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REVIEW ARTICLE

Congenital Deficiency versus Hereditary Persistence of Human Alpha-fetoprotein: A Meta-analysis and Overview of Potential Biomedical Consequences

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Abstract:
Human alpha-fetoprotein (AFP) is a 70 kD tumor-associated fetal protein (oncofetal protein) which has been utilized clinically as a biomarker for both multiple cancers and for fetal defects including neural tube defects and chromosomal abnormalities. Extreme low and high levels of serum AFP in conjunction with gene mutations have now been established in disorders such as congenital deficiency (CD) and hereditary persistence (HP) of AFP. The objectives of the present review are to compare the clinical manifestations and analyze the mutational basis of these two AFP genetic disorders in accordance with presently accepted models of AFP gene regulation. The regulatory elements of AFP gene expression i.e., enhancers, promoters, and silencers are described in light of the genetic alterations expressed by the CD- and HP-AFP patients. By use of structure/function peptide mapping of the full-length AFP amino acid sequence, the potential biomedical consequences exhibited by CD-AFP and HP-AFP patients are presented and discussed. A new prospective on placental dysfunction in these AFP genetic disorders has also been introduced. Using AFP reference levels in maternal serum, cord blood, newborn blood, pediatric and adult serum, AFP serum concentrations are presented that could alert the obstetrician of potential CD-AFP during pregnancy. Knowledge of such serum values could provide tools to allow clinicians to distinguish between adult HP-AFP and malignant/non-malignant diseases such as liver and germ cell tumors, cirrhosis, hepatitis, and various liver disorders. Knowledge of AFP reference levels might serve to guide the clinician in avoiding: a) misguided diagnoses; b) unnecessary treatments and therapies; and c) undue anxieties and stress during pregnant and non-pregnant states.

Keywords: *alpha-fetoprotein, genetic disorder, autosomal recessive, autosomal dominant, gene mutation, pedigree, deficiency, persistence*

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Abbreviations: *HAFP = human alpha-fetoprotein, MOM = multiple of the median, hCG = human chorionic gonadotropin, uE3 = unconjugated estriol, MS = maternal serum, AFAFP = amniotic fluid alpha-fetoprotein, MSAFP = maternal serum alpha-fetoprotein, Ache = acetylcholinesterase*

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Introduction

Human alpha-fetoprotein (HAFP) is a 69 kDa single chain tumor-associated fetal glycoprotein of the albuminoid gene

family that contains 3%-5% carbohydrate and is produced in the yolk sac, fetal liver, and gastrointestinal tract during embryo/fetal development [1, 2]. In the clinical laboratory, HAFP has been employed both as a postsurgical tumor monitoring agent and as a pregnancy fetal defect biomarker for screening neural tube defects and aneuploidies [3, 4]. HAFP produced by the fetus increases steadily in early pregnancy, peaks in fetal serum (3.0 mg/ml) at 10 weeks of gestation, and declines to 50.0 ug/ml in newborns [5] (Fig. 1A). Fetal AFP is transferred to the amniotic fluid (AF) via fetal urination, and amniotic fluid levels parallel those of fetal sera with AF-AFP concentrations albeit being 150 times lower (Fig. 1A) (Table 1). Fetal AFP is transferred to maternal serum (MS) by diffusion and active transport across the placenta and peaks in the MS circulation (150 ng/ml) at 32 to 33 weeks of gestation [5].

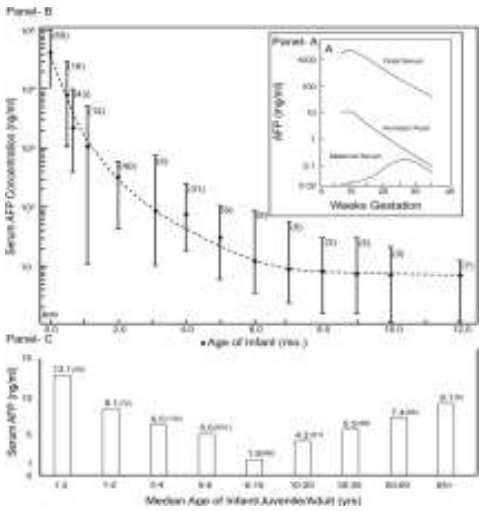

Figure 1. Reference values for alpha-fetoprotein (AFP) in fetal, newborn/infant, juvenile, and adult serum and amniotic fluid are displayed in Panels A, B, and C. *Panel A*: Serum AFP levels are plotted from birth to 12 months in normal infants. Solid black dots and bars indicate the mean and standard deviations of infant serum specimens at the months indicated and expressed as ng/ml. The number of samples (N) is indicated in the parentheses (see References [6-8]). *Panel B*: Reference curves are shown for AFP levels (ug/ml) measured in fetal serum, amniotic fluid, and maternal serum at 5 to 40 weeks of pregnancy. Data was extracted from References [4, 5, 9-13] and plotted as a composite diagram. *Panel C*: Normal serum AFP (ng/ml) levels expressed as a median at the top of each bar of the histogram is displayed. Sample size numbers (N) are indicated in parentheses for each bar of the diagram. Data was extracted and chosen as a composite diagram from References [6, 7, 14-17].

A) Pregnancy Levels: Abnormal AFP concentrations in biologic fluids during embryonic, fetal and neonatal development have been found associated with fetal defects and congenital malformations, largely as a result of genetic disease and/or teratogenic events [20, 21]. Recently, elevated concentrations of AFP during gestation were found to correlate with adverse pregnancy outcome [22, 23]. Following term pregnancy, aberrant serum levels of HAFP have been found to correlate with abnormal growth in newborns, infants, juveniles, and in adults experiencing liver dysfunctions.

Serum levels of AFP that fall outside the normal limits seen in pregnant women have been reported for a multitude of fetal congenital malformations. The first developmental abnormalities found to be associated with abnormal AFP levels were neural tube defects and related spinal disorders; later, the chromosomal abnormalities (trisomies) and various anatomical malformations were reported [9, 24]. The maternal serum AFP (MSAFP) levels associated with fetal defects are abnormally high, whereas the trisomies exhibit abnormally low levels. Although many other congenital anomalies have been described in association with elevated serum AFP, discordant AFP levels have been observed in biologic fluids such as urine, cerebrospinal fluids (CSF), placental extracts, effusions, and ascites fluids [9, 10, 18, 25] (Fig. 1). Elevated AFP serum levels have further been useful in the diagnosis of erythropoietic disorders (anemias), placental disruptions, fetal death, growth restriction/retardation, and preterm labor [23, 25-27].

B) Umbilical Cord Levels: AFP umbilical cord blood levels in normal newborns and those infants at risk with elevated AFP have been previously reported [28, 29]. AFP levels in normal cord blood specimens displayed a median value of 52 μg/ml; hence, a cutoff value of 2.5 times the median (130 μg/ml) has been proposed for elevated levels [28]. However, AFP levels in cord serum have been found dependent on multiple factors, such as gestational age, birth weight, race (ethnicity), diabetic status, mean arterial pressure, and mean venous pressure. Moreover, elevated cord blood AFP have been associated with disorders such as preeclampsia, congenital anomalies, conditions of immaturity, pregnancy-related distress, and hematologic disorders. For a more detailed list, the reader should consult [4, 5, 11, 29].

Table 1: Alpha-fetoprotein (AFP) concentrations in non-affected pregnant women compared to AFP Congenital Deficiency (CD) in pregnant women. Median weekly values are listed by gestational age in maternal sera (ng/ml) and in amniotic fluids (:g/ml). Note that patients with CD have very low or absent levels of AFP.

I. Maternal Sera:

Gestation Week	14	15	16	17	18	19	20	21
Number of observations (N)	250	430	444	392	382	368	321	331
A. Non-affected Mothers*	22.21	25.50	29.41	33.62	38.61	44.50	51.00	58.5
B. CD-AFP Mothers*	1.46	ND	ND	1.16	0.01	0.01	ND	0.01
(N)	(1)			(1)	(1)	(1)		(1)
Multiples of Median (MoM):								
A. Non-affected Mothers*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B. CD-AFP Mothers*	0.06	ND	ND	0.03	0.00	0.00	ND	0.00

*=(ng/mL)

II. Amniotic Fluid:

Gestation Week	14	15	16	17	18	19	20	21
Number of observations (N)	212	268	333	336	268	259	230	200
A. Non-affected Fetuses**	19.21	17.40	13.81	11.20	8.80	7.01	5.52	4.41
B. CD-AFP Fetuses**	0.00	ND	0.10	0.00	2.14	0.50	1.76	2.2
(N)	(1)		(1)		(1)	(1)	(1)	(1)
Multiples of Median (MoM):								
A. Non-affected Mothers**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B. CD-AFP Mothers**	0.00	ND	0.01	0.00	0.24	0.00	0.00	0.50

**=:Ug/ml; ND = Not determined. Normal non-affected pregnancy values were obtained from References [4, 5, 10, 18, 19]. CD-affected pregnancy values were obtained from References 38, 41-43.

C) Newborn Levels: It has been found that different AFP levels in the serum of the newborns decline from day of birth and can be partitioned according to three birthweight categories [5, 11, 30]. Low-birthweight and premature babies at birth displayed AFP levels approximating 100 µg/ml, while the medium-birthweight group averaged 60 µg/ml, and the high birthweight group 40 µg/ml. Following birth, AFP levels attain 20-50 µg/ml by the fourth day in all birthweight categories. When further parsed by gender, specimens in the three birthweight categories did not appear to differ significantly from one sex group to the other. Nonetheless, male specimens in all three groups have a tendency to be slightly higher [13].

D) Pediatric and Adult Levels: Levels of AFP in normal pediatric and adult patients are reported to be less than 10 ng/ml although much variation is observed. Pediatric references intervals have been established in subjects from six months to six years of age by several clinical research groups [6, 7, 14, 17] with AFP levels ranging from 4 ng/ml to 18 ng/ml. However, it should be noted that a scant few children up to two years of age display AFP serum values approaching 70 ng/ml that still fall within normal confidence intervals, reflecting the wide variation observed between individual infants [14]. Under the age of two, it is recommended that serial AFP determination be made on these children. Stable levels of AFP (2-4 ng/ml) are then attained after six years of age in disease-free children. As shown in Fig. 1C, adult AFP levels can rise very slowly as older ages are attained, but normal levels seldom rise above 9.0 ng/ml.

E) Cancer and Non-malignant Disorder Levels: Levels of serum AFP that exceed those of healthy adults (5-9 ng/ml) have been reported in patients with liver cancer and benign hepatic disorders, i.e., viral hepatitis, cirrhosis, and necrosis [15, 16] (Fig. 2). Other tumor cell types found to synthesize AFP were the testicular germ cell tumors (teratomas) and yolk sac tumors of the ovary [31, 32]. Aside from hepatoma and reproductive cancers, AFP secretion has been linked to the gastrointestinal tumors of endodermal origin, especially stomach and pancreas [33]. Less frequently, AFP synthesis has been associated with tumors of pineal/pituitary cysts, hepatoblastomas, hemangioendotheliomas, pancreatoblastomas, hepatic bile duct carcinomas, gall bladder carcinomas, epidermoid cysts, granulosa cell tumors, and others [34-36]. Combined with hCG, AFP serum levels have long been used as a diagnostic aid for the differential diagnosis of seminoma versus non-seminomatous germ cell tumors; germ cell tumors displaying elevated AFP serum levels alone have been found in the former but not in the latter cancers [37].

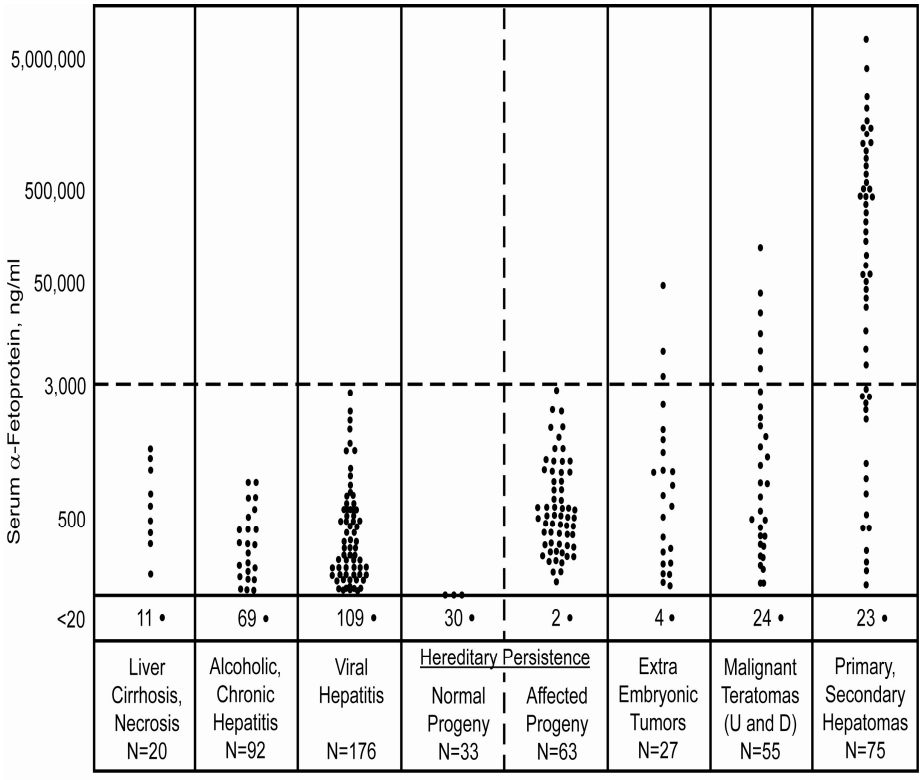

Figure 2. Serum alpha-fetoprotein (AFP) levels (ng/ml) were measured by radioimmunoassay in normal adults and in patients with liver disorders, hereditary persistence of AFP (HP-AFP), and in germ cell and liver cancers. Serum AFP levels of liver cirrhosis, alcoholic and viral hepatitis patients are compared to those with HP-AFP on the left side of the Figure, while embryonic, teratomas, and hepatoma patients sera are displayed on the right side. The total number (N) of samples assayed in each column is shown below each listed disorder. Samples less than 20 ng/ml are displayed by the number in the reticular block above the disorders. Each dot shown in a vertical column fashion indicates one individual sample. Data for the Figure were extracted and plotted from References [3, 31, 38-43].

F) Objectives: To date, there are no published reports in the biomedical literature addressing the potential clinical and biomedical consequences in young and adult patients with CD-AFP and HP-AFP. Moreover, the loss or gain of AFP function in the regulation of these genetic AFP anomalies during human ontogeny has yet to be addressed. In fact, certain AFP biological activities in this survey have not previously been related to any clinical conditions. Thus, the objectives of the present report are threefold. First, the many phenotypic and genetic traits associated with CD- and HP-AFP serum levels of AFP will be presented and surveyed. Second, the use of AFP reference levels as a biomarker of cancer and non-malignant disorders in clinical settings will be compared to those found in CD-AFP and HP-AFP patients. Third, the multitude of congenital malformations, disease states, and biologic activities ascribed to AFP in recent years will be discussed in lieu of the clinical and genetic anomalies

recorded for CD-AFP and HP-AFP patients, with a new focus on placental dysfunctions. The serum levels of these disorders, together with the biological activities of AFP are presented to alert, aid, and guide physicians in distinguishing CD-AFP and HP-AFP from other AFP-associated clinical disorders. For further detailed accounts of the structure, chemistry, and genetics of AFP, the reader is directed to earlier published reviews on AFP [4, 5, 44-46].

CONGENITAL DEFICIENCY OF ALPHA-FETOPROTEIN (CD-AFP)

The first clinical cases of CD-AFP were reported in two patients in 1992 following prenatal triple screening for NTD and Trisomy-21 (T21) [47]. These specimens were of special interest to the screening laboratory in that the samples produced T21 positive screens with the virtual absence of AFP (MSAFP MOM = 0.02-0.05). Moreover, these specimens showed normal levels of hCG and only marginally lowered uE3. Low levels of AFP and uE3 screening values, by themselves, are not indicative of a Down syndrome; however, the combination of the two lowered analytes in the triple test could result in a positive screen at risk for a T21 pregnancy. These patients had no prior histories of pregnancy complications, and the subsequent Stage-II ultrasound, acetylcholinesterase (Ache), AFAFP, and karyotyping (amniocentesis) were found to be unremarkable. At full term, these patients delivered infants with no apparent physical or developmental problems. However, both specimens displayed one unusual biomarker in that MSAFP was very low or virtually absent. As discussed below, extremely low or absent AFP levels have been associated with a rare and supposedly benign genetic condition termed congenital deficiency of AFP (CD-AFP).

The CD-AFP can be considered in cases of unexplained very low or absent maternal serum AFP in the second trimester screening for Down syndrome after a normal karyotype is obtained following amniocentesis. Although thought to be rare, the CD-AFP has now been reported in multiple case histories throughout the biomedical literature including 15 in France, four in Israel, two in the United States, and one in Algeria (see refs. [48-52]). As discussed above, the CD-AFP was first described by Greenberg et al. in 1992 in the United States following routine prenatal maternal serum screens [48]. Greenberg's team reported two infants which demonstrated low or absent AFP in maternal serum, amniotic fluid, cord blood, and in newborn dried blood spots. In both cases, healthy normal infants were delivered with good Apgar scores and normal karyotypes. At 1.0 year of age, both infants were thriving with no apparent physical or developmental abnormalities. Since then, both patients were lost to follow-up and the cause of the AFP deficiencies WAS never determined.

In 1997, Sher and Shohat published a Down syndrome (DS) screening case report which produced a borderline DS screen involving the total absence of MSAFP [52]. The DS screening assays resulted in a MSAFP MoM = 0.00, uE3 MoM = 0.63, and a hCG MoM = 1.63 producing a borderline DS negative risk of 1 in 290. Ultrasound revealed that the genotype was 47 XX + 21 which was confirmatory for Down syndrome. The authors stated that despite publication of previous cases showing that CD-AFP was a benign condition, amniocentesis should be recommended for extremely low AFP screening results. In a later case study, two patients with congenital absence of AFP from first cousin pregnancies were reported by Sharony et al. [50]. In this report, the patients' samples were studied by radioimmunoassay (RIA), immunohistochemical staining, comparative genomic hybridization (CGH), and fluorescence *in situ* hybridization (FISH) analysis. The first patient also displayed inflammatory infiltrate sites located in the placenta (deciduitis) accompanied by Monosomy-16 in trophoblast cells; however, this patient delivered a normal fetus and CD-AFP was genetically confirmed by the CGH and FISH analyses. As discussed in the concluding remarks, placental damage and dysfunction might lead to serotonin-related malformations during fetal brain development resulting in mental disturbances in children affected with AFP genetic disorders [53, 54]. Histochemically-localized AFP was also found to be present in fetal, placental and maternal tissues in this patient. In the second patient described by Sharony, the triple screen demonstrated an AFP MoM = 0.00, hCG MoM = 0.42, uE3 MoM = 0.97 and a cord blood AFP level = 0.00; however, a normal karyotype was present. This patient's test results revealed inflammatory sites in the placenta (chorioamnionitis) accompanied by a placental cell Monosomy-16. Interestingly, the two latter families were unrelated as confirmed by case history and both pregnancies resulted in normal, healthy, term newborns. A further study emerged from Sharony's group in 2004 in which they addressed the genetic etiology of CD-AFP [51]. This study represented a follow-up report of the two previous patients demonstrating an absence of MSAFP (see above); however, this study involved searching for mutations in the AFP gene itself. The group identified a mutation in which the patients' AFP gene demonstrated a frameshift at threonine-294 that led to a

stop codon at AFP glutamic acid sequence #318; this truncation resulted in the absence of the entire third domain and a portion of domain-2 of AFP (Fig. 2). Both of the affected infants were found to be homozygous for the mutation, presented a history of normal development, and were asymptomatic. Thus, the elimination of 48% of the amino acids from the AFP molecule resulted in unremarkable fetal development, a normal term delivery, and subsequent normal postnatal life; these cases serve to display reproductive capability in the males since the fathers were found to have the mutation (Fig. 3). Although CD-AFP is a rare condition, this genetic condition should be taken into account in adults being monitored for post-pregnant serum AFP levels involving surgery, especially in cases involving hepatomas and germ cell tumors (see Fig. 2).

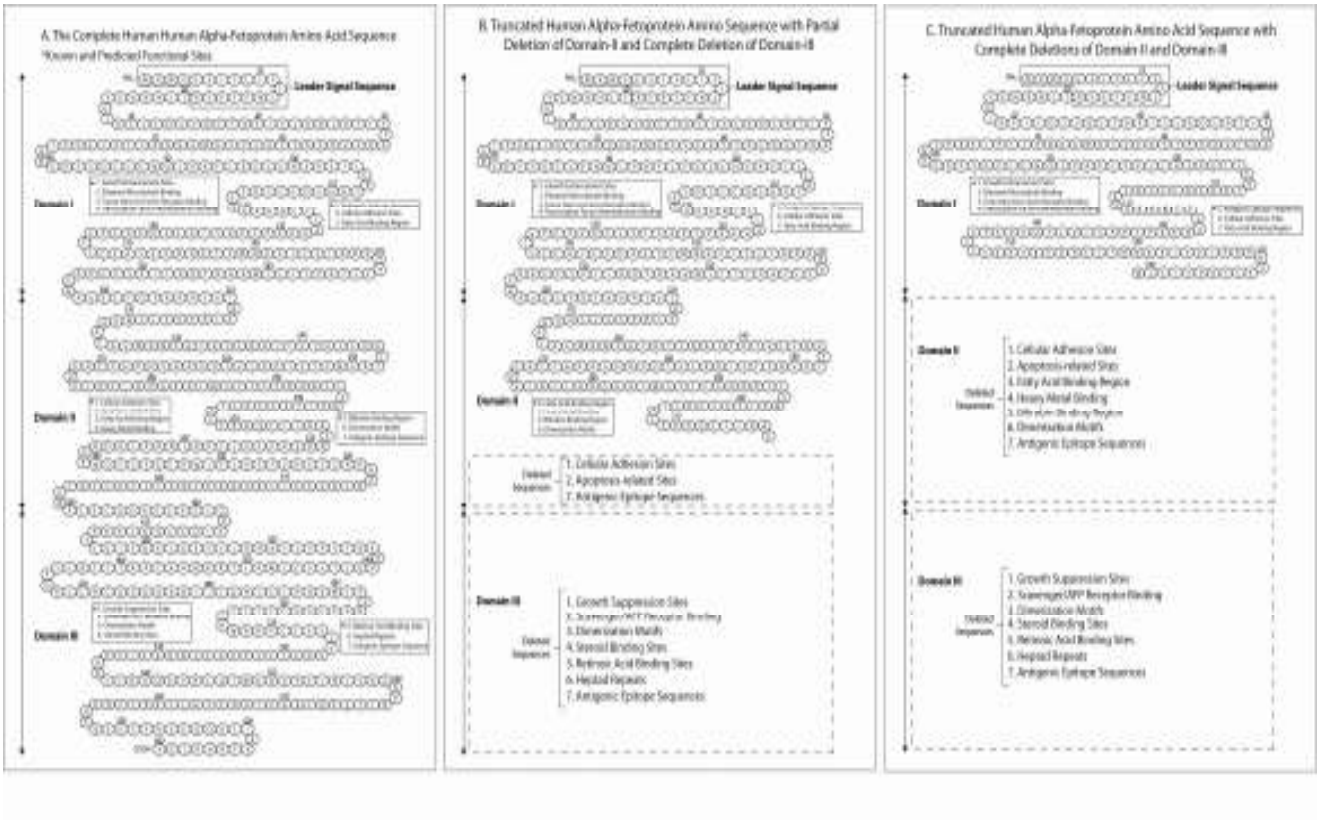

Figure 3. *Panel A*: The complete amino acid sequence of human alpha-fetoprotein (HAFP) is displayed in the single letter amino acid code. As shown, HAFP is composed of three domains determined by intramolecular loops dictated by 32 disulfide bridges. For the sake of clarity, the disulfide bridges are not shown and the reader is directed to Reference 46 for full viewing. The known and predicted functional peptide sequences are shown for each of the triplicate domains as numbered items from 1 to 7 (see Reference 37). The 19-amino acid leader sequence is displayed by the dotted-line box. *Panel B*: The truncated form of the HAFP sequence with partial deletion of Domain-II and the complete deletion of Domain-III is displayed in a patient with Congenital Deficiency of AFP (CD-AFP). Note that the molecule is truncated at Glutamic acid #318 due to the insertion of a stop codon. The potential loss of HAFP functions are indicated by the numbered functional items shown. *Panel C*: The truncated form of the HAFP sequence with the complete absence of Domain-II and Domain-III is demonstrated in another patient with CD-AFP. In this case, note that the molecule is truncated at Tryptophan #181 due to the presence of a stop codon. Again, the potential loss of HAFP biological activities is indicated by the numbered functional items displayed from 1 to 7 for each domain. The peptide structure/function sites were obtained from AFP sequence maps found in References 46, 47, and 49.

Another genetic study of CD-AFP was reported by Petit et al. in which a site mutation in the AFP gene was described following a Down syndrome presumptive positive screen result from a mother undergoing an artificially inseminated pregnancy [49]. These investigators reported a mutation in exon-5 of the AFP gene which resulted in a total absence of AFP both in maternal serum and in amniotic fluid. At 14 weeks gestation, the AFP MoM was 0.06, hCG MoM was elevated at 4.74 MoM, and amniotic fluid AFP was undetectable; however, a normal karyotype was present (46XY). Ultrasound examinations at 14, 22, and 32 weeks proved normal and a healthy infant was delivered at 37 weeks. After PCR-amplification, the AFP gene was sequenced and a new basse mutation was found. It was determined to be a guanine to adenine transition in position #543 which created a premature stop codon in the amino acid tryptophan-#181 position [49]. Thus, the resultant AFP molecule consisted of only 181 amino acids

instead of 509 which constituted nearly 90% the first domain of AFP, but eliminated domains two and three. The second mutation resulted in a frameshift at threonine-#294 followed by a stop codon at glutamic acid #318 [51]. Despite these findings, the infants demonstrated both unremarkable fetal development and normal birth. As discussed above, CD-AFP is a rare, benign trait which can be diagnosed at time of prenatal screening for Down syndrome. Low or undetectable level of AFP (CD-AFP) during gestation has an estimated frequency of 1 in 105,000 [55, 56]. In screening situations, the difficulty lies in distinguishing very low AFP values exhibited by patients at high risk for chromosomal abnormalities from that of CD-AFP. Fortunately, many clinical manifestations can be excluded by cytogenetic and biochemical investigations in conjunction with ultrasound examinations. However, CD-AFP is not detectable until the second trimester since AFP levels are not routinely assayed in the first trimester. It is of interest that CD-AFP can be compared to An-Albuminemia (A-ALB), a very rare metabolic abnormality considered to be benign in infancy but problematic during pregnancy [49]. Moreover, A-ALB is thought to be responsible for severe disorders of later pregnancy such as intrauterine growth retardation, precocious fetal loss, and intrauterine death.

In the two clinical cases of CD-AFP discussed above, analysis of the AFP gene revealed two different DNA mutations. The first mutation was found to affect a mutated amino acid in AFP Domain-I, while the other one was localized to Domain-II of the translated AFP molecule [49, 51]. The mutation affecting AFP Domain-I resulted in expression of a translated AFP consisting of 90% of Domain-I, but lacking both Domain-II and Domain-III (Fig. 3A). As shown from structure/function mapping of AFP peptide sequences [42, 57] , lack of synthesis or removal of these two AFP domains in the amino acid translated version could hypothetically deprive the AFP molecule of participation in biologic activities such as fatty acid/retinoic acid binding, hydrophobic ligand affinity, dimerization motif and extracellular matrix (ECM) sites, apoptosis, and homeodomain homology regions [57]. In comparison, the second genetic mutation resulted in a frameshift at threonine #294 that produced a stop codon at glutamic acid #318; this resulted in elimination of the entire Domain-III and 35% of Domain-II (Fig. 3). This truncated form of the AFP molecule could be proposed to be deficient in physiological properties related to ECM binding, homeodomain recognition, apoptosis, receptor binding, chemokine signaling, and dimerization partnering [57]. It has been demonstrated that AFP Domain-I constitutes the “humanized domain” of the molecule to distinguish it histocompatibly from other mammalian species [12, 58, 59], and this domain determines WHAT human-specific antibodies will be produced [60]. While Domain-II is less human specific, Domain-III is similar to many other mammalian species and readily cross-reacts immunologically with gorilla, chimpanzee, horse, dog, and others [60]. In view of these published observations, the antibody detection of a remnant-remaining Domain-I in CD-AFP would be greatly diminished due to the different tertiary conformations that such an AFP fragment might assume. Once a departure in tertiary structure of Domain-I from that of the compactly folded native AFP molecule is produced, most commercial antibodies (kits) are not capable of detecting altered peptide fragments of Domain-I and could result in the apparent clinical absence of serum AFP.

While the first AFP gene mutation was found in first Domain-I, the second one was located in Domain-II of the translated AFP molecule. This translated AFP molecule contained all of Domain-I, but lacked a portion of Domain-II and all of Domain-III. As seen in Fig. 4, removal of these two portions would have deprived the AFP molecule of functions which potentially could include extracellular matrix (ECM) binding, apoptosis regulating regions, fatty acid (FA) retinoic acid (RA), and hydrophobic binding sites, dimerization sequences, homeodomain (Notch, HOX, Wnt, Crumbs, Pou) homology motif expression, and receptor binding sites [57]. This truncated form of the AFP molecule would only retain a portion of the ECM binding sequences, homeodomain motifs, loss of FA/RA binding sites, dimerization sequences, apoptosis induction regions, growth factor receptor sites, and chemokine-like sequences. For a complete mapping of the AFP structure/function peptide sequences, the reader is directed to a published HAFP atlas [57].

Due to the high level of expression during embryo/fetal development, it was previously assumed that AFP was essential throughout the course of mammalian development. However, the above clinical cases would argue that AFP is not required for the successful completion of pregnancy and for the birth of a viable newborn. Interestingly, the development of an AFP gene knockout mouse model is consistent with the present clinical observations in that AFP does not appear essential for development during pregnancy [55]. The AFP-gene knockout study in mice showed that neither the embryos/fetuses nor the maternal placental tissues were dependent on AFP for the successful completion of pregnancy and a full term birth. Indeed, the mutant

homozygous adult male mice were both viable and fertile as were the fathers in the present human clinical studies. It is noteworthy that the rodent AFP-null female mice were infertile due to subsequently-discovered dysfunctions in the hypothalamic/pituitary axis due to the absence of AFP, resulting in an anovulatory state [55, 56]. This lack of ovulation result was predictable because it had been mimicked in previous studies using anti-AFP antibodies injected into the brains of newborn mice [61, 62]. However, examination of the pedigree infertility history from females in the presently affected families has not yet been determined; so to our knowledge, the females in these studies have not yet reached the age of fertility (maturity) to compare it to the rodent model. Similar to the hereditary persistence of AFP (discussed below), CD-AFP is presently considered to be a benign condition because anatomic deformities are not observed at term even though pregnant women still experience pregnancy complications. In view of these clinical observations, the benign state of the CD-AFP disorder should be placed in question (see Concluding Remarks).

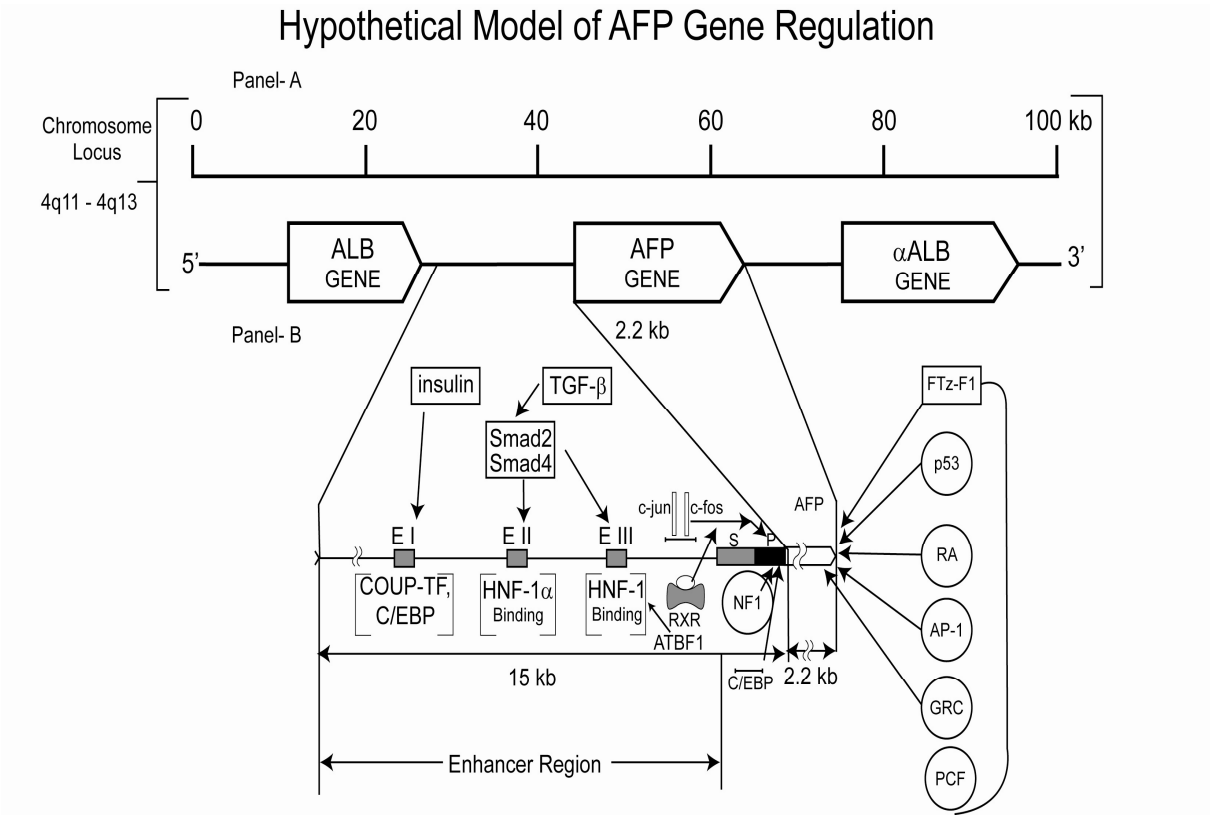

Figure 4. A hypothetical model of human alpha-fetoprotein gene regulation is displayed in the Figure. *Panel A*: The human genes of the albuminoid family consisting of the albumin (ALB) gene, alpha-fetoprotein (AFP) gene, and alpha-albumin (αALB) gene are located in a tandem arrangement on chromosome locus 4q11-4q13 within an approximate 80 kilobase (kb) segment. While the ALB gene is located at the 5' region, the αALB gene has been localized at the 3' region of this site with the AFP gene in the middle. *Panel B*: In an expanded telescopic view, the AFP gene regulatory region structure has been depicted. This region consists of the promoter (P), silencer (S) and the E1 to E111 minimal enhancer sites of the AFP gene. The transcription hierarchy of the hepatocyte nuclear factors (HNF-1β, HNF-1α, and HNF-1) which bind to the enhancer sites are depicted. The AFP gene promoter regulatory sites can be modulated by c-Jun/c-Fos (AP-1), C/EBP, RXR transcription factors, and GRC. The transcription factors participating in the regulation of AFP gene expression are p53, RA, NF-1, AP-1, PCF, and FTz-F1. Transcription factor abbreviations are as follows: GRC = glucocorticoid; RA = retinoic acid; AP-1 = activator Protein-1 composed of c-Fos and c-Jun transcription factors; PCF = promoter-linked coupling element; C/EBP – Ccaats-enhancer binding protein; NF-1 = non-tissue-specific factor; p53 = tumor suppressor protein that regulates cell cycle, FTz-F1 = AFP transcription factor; SMAD-2 = transcriptional modulators that are mediated by transforming growth factor-beta activity; COUP-TF = chicken ovalbumin upstream promoter-transcription factor; RXR = retinoic-X receptor; ATBF1 = AFP transcription (enhancer) binding factor. Data for the figure were obtained from References 70-76.

Comments regarding the CD of AFP in Amniotic fluid (AF) are worthy of further discussion. Laboratories which report low or absent AFP might respond by further actions or seek additional pursuits. Examples of such pursuits might be: 1) determine if an AFP specimen may have been urine; 2) suggest Stage II diagnostic ultrasound; 3) seek whether the result is consistent with an ongoing pregnancy; 4) perform a Fern test (see below); 5) determine if CD-AFP is present in family members; and 6) analyze for an AFP gene mutation. The verification that the specimen was an amniotic fluid sample instead of urine is ascertained by performing a total protein quantitation (Bradford) test on the sample. Amniotic fluid displays a total protein value of 5-8 mg/ml, while urine contains little or no protein unless proteinuria is present (detectable with a dipstick bromophenol-blue test). Some labs recommended a Fern test be employed which can be used in two fashions; first, it can be used to detect leakage of amniotic fluid from the placental membranes; and second, it is an indicator of elevated estrogenic activity in cervical mucus smears indicating that ovulation has occurred. Due to inconclusive results, some labs may elect not to provide the physician with an AF MoM or its interpretation until a confirmation of CP-AFP is established.

In summary, it can be seen from the preceding paragraphs that the Down syndrome (DS) positive screen from a CD-AFP women can potentially produce a false positive DS result. A suspected CD-AFP with an actual DS screen outcome was published by Sher and Shohat; however, that case did result in a confirmed positive screen for Down syndrome [52]. By analysis of the triple test result, it was evident that the MoM screening results predicted DS as follows: AFP MoM = 0.0, uE3 MoM = 0.63, and hCG MoM = 1.63. Unlike CD-AFP cases found in the literature [49, 50], the Sher and Shohat patient case report was a positive screen Down's patient as confirmed by karyotyping. Thus, a CD-AFP is one condition that should be ruled out when a very low or absent AFP screen result is encountered following triple or quad assay for Down syndrome. Nonetheless, when MSAFP is very low or absent, an amniocentesis should be offered to patients despite a normal screen result.

HEREDITARY PERSISTENCE OF ALPHA-FETOPROTEIN (HP-AFP)

When a sample is screen positive for NTD with an unexplained elevated MSAFP but is not later confirmed, the specimen is of special interest to the screening laboratory. The follow-up actions for such specimens are the following: genetic counseling, ultrasound, amniocentesis, and repeat testing of the sample. If such specimens have an unexplained elevated MSAFP but lack an abnormal Ache and/or fetal hemoglobin result from AF analysis, such results are problematic for screening laboratory follow-up. Such pregnant women are referred to a tertiary care medical center for follow-up and review of family history to determine possible endocrine reproductive problems such as accelerated body growth, advanced bone growth, and precocious puberty; such studies might also include physician consultation with immediate family members. If other family members also exhibit elevated AFP levels, the patient could be a candidate for Hereditary Persistence of AFP, an autosomal genetic disorder. When amniocentesis and ultrasound results in such patients show a normal karyotype and unremarkable level-II diagnostic sonography for NTD or other structural or anatomic anomalies, the course of action for such patients could be as a presumptive positive case for HP-AFP. Due to the advanced body/bone growth and early puberty in some HP-AFP women, an endocrinologist might suggest that other family members undergo a serum test for AFP and possibly other reproductive biomarkers. Serum elevated adult AFP levels have been found in multiple family members spanning over three generations following pedigree blood testing; such patients (called probands) can subsequently be diagnosed as Hereditary Persistence of AFP (HP-AFP).

Unexplained elevated levels of MSAFP in prenatal screening programs without a confirmed diagnosis of NTD causes a concern for the prenatal screening laboratory. Patients having such screen results might involve a rare benign autosomal dominant trait with complete penetrance exemplified by HP-AFP. The first clinical case of HP-AFP was described in 1983 from a prenatal screening program for spina bifida [63], [64]. A 38 year old Caucasian woman in her 21st week of pregnancy displayed unexplained highly elevated levels of MSAFP. Ultrasound revealed a normal singleton fetus at the correct gestational age together with normal and amniotic fluid AFP levels. The full-term newborn baby was anatomically unremarkable at time of delivery with no visible malformations or clinical disabilities. Subsequently, 70 family members of this patient were serum tested and 21 members (including nine males) were found to have elevated levels of serum AFP in adulthood. Since that time, nineteen other families have been identified in the biomedical literature, some cases of which displayed testicular abnormalities in the males. It is of interest that females frequently displayed various reproductive endocrine problems. The inherited trait can be present in

both male and female descendents within a given family line, however, females seldom demonstrate life-threatening clinical problems. Both males and females have been reported to be reproductively fertile. Elevated AFP levels in adults without HP-AFP may be present in a variety of conditions other than pregnancy which include liver and germ cell disorders, cirrhosis, hepatitis, malignancies, anemias, immunodeficiencies, and ataxia telangiectasias [8, 40, 43] (Fig. 2). Since HP-AFP can be a cause of persistently elevated AFP levels into adulthood, the condition can be discerned by the testing of serum AFP in first and second generation family members followed by molecular DNA studies to determine the genetic basis of the trait. However, adult elevated AFP levels may be difficult to distinguish in HP-AFP patients already being blood tested for malignancies, liver dysfunctions, and pregnancy (Fig. 2).

In the adult non-pregnant population, AFP has served as a biomarker for hepatocellular carcinomas, hepatitis, cirrhosis, liver dysfunction, and non-seminomatous germ cell tumors [39, 41, 65, 66]. Serial levels of serum AFP are monitored following surgical tumor ablation and these usually recede to normal adult AFP levels which are less than 10 ng/ml. Patients with non-receding AFP values are presumed to have recurrent or persistent hepatic disease and are usually treated with chemotherapy at least in cases of hepatoma. This standard of care, however, does not always take into account the presence of HP-AFP, which could manifest as a continuously elevated serum AFP levels in serial determinations. Such a case was reported in a 20 month old male child (AFP = 2,659 ng/ml) who underwent surgery for a testicular yolk sac tumor and needlessly received multiple rounds of chemotherapy [39, 67]. The authors of this report proposed that, in retrospect, levels of AFP in family members should be assayed before deciding on chemotherapy based on mildly or moderately elevated AFP in the patient. The AFP blood test is a relatively simple and inexpensive test that could avoid unnecessary exposure to potentially toxic treatments. Several other cases of males with testicular seminoma [68], benign testicular cyst [69], testicular pain [70] and metastasizing seminoma cases have been described [70, 71]. In other instances, persistently elevated AFP levels were found in an apparently healthy 43 year old man and subsequently found in three of his first degree relatives; these included two siblings, and one daughter of reproductive age [72]. In a third case, a 39 year old man with a testicular fibroid nodule demonstrated HP-AFP as did 5 of 13 relatives within a three generational span [73]. The reported adult AFP levels in these cases ranged from 18 to 198 ng/ml with no overt disease or functional abnormalities. Finally, HP-AFP was reported in a 42 year old man (AFP = 43 ng/ml) undergoing removal of a testicular tumor stage 1 Seminoma [69]. AFP levels in his mother and sister ranged from 32 to 65 ng/ml. AFP is elevated in cases of non-seminoma tumor, but not in seminoma tumors and, in such instances, HP-AFP should be ruled out prior to the diagnosis and treatment for the presence of presumptive testicular tumors.

By the use of lectins such as Concanavalin-A (Con-A), the tissue origin of AFP can be determined due to the structure of the sugar chain on AFP; this can be used to rule out liver involvement. On Con-A affinity columns, the AFP eluent exhibits “binding” and “non-binding” fractions. If the fetal protein originates from a germ cell or gastrointestinal (GI) organs, a large non-binding form of AFP is observed. However, if the majority of the eluent is a Con-A binding fraction, this indicates that AFP originated from dysfunctional liver or a hepatoma. In effect, the Con-A binding test can be used to differentiate between yolk sac (GI) AND liver tumors [74]. In the mothers and adult sisters of AFP lectin-tested male patients with testicular dysfunction and germ cell tumor, the non-bound serum AFP values can range from 32 to 65 ng/ml.

AFP belongs to the albuminoid gene family and all members are tandemly linked in the 4q subcentromeric region of the same DNA-strand which maps to the locus 4q11–4q13 [75] (Fig. 4). The human AFP gene is about 2.2 kilobase pairs long and contains 15 exons and 14 inrons [76]. In experimental animal models and human clinical studies, it has been demonstrated that AFP gene expression is regulated largely at the transcriptional level in which a regulatory region resides upstream on the 5’ side of the AFP gene (Fig. 4). Within this region, a tissue-specific promoter, three independent enhancers, and a gene silencer region have been localized [77]. All three enhancers of the AFP gene exert an additive action on the promoter. The gene silencer seems to be responsible for the decrease in post-pregnancy AFP gene expression in adult liver as reported in man and other mammals [78]. A multitude of tissue-specific transcription factors, including hepatocyte nuclear factors (HNFs), retinoic acid (RA), non-specific tissue factor (NF-1), AP-1 (jun/fos), Ftz-Fl (FTF), and others have been shown to bind to the AFP gene enhancer and promoter elements [77]. However, the mechanisms determining regulation of the AFP gene at the synthesis level during ontogenesis and carcinogenesis have yet to be fully explained. Finally, little is known concerning the negative regulators of gene expression,

other than glucocorticoid hormone-receptor complex in non-hepatic organs and in adult liver [79, 80].

A gene sequence analysis in the five-prime flanking region of the AFP gene was discovered in a family with HP-AFP which revealed a mutation in a gene expression enhancer element (G-to-A transition at position -119) responsive to the binding of the Hepatocyte Nuclear Factor-1 (HNF-1) [81]. In a competitive gel retardation assay, the mutant sequence was found to bind HNF-1 more tightly than did the wild type base pairs. They also found that 5'-flanking sequences of the human AFP gene containing the G-to-A substitution directed a higher level of CAAT expression in transfected human hepatoma cells than did the wild type sequences. Their results served to emphasize the importance of the HNF-1 binding site of DNA in the developmental regulation of the human AFP gene [82].

A second case report of HP-AFP in a Spanish family concerned a 48 year old woman suffering from asthenia (fatigue and weakness) that also included HP-AFP in 8 of 16 family members over three generations [83, 84]. Molecular genetic analysis from that woman and the affected family members revealed the classical G to A substitution at the revised position -119 of the 5'- flanking region and this mutation was absent in all members showing normal AFP levels. The AFP serum levels in the first generation family members ranged from 364 to 881 ng/ml, while those of the next generation were 240 to 583 ng/ml. Further molecular studies of HP-AFP from various ethnic groups involved a family from Bengali (India) and one of Italian descendents [85]. The Bengali family showed a reported distal mutated promoter G to A substitution, while the Italian members exhibited a C to A (-55) and a C to T (-65) mutation toward the proximal HNF-1 binding region of the promoter. However, gel shift and transfection experiments failed to show any biological effect of the latter HNF-1 substitution associated with the C to A mutation. Thus, at least two and possibly three different mutations present in the HNF-1 binding sites of the AFP gene promoter were shown to result in HP-AFP.

In contrast to the above discussion, a large Taiwanese family demonstrating the HP-AFP trait did not exhibit the single nucleotide polymorphism (SNP) of previous studies involving the AFP promoter G to A (-119) mutation. Seven members of this Asian family with the HP-AFP trait also failed to show changes in the AFP promoter of the C to A and C to T sequences previously reported [84]. This observation remains unexplained at present. However, a further HP-AFP study of two other unrelated Japanese families did exhibit the cannonical G to A substitution at nucleotide -119 in the HNF-1 binding site of the AFP promoter [86]. In addition to these clinical cases, the latter investigators showed that the nucleotide substitution in the AFP promoter significantly stimulated its transcriptional activity in cultured human hepatoma cells and in adult mouse liver cells *in vivo*. Overexpression of HNF-1 stimulated wild type and various AFP promoters in both liver tumor and non-tumor cells, and their activation was both up- and down-regulated by nuclear factor-1 (NF-1) overexpressions. It was found that the HNF-1 binding site stimulation led to induction of AFP gene expression in adult liver cell gene regulation, while NF-1 regulated transcription of the AFP gene only during liver development. Following these reports, a procedure has been developed and reported for the rapid determination of the AFP gene promoter mutations in HP-AFP patients [85].

In summation, it has been shown that persistently unexplained elevated AFP levels in pregnancy and in normal adulthood could be suspect for the presence of the HP-AFP disorder. Even though further HP-AFP cases have yet to be reported in prenatal screening programs for NTD and Trisomy-21, this trait may have long gone undetected in pregnancies experiencing "unexplained MSAFP levels" due to the omission of familial (sibling) testing. Since the first case was described in 1983, many adult HP-AFP affected female family members of child-bearing age have been identified [87]. It is reasonable to assume that some positive NTD screens of unexplained etiology may have gone undetected because family members of first and second degree generations had not undergone AFP serum sibling testing and/or AFP promoter mutation DNA analysis. Determinations of AFP-levels in suspected HP-AFP could prevent unnecessary anxiety in pregnant women and inappropriate clinical treatments and decisions especially in males with reproductive and urological disorders and in females with growth and puberty dysfunctions (see Houwert for Review [64]).

CONCLUDING REMARKS

It is implicit in the preceding treatise that AFP gene mutations introducing stop codons to the AFP gene led to CD-AFP in pregnancy, while mutations involving HNF-1 enhancer binding to the gene promoter site led to high levels of AFP gene expression in HP-AFP of adults. Thus, both CD-AFP and HP-AFP exhibit altered genetic mutation as the basis for these clinical manifestations. The CD-AFP in patients is supposedly a benign condition that would likely remain undetected in non-pregnant adults and is detected only in pregnant females having undergone a

prenatal NTD/Down syndrome screen; this is because the human AFP gene is down regulated (silenced) in adult life to exhibit vanishingly low serum levels (<5 ng/ml). Patients could be compromised if adults with the CD-AFP condition required post-surgical tumor monitoring of AFP as a marker for various cancers and hepatic disorders. As discussed above, molecular DNA analysis of the AFP gene can be pursued by assays such as CGH and FISH analysis.

In the case of HP-AFP, pregnant women might display unexplained elevated levels of MSAFP producing a screen positive result in NTD prenatal screening programs. In a worst case scenario, serum levels in adult males with HP-AFP could mimic clinical conditions of germ cell cancer and testicular dysfunction, while non-pregnant women might exhibit endocrine dysfunctions such as exaggerated bone growth and height stature and precocious puberty. Without family pedigree measurement of AFP serum levels, such clinical findings could lead to unexplained and persistent elevation of AFP which might initiate inappropriate exploratory surgery, erroneous clinical decisions, misdiagnosis, and unnecessary and unjustified clinical treatments. In at least one clinical case discussed above, unnecessary chemotherapy was administered prematurely to treat cancers of the liver and/or testes. If unexplained elevated AFP serum levels can be serially documented in a patient, the following options are available: a) serum AFP levels can be tested in multiple family members; b) active liver inflammation be ruled out, c) transaminases and liver enzyme profiles can be measured; and d) malignant tumor growth determination be made by various imaging techniques, biopsy, and biomarker serum testing. If elevated AFP levels are confirmed spanning two generations of family members, molecular DNA analysis could be pursued by several methods including: a) gene sequencing; b) restriction fragment length polymorphism determinations; c) competitive gel retardation analysis; and d) CD-AFP PCR exon amplification; and e) AFP gene restriction analysis. In spite of the apparent normal pregnancy outcomes, the benign condition of CD-AFP in patients should be ruled out. Such is necessary due to the occurrence of maternal family case histories of seemingly minor medical disorders and complications reported in past and present pregnancies in the proband patient. Such clinical complications reported in several families have included pre-eclampsia, induced labor, newborn low birthweight, low AFP and albumin levels, low serum total protein, and abnormal heart rhythm at birth (Table 2). Placental malformations/anomalies from the patients have encompassed the presence of small, thick placental discs, plasma cell deciduitis, Monosomy-16, lymphohistocytic fibrosing villitis, eye problems including myopia (short-sightedness), strabismus (non-parallel eye formation), and epilepsy (Table 2).

It should be noted that the clinical manifestations exhibited by CD- and HP-AFP patients may not be as benign when viewed in the totality of all family members (Table 2). Although many of the effects observed in CD-AFP patients are found during fetal and placental development, such defects could affect the perinate, the newborn, and infant and/or juvenile children. For example, cardiac abnormalities as seen in Table 2 are often observed in infants delivered from pregnancies associated with chromosomal abnormalities such as Down syndrome, which displays low MSAFP levels [88, 89]. The eye anomalies observed in infants with CD-AFP may be related to a previous report showing AFP involvement with fetal eye development [90], while recent studies have revealed high AFP levels present in normal human fetal vitreous humor fluids [91, 92]. Chromosomal abnormalities might also be involved with the presence of Monosomy-16 in trophoblast cells of the placenta (but not the fetus) found in two of the present case studies described in this review [74, 75]. Placental inflammation, damage, dysfunction and chromosomal abnormalities reported for patients in the present review may be involved in the etiology of clinical consequences not previously addressed. It was recently reported that placental-derived (not maternal) serotonin is required for frontal lobe fetal human brain development [54]. Before week 20 of pregnancy, the lack of placental-derived serotonin due to inflammation or genetic mutations may cause the fetus to establish altered neural networks that could produce susceptibility to schizophrenia, bipolar, and autism disorders [53]. In other instances, the cellular interactions between cells of the innate immune system (i.e., NK cells) and alpha-fetoprotein have long been recognized [58, 93-95], while the effect of AFP on placental cell growth and proliferation is also well-documented [96]. Several CD-AFP patients were reported to exhibit placental disorders such as lymphoreticular and histocytic infiltration, villitis, and deciduitis. Since AFP has been confirmed to be a regulator of the immune response and to influence placental growth [95, 96], an involvement of AFP in the host (mother) to homograft (fetus) relationship can be considered. It has been proposed that AFP could be at least one factor that allows the fetus to grow as a homograft in the uterine tissues in what has been described as a “controlled state of inflammation” and the induction of an immunologically privileged site [4].

Table 2: Clinical manifestations and outcomes exhibited among various patients with Congenital Deficiency of Alpha-fetoprotein (CD-AFP) or Hereditary Persistence of Alpha-fetoprotein (HP-AFP). It should be noted that these manifestations are found spread over multiple family members and these disorders may not be as benign as once believed.

I. CD-AFP	II. HP-AFP
1. Fetal/Maternal Effects	**1. Juvenile/Adult Effects**
a) Lifelong low AFP levels b) Preeclampsia, Induced labor c) Low birthweight d) Lifelong low albumin and Total serum protein levels e) Transient abnormal heart rhythm f) Myopia (short-sightedness) g) Strabismus (non-parallel eye formation) h) Presence of epilepsy in one patient	a) Lifelong high AFP levels b) Tall stature, height c) Increased body weight d) Advanced bone age e) Accelerated body growth f) Reproductive-associated disorders g) Acute epididymitis h) Premature puberty (females) i) Non-liver (germ cell) malignancies
2. Placental Effects	**2. Testicular Effects**
a) Trophoblast Cell Monosomy-16 b) Small, thick placental discs c) Plasma cell deciduitis of placenta d) Lympho-histiocytic fibrosing villitis e) Sparse, nucleated RBCs in a small placenta f) Chorioamnionitis	a) Benign testicular disease b) Testicular pain, cysts c) Testicular nocules d) Testicular tumors (teratomas, yolk sac) e) Metastatic seminomas
3. AFP Gene Mutation Effects	**3. AFP Gene Mutation Effects**
a) Frameshift at amino acid Thr-294 followed by 24 irrelevant amino acids and a stop codon at Glu-318 b) Frameshift with stop codon at amino acid Trp-181 c) Reduction in AFP mRNA stability, with mRNA probably degraded	a) G to A (-119) substitution in HNF-1 distal binding site in AFP gene promoter (causes increased binding) b) C to A (-55) substitution in HNF-1α proximal binding site in AFP gene promoter c) C to T (-65) mutation in HNF-β binding site creating a CCAAT box, a target for enhancer binding protein

Regarding HP-AFP, a neonatal/childhood disorder termed Beckwith Weideman Syndrome (BWS) exhibits high AFP serum levels and an organ/tissue overgrowth condition termed macrosomia [97, 98]. Although many of these infants do not have cancer at presentation, many are prone to develop Wilm's tumor and other childhood cancers such as hepatoblastomas, neuroblastomas, and rhabdomyosarcomas. Furthermore, the BWS child can present with overgrowth disorders resulting in macroglossia, gigantism, congenital abdominal wall defects, oversized adrenal glands, and neonatal hypoglycemia. Elevated AFP levels have also been reported in a condition called Simpson-Golabi-Behmel syndrome, which is an x-linked condition with pre- and postnatal overgrowth, characteristic facies, visceral and skeletal anomalies, and a marked macrosoma [99]. In reproductive disorders of HP-AFP patients, AFP has also been reported to be involved in regulation of the prepubertal reproductive tract, and in particular, genital function [100, 101]. During the neonatal/juvenile period in mammals, AFP has been shown to participate in the onset of puberty, oocyte proliferation, ovulation, and regulation of the sex cycle and the ovarian-hypophysis feedback axis [102-106]. In association with testicular function, AFP levels in infants have been reported to modulate activities such as spermatogenesis, development of cryptorchidism, seminal vesicle/epididymal function, and the growth and progression of teratomas, testicular,

and germ cell malignancies [107-110]. Indeed, some of the HP-AFP patients in the present report were found to exhibit testicular pain, cysts, nodules, tumors, and male sex accessory gland inflammatory states (Table 2). Therefore, even though the clinical disorders seen in CD-AFP and HP-AFP patients are presently considered to be benign, physicians and healthcare providers should be aware of the potential for collateral health complications and side effects that may affect the well-being in such patients. Finally, due to the potential for placental dysfunction regarding brain neurotransmitter synthesis, transport, and passage in AFP genetic disorders, clinicians should be aware of the potential for mental health problems relating to anxiety, depression, and stress disorders.

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