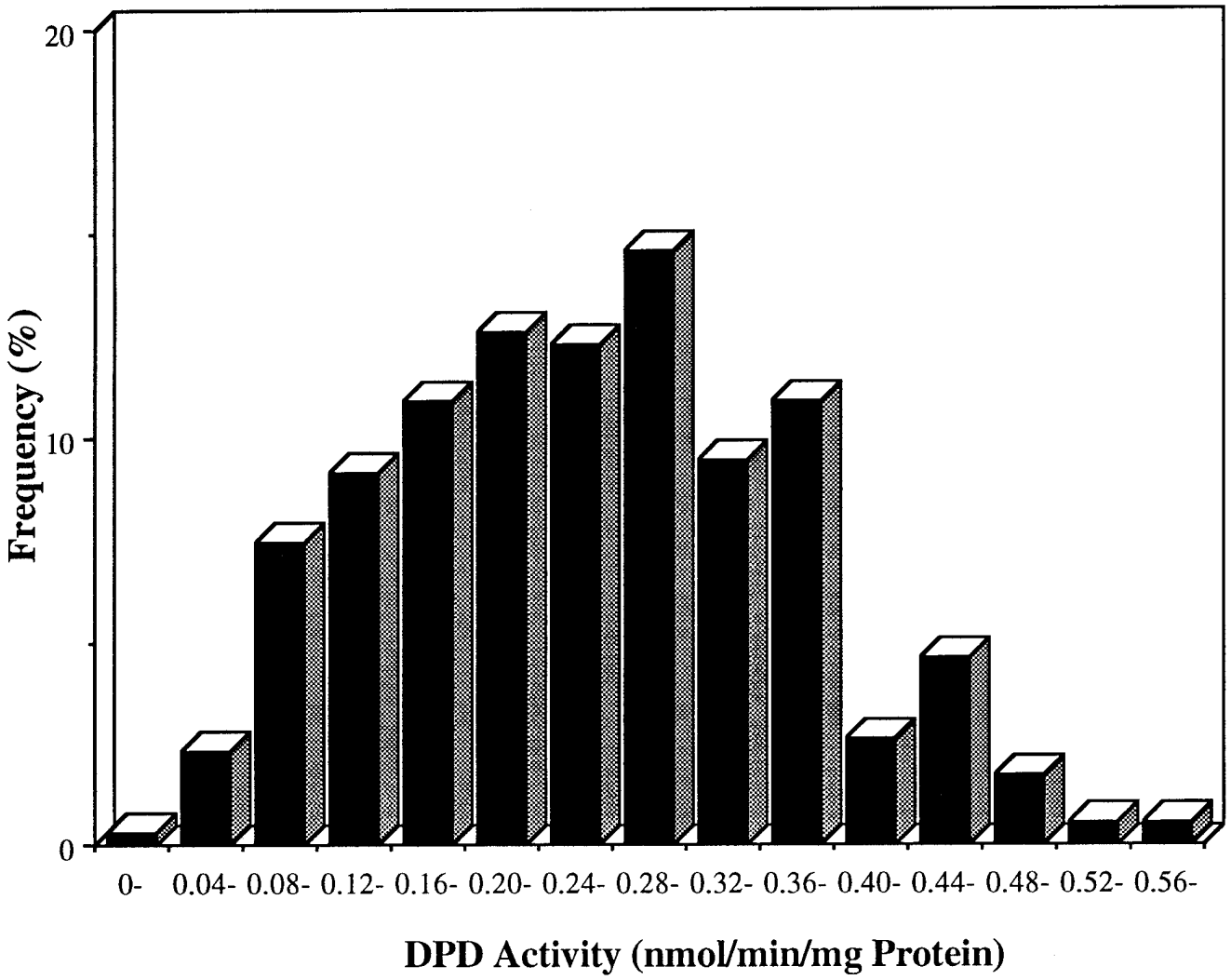

Fig.1 Population Distribution of PBM-DPD Activity in Breast Cancer Patients. Statistical analysis demonstrated that PBM DPD activity follows a normal distribution (Guassian distribution).

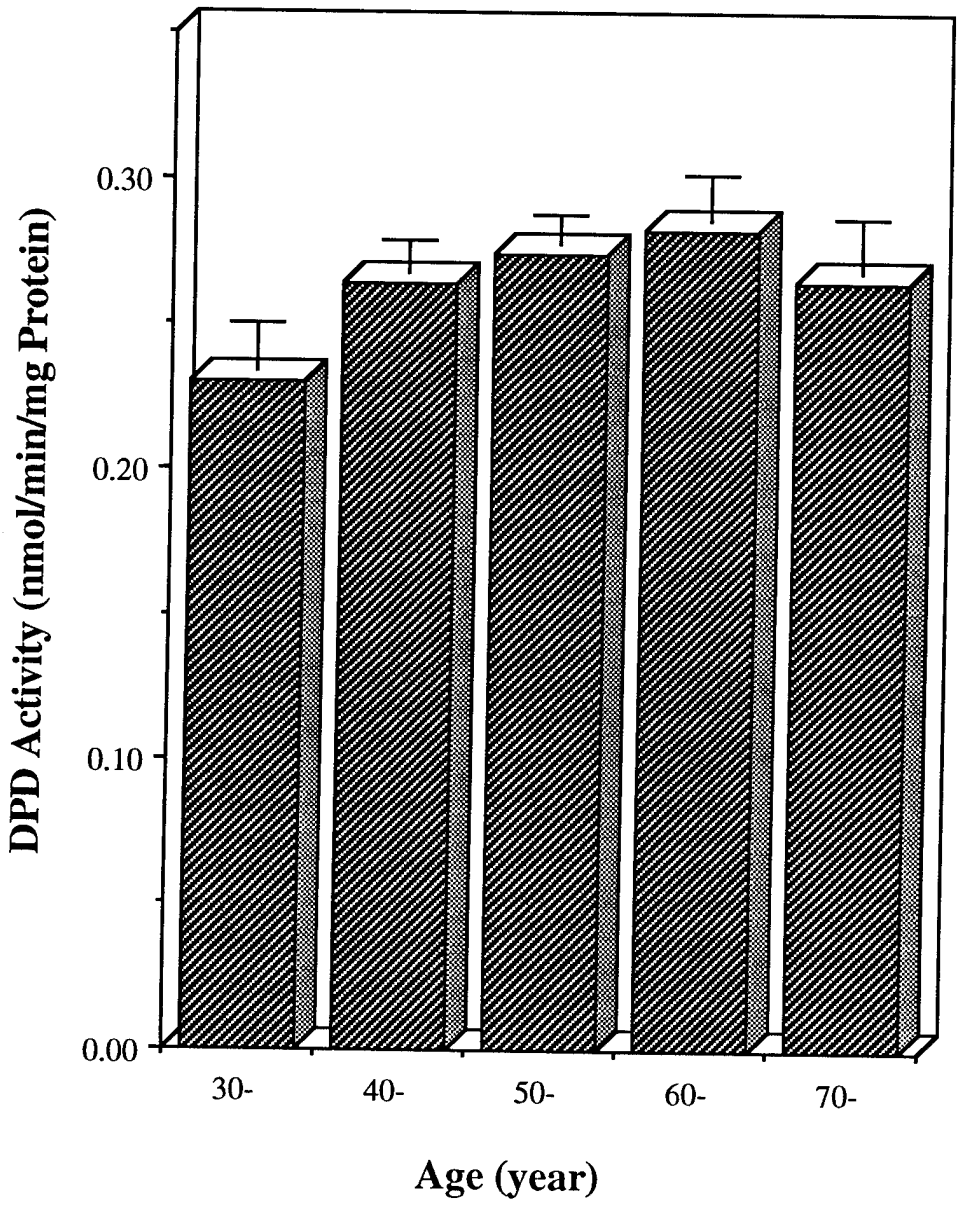

Fig.2 Population Distribution of PBM-DPD Activity in Breast Cancer Patients by Age.

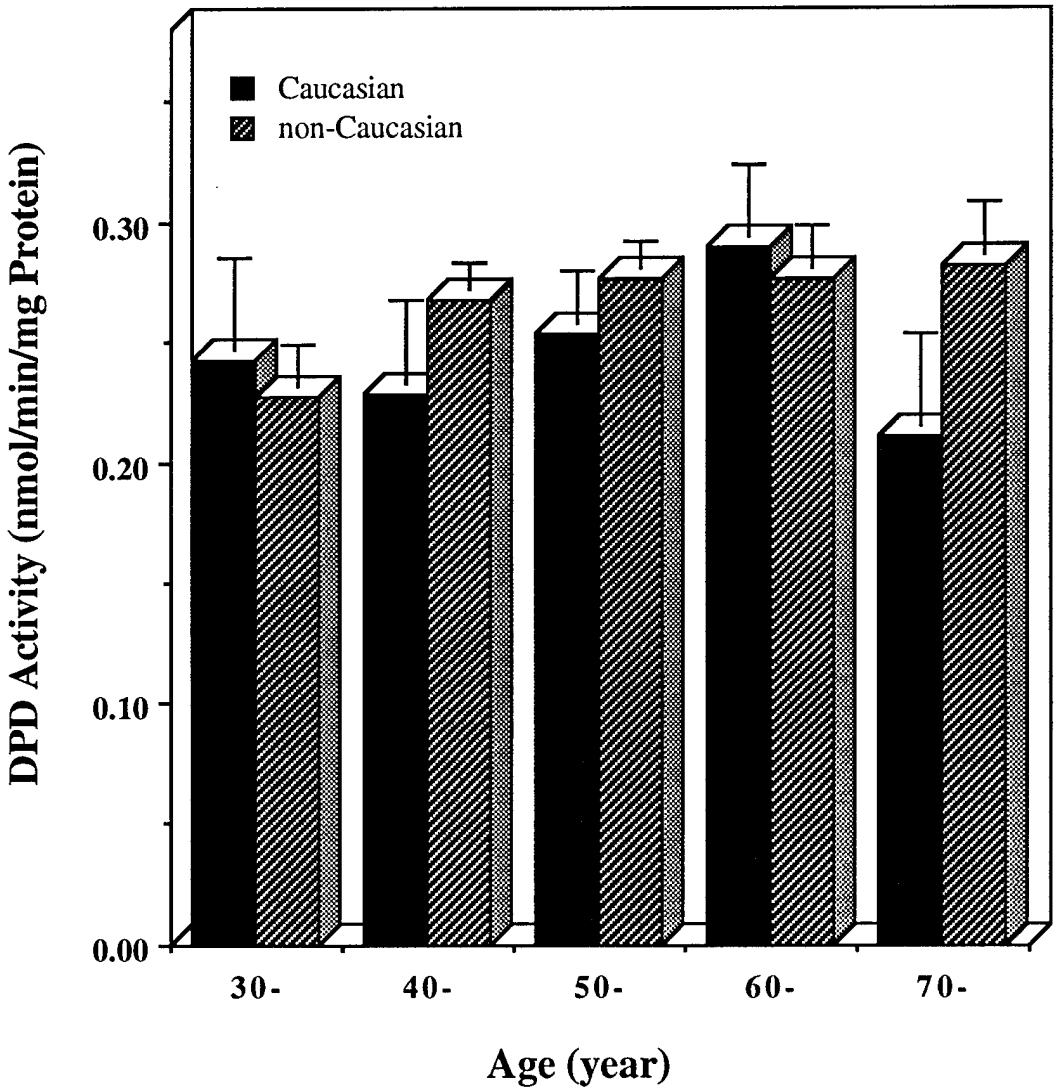

Fig. 3 Population Distribution of PBM-DPD Activity in Breast Cancer Patients. This figure illustrates the comparison of mean (± S.E.) DPD activities between african americans and caucasians.

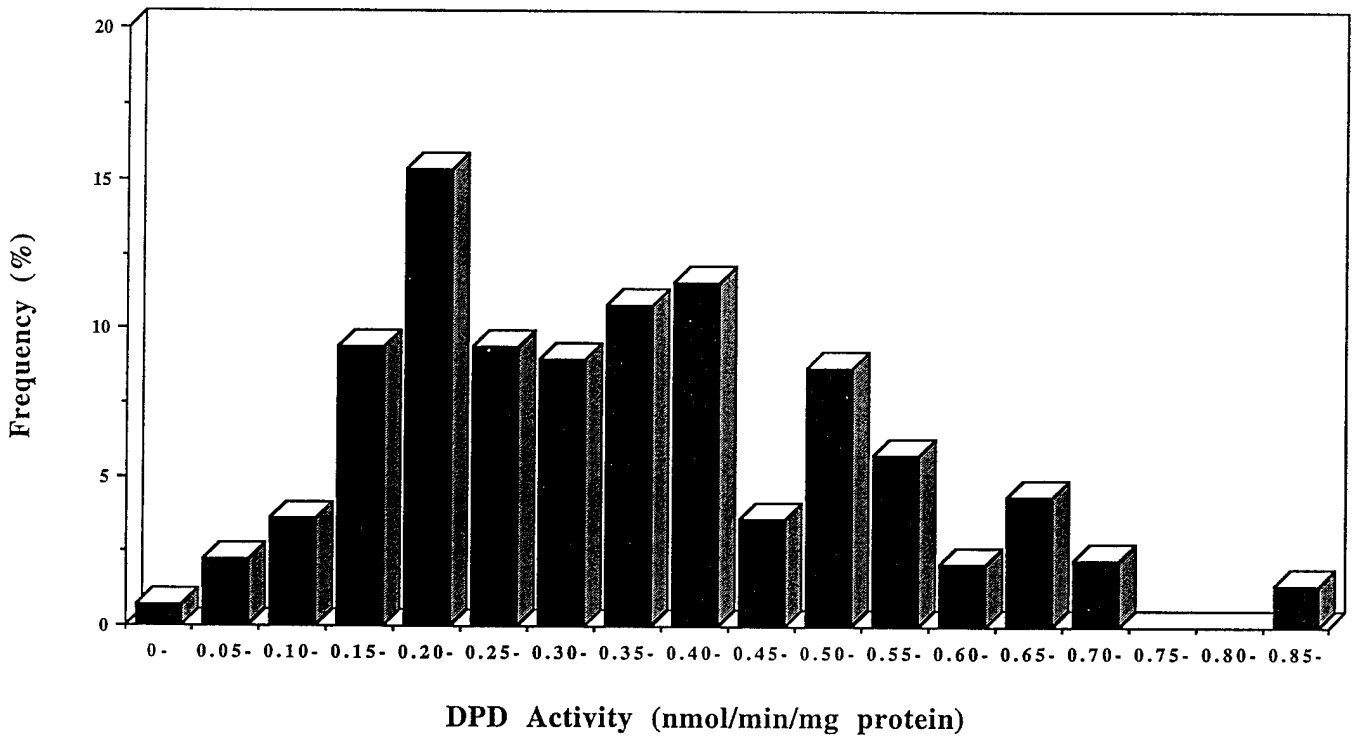

Fig.4 Population Distribution of Liver DPD Activity
Statistical analysis demonstrated that liver DPD activity follows a normal distribution (Guassian distribution).

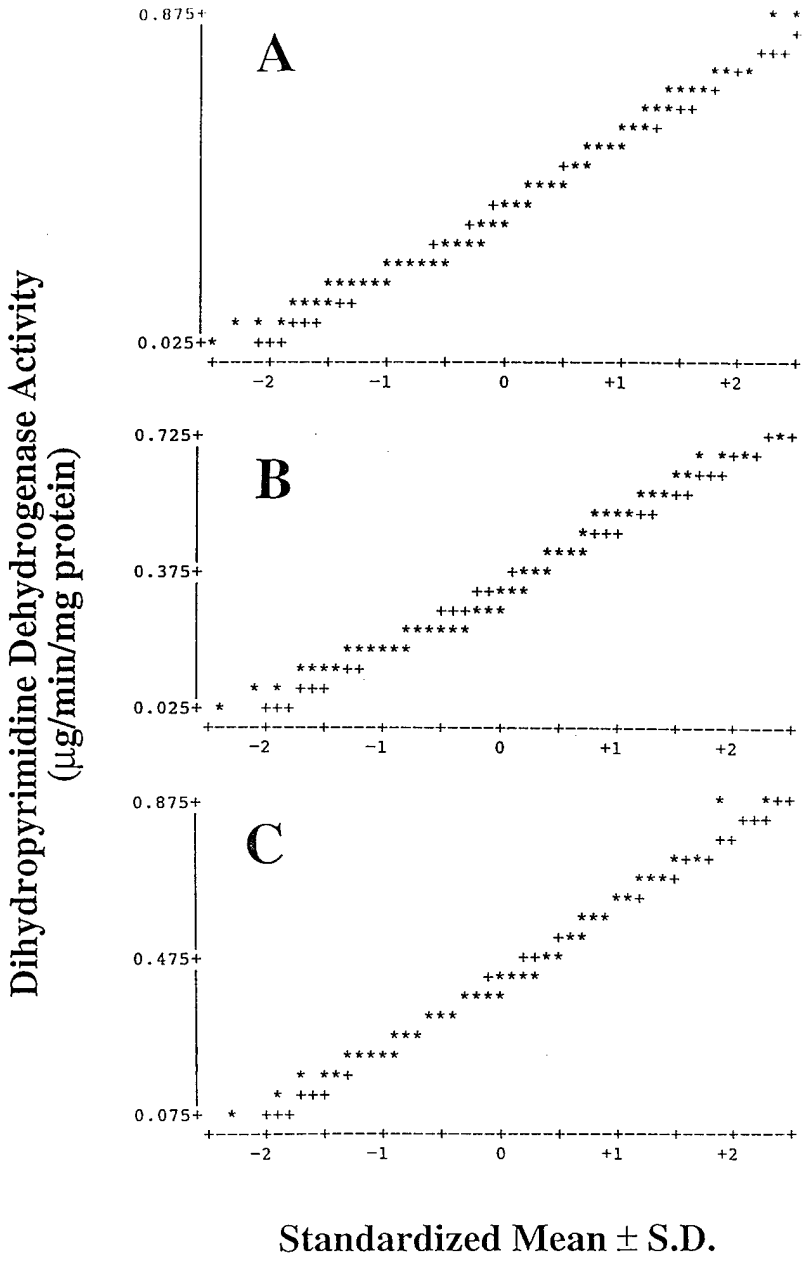

Fig. 5 Probability Testing of Distribution of Liver DPD Activity
Panel A: Total subjects; Panel B, male subjects; Panel C, Female subjects.

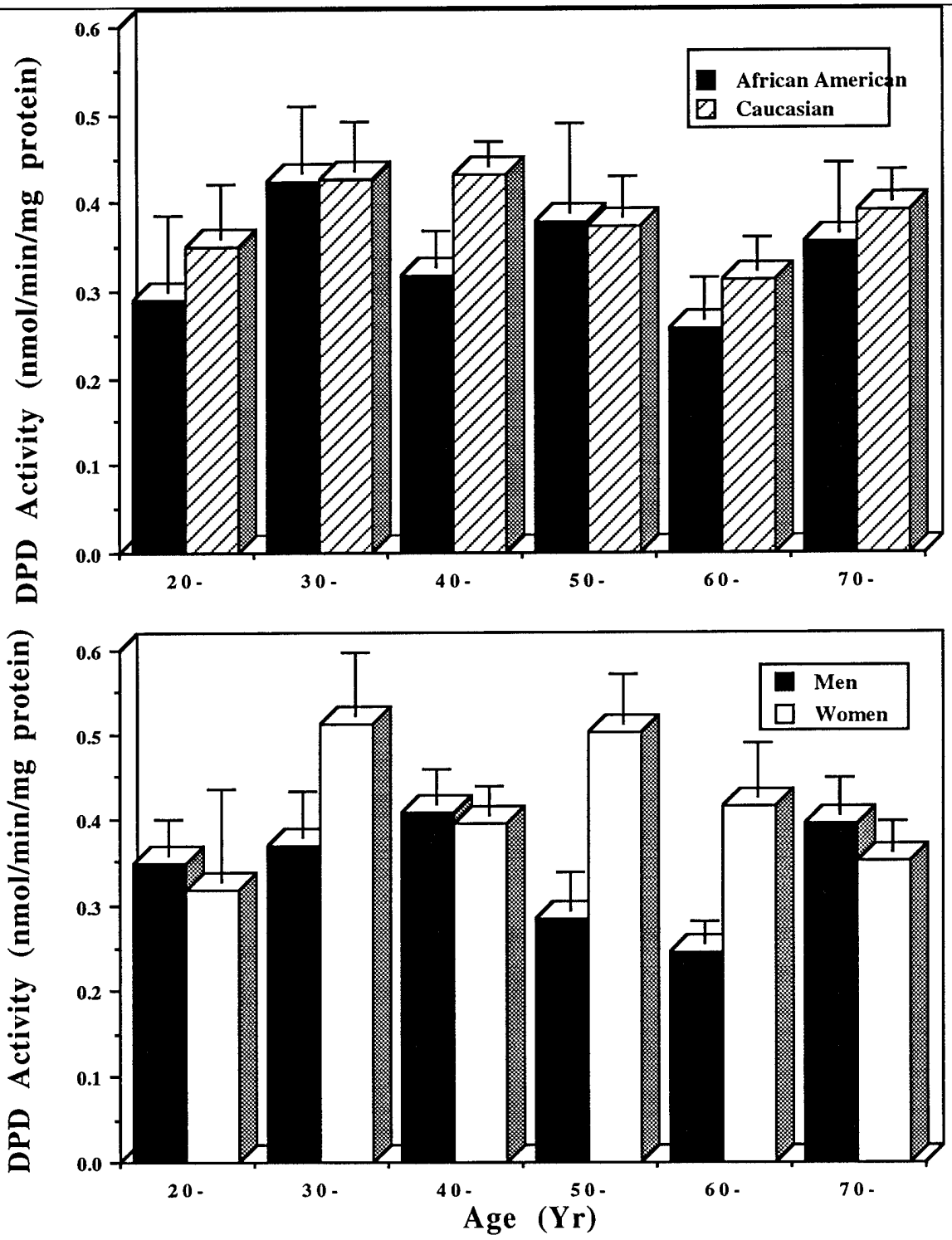

Fig. 6 Population Distribution of Liver DPD Activity by Age.
Top panel illustrates the comparison of mean (± S.E.) DPD activities between african americans and caucasians, and bottom panel presents the comparison of mean (± S.E.) DPD activities between males and females.

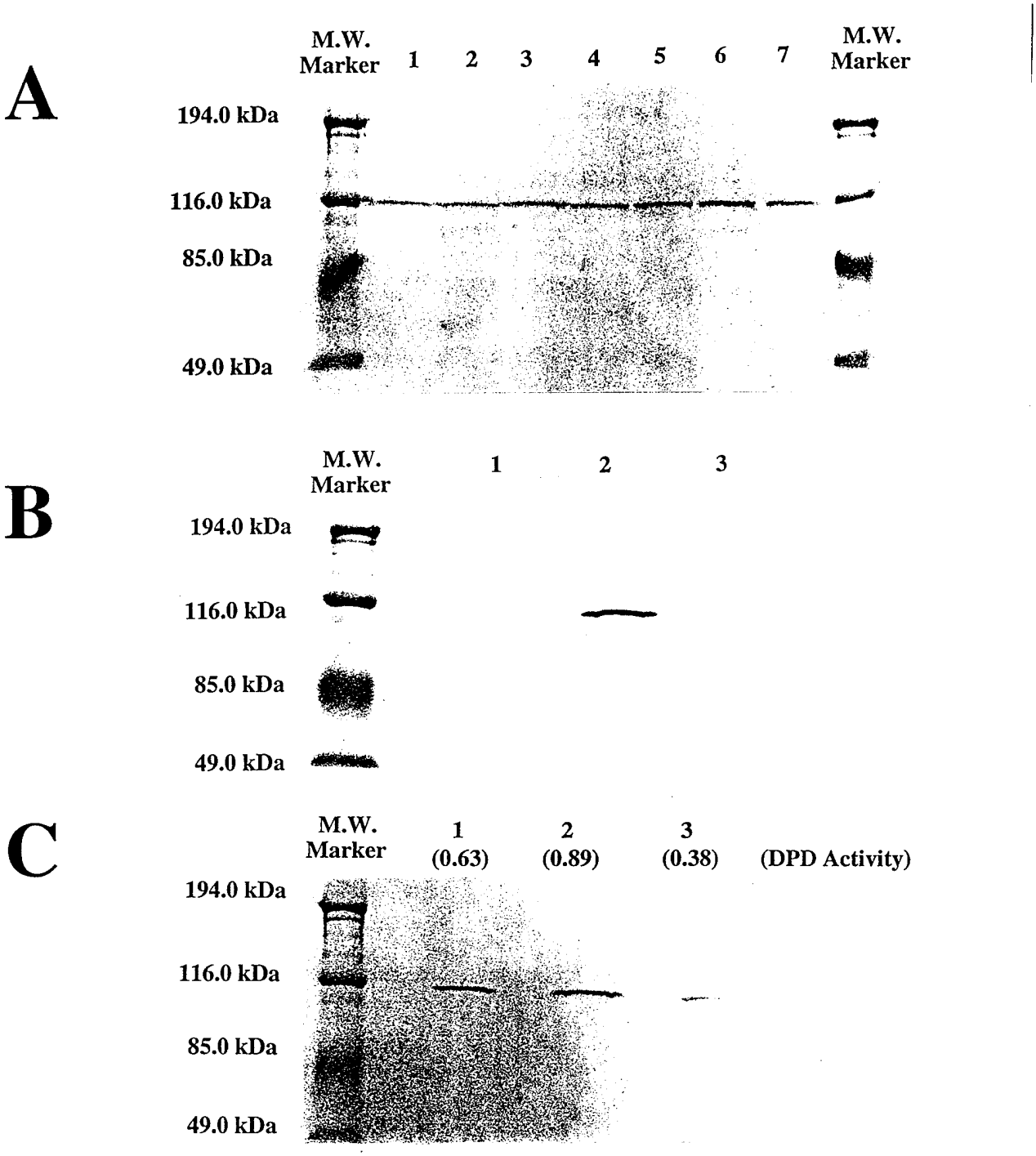

Fig. 7 Immunoblot Analysis of Liver Dihydropyrimidine Dehydrogenase
Each lane contains 200 μg of crude liver cytosol. *Panel A*: 7 representative samples with DPD activities between 0.30 and 0.55 nmol/min/mg; *Panel B:* Two samples with extremely low DPD activities (< 0.05 nmol/min/mg); *Panel C:* Three samples with DPD activities being 0.38, 0.63, and 0.89 nmol/min/mg. The correlation between the DPD activity and the DPD protein band density on western blot analysis is illustrated in Figure 8.

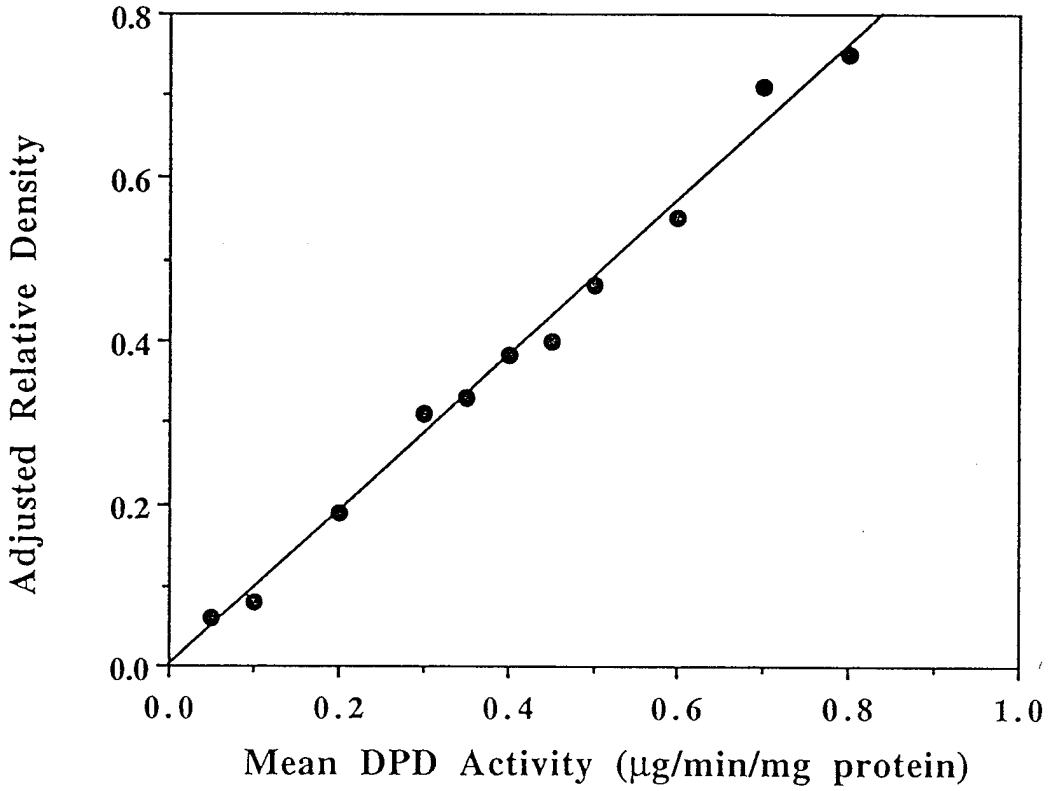

Fig. 8 Correlation Analysis of DPD Activity and DPD Protein Band Density on Immunoblot Analysis

Linear correlation analysis indicates that there is a correlation between liver DPD activity and the density of DPD protein on the immunoblot analysis (R^2=0.992, $p<0.01$).