

Persistent α -Fetoprotein Elevation in Healthy Adults and Mutational Analysis of α -Fetoprotein Promoter, Enhancer, and Silencer Regions

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Background/Aims: α -Fetoprotein (AFP) is normally <10 ng/mL in adults without malignancy or liver regeneration. However, hereditary or nonhereditary persistence of AFP in healthy adults may be encountered in clinical practice. This study describes four cases of persistent AFP elevation in healthy adults and investigates mutations in key transcription regulatory regions of the AFP gene as potential drivers of AFP overexpression. **Methods:** Four healthy adults with persistently elevated AFP levels (12.1 to 186.1 ng/mL) for >1 year, and 20 controls with low AFP levels (<0.61 to 2.9 ng/mL) were included in the study. AFP levels were collected from the families of two of the patients. We sequenced five regions that are critical for AFP expression: a promoter, two enhancers, and two silencers. **Results:** One of the two cases in which family information was represented is the first case of hereditary persistence of AFP in South Korea. Mutations related to AFP overexpression were not found in the transcription regulatory regions among the four patients. **Conclusions:** Persistent AFP elevation is a heterogeneous condition with or without a hereditary pattern and may be caused by factors outside of transcription regulatory region changes. Further research on the mechanism of AFP elevation is needed. (*Gut Liver* 2017;11:136-141)

Key Words: Alpha-fetoprotein; Biomarkers; DNA mutational analysis

INTRODUCTION

α -Fetoprotein (AFP) is a plasma protein highly produced by the fetal yolk sac, liver, and gastrointestinal tract at levels around 300,000 ng/mL¹ in the fetus. Postnatal repression of

AFP leads to low levels (<10 ng/mL)² in healthy adults. However, AFP level is elevated in a variety of clinical conditions such as pregnancy, liver cirrhosis, hepatocellular carcinoma and testicular carcinoma,² making AFP an important tumor marker for related malignancies.

Nonetheless, AFP elevation may be encountered in other circumstances. First, AFP levels are commonly measured by sandwich immunoassays, which may falsely report elevation due to heterophilic antibodies crosslinking with immunoassay antibodies. In the case of true AFP elevation in a healthy adult, it may be caused by the hereditary persistence of α -fetoprotein (HPAFP).¹ HPAFP is a rare, benign condition in which AFP elevation is persistent without any clinical abnormality, and it is inherited in an autosomal, dominant fashion. Until now, there have been 20 reported cases of HPAFP in the literature, and six of these were linked to two mutations in AFP promoter region: a -119 G>A substitution and a -55 C>A substitution.³⁻⁶ However, some families with HPAFP remain mutation-free for the promoter region, suggesting that other factors such as enhancer or silencer regions of AFP gene may contribute to AFP elevation.⁷⁻⁹

Separately from HPAFP, rare cases of persistent AFP elevation but with no related family history may be encountered in the clinical setting. These cases suggest the presence of a nonhereditary persistence of AFP. Since AFP elevation often prompts further clinical workup to exclude pathological conditions and provokes much anxiety in patients, it is important to be aware of AFP elevation without clinical abnormalities in healthy adults. In addition, the mechanism underlying AFP elevation in such cases should be elucidated. Thus, in this study, we described hereditary and nonhereditary cases of persistent AFP elevation in healthy adults from South Korea and broadened the

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scope of HPAFP mutation analysis by targeting AFP enhancer, silencer, and promoter regions.

MATERIALS AND METHODS

1. Cases and controls

Four adult cases with persistently elevated AFP (AFP1 to AFP4) with no signs of accommodating clinical abnormalities were prospectively included in this study at the Seoul National University Bundang Hospital (SNUBH) from September 2010 to Mar 2016. The Institutional Review Board (IRB) at SNUBH approved this study, and written informed consent was received from all participants (IRB number: B-1504/294-304). Familial information from AFP1 and AFP2 was obtained to construct pedigrees (Fig. 1). A control set of twenty healthy controls with low AFP levels (<0.6 to 2.9 ng/mL) collected from repository samples was included (IRB number: B-1307/210-006). They were healthcheck examinees at SNUBH with normal AFP levels (<2.9 ng/mL) and showed neither clinical symptoms nor laboratory and radiological abnormalities. Serum AFP level was measured by an automated enzyme-linked chemiluminescent immunoassay (Roche Diagnostics, Mannheim, Germany).

2. PCR amplification and sequencing

Blood from subjects were centrifuged for 15 minutes at 3,000 rpm at 4°C. DNA was extracted from the buffy coat with QIAamp DNA Blood Mini Kit (QIAGEN Inc., Hilden, Germany). Five areas upstream of the AFP gene were amplified: short promoter (-182 to +112),¹⁰ enhancer domain A (-4120 to -3756), enhancer domain B (-3492 to -3300),¹¹ distal silencer (-1822

to -1414), and a full promoter region (-952 to +28), which included the proximal silencer (-402 to -169).¹² Primers used for each section are listed in Table 1. Polymerase chain reaction (PCR) conditions were adapted from a previous report.⁶ Briefly, the reaction solution included 100 ng of DNA, 0.1 μ mol/L of each primer, 2.5 U Taq DNA polymerase, 1 \times PCR buffer, and 200 μ mol/L of each deoxynucleotide triphosphate (Taq, PCR buffer, and deoxynucleotide triphosphate from i-StarTaq™ DNA polymerase; iNtRON Biotechnology, Seongnam, Korea) for a total of 50 μ L. PCR conditions were 95°C for 6 minutes and 29 cycles (enhancer domain A, promoters, and silencers) or 35 cycles (enhancer domain B) of 95°C for 20 seconds, 56°C (enhancer domain A, promoters, and silencers) or 45°C (enhancer domain B) for 1 minute, and 72°C for 1 minute. PCR products were purified using a commercial kit (Bioneer Corp., Daejeon, Korea) and sequenced by a commercial sequencing company (Bioneer Corp.). The genome reference consortium human (GRCh) 38 primary assembly (GenBank accession number: CM000666.2) was used as the reference for sequence comparison.

RESULTS

1. Case descriptions

All cases were referred to our clinic with evidence of elevated AFP serum levels in routine blood lab workups. We used the following criteria to define cases of persistently elevated AFP: (1) adults with >10 ng/mL serum AFP exhibited at least three times and persisting for at least 1 year and (2) AFP elevation despite lack of evidence for related malignant and nonmalignant clinical conditions previously associated with AFP elevation. The

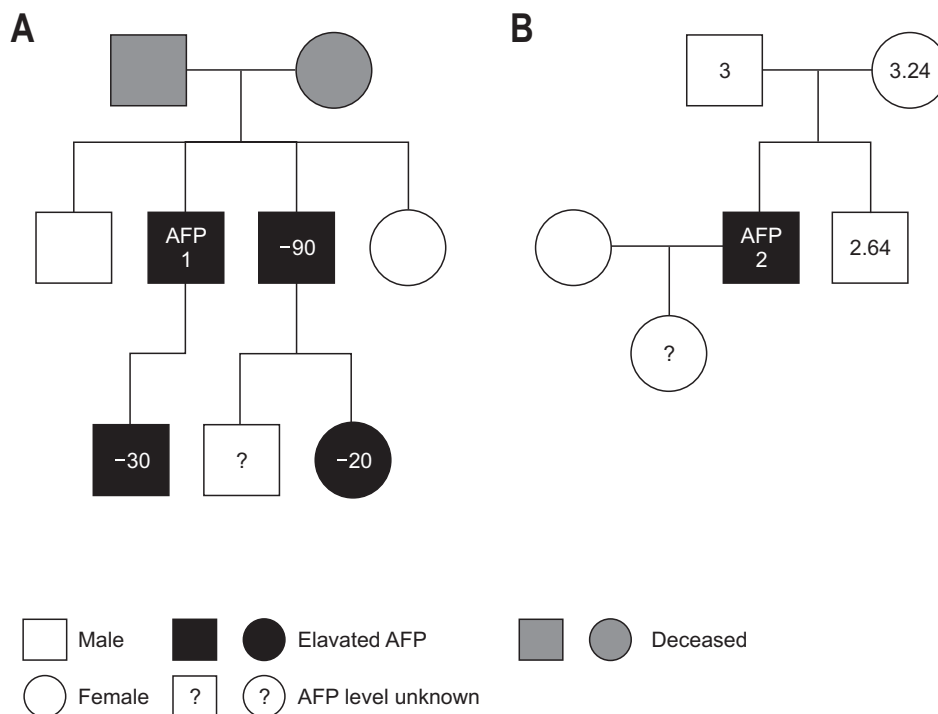


Fig. 1. Pedigree for AFP1 and AFP2 with AFP values. Pedigrees with AFP values for (A) the AFP1 family and (B) the AFP2 family. All AFP values are reported in ng/mL. AFP, α -fetoprotein.

Table 1. Primers and Product Size for Polymerase Chain Reaction Amplification of the Target Regions

Target region	Primer sequence	PCR product length (bp)
Short promoter*	5'CTATGCAGTGTCTGGATTC3' 5'GCCCCAAAGAGCTCTGTGTC-3'	294
Enhancer A [†]	5'-CAGATTGAATTATTTGCCTGTC-3' 5'TTGTAGCCATGTGAGTGGTTGAGAAAAATA-3'	364
Enhancer B [†]	5'-CCTGATTAATAATTACACTA-3' 5'-TAGGAAGTTTTTCGCAATAATA-3'	192
Full promoter [‡]	5'-TTTAGAAAATCCTGGTGCC-3' 5'-TGTATTGGCAGTGGTGAAG-3'	980
Distal silencer	5'-CTTGGGATATTTGCCCATG-3' 5'-CATACTGCAAGCTACTAGGGC-3'	408

PCR, polymerase chain reaction; bp, base pair.

*Primer TT1 and TT2 from McVey *et al.* (1993);¹⁰ †Primer sequence from Nakabayashi *et al.* (2004);¹¹ ‡Primer FTPpro980_F and FTPpro980_R from Chen *et al.* (2007).¹³

Table 2. Summary of AFP Cases with AFP Ranges and Follow-Up Dates

Case	Age/sex	ALT, IU/L	AFP range, ng/mL	HBsAg/anti-HBs	anti-HCV	Follow-up dates, mo/yr
AFP1	61/male	33	21.8–31.1	-/+	-	9/2010–3/2016
AFP2	31/male	16	115.4–186.1	-/+	-	9/2010–1/2016
AFP3	40/female	15	10.1–12.6	-/+	-	1/2015–3/2016
AFP4	45/male	32	19.8–27.6	-/-	-	11/2014–2/2016

AFP, α -fetoprotein; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBs, hepatitis B surface; HCV, hepatitis C virus.

range of AFP levels, gender, sex, alanine aminotransferase (ALT) level, and dates of AFP measurement were listed in Table 2. Abdominopelvic computed tomography (CT) or ultrasound (US), gastroendoscopy, and urological or gynecological examinations confirmed the lack of malignancy.

AFP1 is a 60-year-old male with negative hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (anti-HCV), with an ALT level of 33 IU/L. A liver ultrasound revealed no signs of malignancy but a mild fatty liver and gallbladder stones. Testicular examinations revealed no remarkable findings. AFP remained elevated during follow-up duration with no malignancy from September 2010 to February 2015. Family members' AFP levels were obtained. The pedigree (Fig. 1A) confirmed an autosomal dominant hereditary pattern, indicating the first case of HPAFP from South Korea.

AFP2 is a 30-year-old man with normal aspartate aminotransferase (AST) and ALT levels, and he was negative for HBsAg and anti-HCV but positive for anti-HBs. Extensive imaging work up of the liver (CT, magnetic resonance imaging, and positron emission tomography computed tomography) and testis (sonography) revealed no abnormalities. AFP2 did not develop any abnormal clinical manifestations during follow up (September 2010 to January 2015), but continued to have elevated AFP levels of 115.4 to 186.1 ng/mL. AFP levels were determined for the father, mother, and brother. However, all family members except for AFP2 had normal AFP levels (Fig. 1B). This case pre-

sented the existence of non-hereditary cases of persistent AFP elevation.

AFP3 is a 39-year-old nonpregnant female who experienced transient mild gastroenteritis. Following blood work up revealed elevated AFP and negative HBsAg, anti-HCV, and anti-hepatitis A virus antibody, with an AST/ALT of 22/15 IU/L. An abdomen and pelvis US revealed no abnormalities. She underwent abdominopelvic CT and gastroscopy at 1 year after her visit, all of which showed normal results.

AFP4 is a 44-year-old male who was referred to our clinic in November 2014 for elevated AFP levels, but had persistently elevated AFP recorded since November 2011 with no signs of malignancy. He had an ALT of 32 IU/L with negative HBsAg and anti-HCV. An abdominal and pelvis CT showed a tiny hepatic hemangioma, but no other remarkable findings were found.

2. Mutation analysis results

Mutational analysis of AFP transcriptional regulatory areas revealed no mutations unique to AFP1 to AFP4 when compared to the reference or controls.

DISCUSSION

This study reports four South Korean cases of persistent AFP elevation without associated clinical abnormalities. Of these

four cases, one case was confirmed as HPAFP, and the other case indicated the presence of a nonhereditary persistent elevation of AFP. Furthermore, as major changes of AFP expression are thought to be from changes in AFP transcription,¹⁴ this study sought to investigate mutations in enhancer and silencer regions, in addition to the previously investigated promoter region, that may drive persistent AFP elevation. Nonetheless all four cases of elevated AFP did not have mutations in these regions, suggesting that factors outside of these AFP transcription regulatory regions may lead to persistent AFP production.

HPAFP, a benign, autosomal dominant condition may be caused by a mutation in the AFP promoter region that increases hepatocyte nuclear factor 1 (HNF-1) binding affinity, raising AFP transcription levels.⁶ To our knowledge, there have been only 20 cases of HPAFP reported, and among them, nine cases have conducted mutational analysis of the AFP promoter region. As summarized in Table 3, six cases revealed mutations located at HNF-1 binding sites within the promoter region of the AFP gene (promoter positions -119 and -55). By increasing binding affinity, these mutations increase AFP transcription.⁶

Table 3. Reported Cases of HPAFP with Mutation Analysis

Author (year)	AFP level, ng/mL	Mutation
Ferguson-Smith <i>et al.</i> (1983) ³	Grossly raised	-119 G>A ¹⁰
Cochran <i>et al.</i> (1999) ⁷	61.3–152.9	NF
Blesa <i>et al.</i> (2003) ⁴	1,500–3,564	-119 G>A
Alj <i>et al.</i> (2004) ⁶	1,084	-119 G>A
Alj <i>et al.</i> (2004) ⁶	217	-55 C>A
Yeh <i>et al.</i> (2004) ⁸	143	NF
Nagata-Tsubouchi <i>et al.</i> (2005) ⁵	516	-119 G>A
Nagata-Tsubouchi <i>et al.</i> (2005) ⁵	1,200	-119 G>A
Waseda <i>et al.</i> (2012) ⁹	19–27	NF

HPAFP, hereditary persistence of α -fetoprotein; AFP, α -fetoprotein; NF, none found.

Three families with HPAFP however do not have these reported mutations within the AFP promoter. Furthermore, AFP1 and his relatives represent a HPAFP case with neither of the two mutations (a -119 G>A substitution and a -55 C>A substitution) previously reported in the AFP promoter of HPAFP families.⁶ This supports the heterogeneous etiology of HPAFP.

Though the promoter has been the focus of mutational investigation in HPAFP cases, several areas outside of the promoter have been reported to regulate AFP transcription. These are two enhancer regions, which have binding sites for multiple liver-enriched transcription factors (LETFs),¹¹ and two silencer regions, which have a repeated suppressor element,¹² upstream of AFP (Table 4). Thus, it is possible that mutation negative families may have mutations within these previously uninvestigated regulatory areas of AFP. Nonetheless, as with the three other cases in this study, AFP1 did not show any unique mutations within other transcription regulatory regions examined in this study, suggesting that other factors contribute to HPAFP and AFP elevation.

This study also showed that there are cases of nonhereditary AFP cases in patients with no related clinical abnormalities. The AFP2 case did not reflect HPAFP. Although AFP2 showed persistently high levels of AFP elevation (115.4 to 186.1 ng/mL) from September 2010 to January 2016 without any signs of malignancy, his father, mother and brother all had normal AFP levels. This showed that there are incidences of elevated AFP that do not occur in a hereditary fashion like HPAFP.

This etiological heterogeneity may be linked to the heterogeneity found in the level of AFP elevation. There seems to be a threshold of AFP level that is associated with a mutation.² Previous studies show that compared to mutation positive cases (516 to 3,564 ng/mL for -119 G>A and 217 ng/mL for -55 C>A), mutation negative cases (19 to 152.9 ng/mL) exhibit lower levels of AFP.^{3–9} This study supports this claim as AFP1 to AFP4 had levels that fit in the latter group and were mutation negative at -119 and -55 promoter positions. It is possible

Table 4. Areas of Interest within the Transcription Regulating Regions of AFP

Transcriptional element	Region of interest	Region description
Promoter	-130 to -118, -59 to -47	HNF-1 binding sites ⁶
Enhancer A	-4114 to -4107, -4094 to -4081, -4043 to -4025, -3915 to -3902, -3886 to -3876, -3878 to -3866, -3783 to -3770	LETf binding sites ¹¹
Enhancer B	-3489 to -3476, -3473 to -3462, -3448 to -3435, -3319 to -3306	LETf binding sites ¹¹
Proximal silencer	-317 to -301	Suppressor element ¹²
Distal silencer	-1808 to -1791, -1784 to -1768, -1770 to -1754, -1742 to -1727	Suppressor element ¹²

AFP, α -fetoprotein; HNF, hepatocyte nuclear factor; LETf, liver-enriched transcription factor.

that specific changes lead to different ranges of AFP elevation. For example, a point mutation at position -55 leads to a 250-fold increase of HNF-1 binding affinity, causing AFP levels that range from 216.9 to 698.8 ng/mL.⁶ Meanwhile, the multifactorial interaction of enhancers, silencers, and the promoter¹⁴ are the primary factors that repress AFP levels of 300,000 ng/mL¹ in the fetus to lower than 10 ng/mL in adults.¹⁵ Clearly, these are levels of change that were not evident in the four cases reported here, suggesting that a more subtle change outside of the transcriptional region may have caused their AFP elevations.

Outside of the transcription regulatory region, several other alternations may be responsible for AFP elevation. Although changes in AFP expression are primarily attributed to the transcription stage, posttranscriptional changes may influence AFP production. It is also possible that rather than the transcription regulating regions themselves, changes within LETFs, such as HNF-1 or CCAAT/enhancer binding proteins,¹⁶ all of which have several binding sites in AFP regulatory regions and share complex interactions with one another, may impact AFP expression. AFP elevation may also root from an unlinked regulator identified as α -fetoprotein regulator I, which is known to help regulate AFP repression via interactions with the promoter.¹⁷

Though very rare in commercial AFP assays used in our study and previous studies, heterophilic antibodies may interfere with many immunoassays in clinical practice. The possibility of a false positive AFP elevation by a heterophilic antibody was excluded in AFP3 by the simultaneous examination of the patient's preblocked and unblocked sera. This was completed with a blocking agent against a heterophilic antibody, which was provided by Roche diagnostics.

Finally, only 20 cases of HPAFP have been reported since its discovery in 1983. In our study, we were able to only confirm one of two studied cases (AFP1 and AFP2) as HPAFP. This may suggest that while HPAFP is a rare condition, AFP elevation, regardless of hereditary pattern, may be a more common phenomenon as we were able to detect four cases of AFP elevation within 5 years. The higher number of patients at risk for chronic liver disease and liver cancer who are routinely checked for AFP levels in South Korea may contribute to this higher rate of detection.

We conclude that persistent AFP elevation is a heterogeneous condition that is not only the result of changes in AFP transcription regulatory regions. It is not always hereditary, and it may present a wide range of AFP levels. Due to AFP's significance as a tumor marker for many malignancies, it is important to recognize this condition to prevent inappropriate clinical intervention. Future studies should look at changes outside of AFP regulatory regions with an even broader scope through whole genome or exome sequencing.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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REFERENCES

- Li X, Alexander S. Hereditary persistence of alpha-fetoprotein. *Pediatr Blood Cancer* 2009;52:403-405.
- Houwert AC, Giltay JC, Lentjes EG, Lock MT. Hereditary persistence of alpha-fetoprotein (HPAF P): review of the literature. *Neth J Med* 2010;68:354-358.
- Ferguson-Smith MA, May HM, O'Hare E, Aitken DA. Hereditary persistence of alphafetoprotein: a new autosomal dominant trait identified in an antenatal screening programme for spina bifida. *J Med Genet* 1983;20:458.
- Blesa JR, Giner-Durán R, Vidal J, et al. Report of hereditary persistence of alpha-fetoprotein in a Spanish family: molecular basis and clinical concerns. *J Hepatol* 2003;38:541-544.
- Nagata-Tsubouchi Y, Ido A, Uto H, et al. Molecular mechanisms of hereditary persistence of alpha-fetoprotein (AFP) in two Japanese families A hepatocyte nuclear factor-1 site mutation leads to induction of the AFP gene expression in adult livers. *Hepatol Res* 2005;31:79-87.
- Alj Y, Georgiakaki M, Savouret JF, et al. Hereditary persistence of alpha-fetoprotein is due to both proximal and distal hepatocyte nuclear factor-1 site mutations. *Gastroenterology* 2004;126:308-317.
- Cochran PK, Chauvenet AR, Hart PS, et al. Hereditary persistence of alpha-fetoprotein in a child with testicular germ cell tumor. *Med Pediatr Oncol* 1999;32:436-437.
- Yeh SH, Kao JH, Chen PJ. Heterogeneity of hereditary persistence of alpha-fetoprotein. *Gastroenterology* 2004;127:687.
- Waseda Y, Tanaka H, Nakagomi K, Goto S, Ido A. A case of hereditary persistence of alpha-fetoprotein: diagnostic usefulness of the subfraction profile. *Jpn J Clin Oncol* 2012;42:767-769.
- McVey JH, Michaelides K, Hansen LP, et al. A G-->A substitution in an HNF I binding site in the human alpha-fetoprotein gene is associated with hereditary persistence of alpha-fetoprotein (HPAFP). *Hum Mol Genet* 1993;2:379-384.
- Nakabayashi H, Koyama Y, Suzuki H, et al. Functional mapping of tissue-specific elements of the human alpha-fetoprotein gene

- enhancer. *Biochem Biophys Res Commun* 2004;318:773-785.
12. Nakabayashi H, Hashimoto T, Miyao Y, Tjong KK, Chan J, Tamaki T. A position-dependent silencer plays a major role in repressing alpha-fetoprotein expression in human hepatoma. *Mol Cell Biol* 1991;11:5885-5893.
 13. Chen GG, Ho RL, Wong J, Lee KF, Lai PB. Single nucleotide polymorphism in the promoter region of human alpha-fetoprotein (AFP) gene and its significance in hepatocellular carcinoma (HCC). *Eur J Surg Oncol* 2007;33:882-886.
 14. Spear BT. Alpha-fetoprotein gene regulation: lessons from transgenic mice. *Semin Cancer Biol* 1999;9:109-116.
 15. Camper SA, Tilghman SM. Postnatal repression of the alpha-fetoprotein gene is enhancer independent. *Genes Dev* 1989;3:537-546.
 16. Supriatna Y, Kishimoto T, Furuya M, et al. Expression of liver-enriched nuclear factors and their isoforms in alpha-fetoprotein-producing gastric carcinoma cells. *Exp Mol Pathol* 2007;82:316-321.
 17. Peyton DK, Huang MC, Giglia MA, Hughes NK, Spear BT. The alpha-fetoprotein promoter is the target of Afr1-mediated postnatal repression. *Genomics* 2000;63:173-180.