Clinical and Applied
Thrombosis/Hemostasis
16(6) 679-683
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DOI: 10.1177/1076029609338045
http://cath.sagepub.com

Factor V Leiden in an Urartian, Dating Back to 1000 BC

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Abstract

Factor V Leiden (FVL) is the most common monogenic disorder that causes activated protein C (APC) resistance, creating hypercoagulation. The mutation shows an uneven geographic distribution, significantly high in European populations. The mutation is believed to have originated approximately 20 000 years ago probably from a geographic region close to Anatolia. This fact makes it noteworthy to search for the mutation in ancient populations that once lived in this area. One of these civilizations, Urartu was centered around Van Lake in Eastern Turkey. The archeological remains from the excavations of the region are dated back to 1000 BC. Teeth, taken from the excavations of Van Yoncatepe fortress, were taken into DNA analysis considering all the precautions for ancient DNA analysis. Multiplex STR (Short Tandem Repeats) analysis were performed both to determine the gender of the samples and to conclude that the samples are preserved from modern DNA contamination. After getting an 80% amplification success for amelogenin, a melting curve analysis using lightcycler was performed to determine the FVL genotype of each sample. Of the 60 samples, 1 gave a positive amplification result for *FV* gene and was found to be heterozygous. To date, the age of this mutation was estimated based on statistical calculations using haplotype frequencies; here for the first time, we report FVL in an ancient population of 3000 years.

Keywords

factor V Leiden, Urartian, ancient DNA, selective advantage, orthograte entrance technique

Introduction

Factor V Leiden ([FVL] 1691G-A) is an autosomal dominant mutation that causes activated protein C (APC) resistance, thus, yielding to a hypercoagulation disorder.[1] The heterozygote carriers of the mutation have approximately a 5-fold increased risk of thrombosis, whereas homozygotes are subject to a 20- to 80-fold increased risk.[2] The mutation shows an uneven geographic distribution, ranging from nonexistence in natives of Africa, America, Asia, and Australia, to high existence reaching at 15% in some areas of Europe.[3,4] The high prevalence of the mutation among all whites makes it plausible to ask whether FVL had caused selective advantages during evolution, some of which are assumed to be the reduction of the risk of hemorrhage after delivery, the improvement of hemoglobin status, and the reduction of the mortality rate in relation to severe sepsis.[5-7] It is also possible to assume that the hypercoagulation effect caused by the mutation may have produced a survival advantage for fighter ancestors.[8] Mutation age through haplotype analysis for FVL has been estimated to be 21 340 years coinciding the last Pleistocene glaciation, which can be characterized by extensive population growth, migration all over the world, and development of agriculture. There are various assumptions about the origin of the mutation. Firstly, the high prevalence of the mutation in Middle East leads to the possibility that the founders of FVL mutation lived around this region.[5] Yet, another study designed to determine the origin of FVL reveals that Mediterranean and Indian individuals constitute a single haplotype for the mutation. This is compatible with the hypotheses in which Mediterranean and Indian individuals are considered to have been descended from the same ancestral population, later spreading the mutation both in Europe and in India.[9] Finally, another study on population genetics of the mutation shows that during the Neolithic period, FVL in Europe was probably expanded from Turkey, Anatolia.[3] Studies on the frequency of FVL existence in Turkey report that the frequency ranges from 3.5% to 15% in different parts of the country.[10,11]

Turkey, stretching between 2 continents, Europe and Asia, connecting East and West, played a major role in the world history.[12] A significant number of the earlier civilizations were constructed within these geographical boundaries, one of which is Urartu, an Iron Age kingdom in Eastern Anatolia. The

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Kingdom of Urartu was located in the mountainous plateau between Asia Minor, Mesopotamia, and the Caucasus mountains, and was centered around Lake Van the in present-day Eastern Turkey.[13] The archeological samples taken from excavations of Van Yoncatepe region date back to 1000 BC.[14]

Because FVL is believed to have originated somewhere close to Anatolia approximately 20 000 years ago, the existence of mutation in this geography is expected to be high. The aim of this study was to determine the prevalence of FVL in Urartu, an ancient Anatolian population of 3000 years.

Materials and Methods

Ancient teeth were taken from the excavation of Yoncatepe (AD 1000), Eastern Turkey (n = 60).[14] Several precautions were taken to prevent contamination during this study. Samples were chosen among hundreds of ancient teeth based on their morphological integrity. Prior to extraction, the teeth samples had been washed with 10% SDS (Sodium Dodecyl Sulfate) and distilled water. Then, each sample was exposed to UV light for 10 minutes.[15] In addition, the orthograte surface of the teeth were cleaned with Mikrozid AF (Schulke-Mayr, England), a sterilization agent used in dental practices.

Orthograte entrance technique, in which the method used in endodontic treatment is simulated, was used to get pulp and dentin powder. This technique was presented as an advantageous alternative to get samples from ancient teeth without contamination and sample destruction (Alakoc YD, PhD and Aka PS, PhD (accepted, Forensic Science International, 2009)).

Before DNA was extracted through conventional phenol chloroform extraction method, pulp samples were decalcified at 4°C for 10 minutes. The quantification was performed using Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, USA).

At every step, disposable gloves, laboratory bonnets, and masks were used. Nondisposable dental equipments used to extract pulp tissue were sterilized in B type sterilizator (Euronda sterilizator, Vincenza-Italy SN EEG060546) at 134°C. After extracting the pulp tissue of each sample, the working area was cleaned by Mikrozid AF solution. DNA extraction, quantitation, amplification, and data analysis were performed in separated laboratory areas. The separate hoods used for DNA extraction and polymearase chain reaction (PCR) preparation were irradiated with UV rays before and after the experiment. Disposable sterile tubes, filtered tips, sterile reagents, and solutions were used. Negative controls were included in each amplification.

Two different sets of amplifications were arranged from each sample. Initially, multiplex STR amplification was performed using AMFlSTR SGM Plus Primer set (Applied Biosystems, CA, USA) on 9600 GeneAmp PCR system. The PCR products were analyzed in ABI 310 genetic analyzer. The AmpFlSTR SGM Plus PCR Amplification Kit used for human identification amplifies 10 different sized STR loci plus the gender marker Amelogenin. Furthermore, FVL mutation analysis was performed by real-time PCR application based on melting curve analysis using Lightcycler (Roche Molecular Biochemicals, Manheim, Germany). All the stages of the study were performed by a FVL negative female.

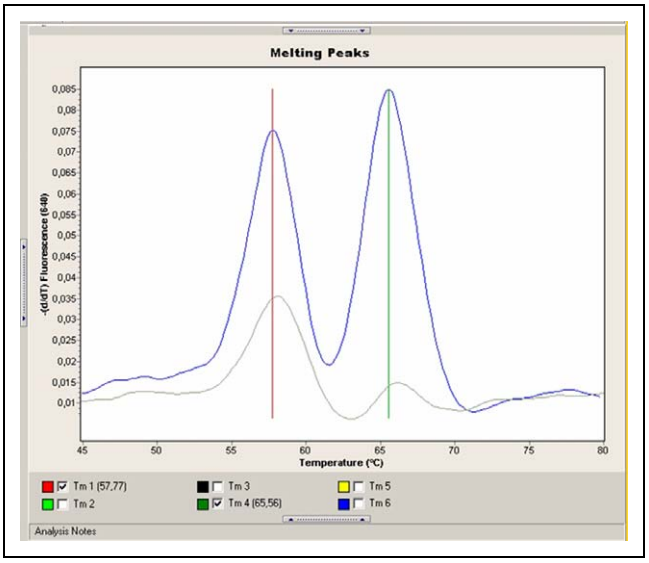


Figure 1. A melting curve analysis result representing both the Urartian sample and the positive control. The projection of melting curves of both samples fit properly.

Results

The DNA quantities of the 60 samples according to nanodrop ND-1000 spectrophotometer changed between a minimum of 1.84 ng/μL and a maximum of 154.79 ng/μL. Of these 60 samples, 48 were positively amplified for 103 to 109 base paired (bp) amelogenin. The 123-170 bp D8S1179 region was successfully amplified only in a few samples. A product larger than 170 bp could not be amplified. Using FVL amplification kit, only one out of all samples could successfully be amplified (222bp), representing a heterozygote profile (figure 1). It was previously determined by the former study that this sample belonged to a male (Figure 1).

Discussion

Factor V Leiden is the most common monogenic disorder in European populations, reaching carrier rates of up to 15% among Greeks and Swedes. The fact that the mutation is rarely seen in other regions of the world but it is highly frequent in Europe makes the matter worth examining.[3,4,16-22] The alteration among different ethnic groups suggests that the FVL came about after the separation of Mongoloids and Caucasians.[18] This idea gives rise to the conclusion that a point mutation occurred recently in the founder population of Europe and was spread throughout the continent by migration.[18,23] Actual distribution of haplotype frequencies support a single origin for FVL, estimated to have emerged 21 000 to 34 000 years ago.[24] The estimated age of the mutation based on haplotype analysis

fits well with the divergence of the modern Europeans 40 000 years ago, calculated approximately based on genetic, linguistic, and archeological evidence.[3,24] Low frequency of the mutation in French Basques, known to be derived from pre-Neolithic Europeans, supports the idea of diffusion of the mutant allele in Europe through the migration of Neolithic farmers.[3,25] It is also possible to speculate that the founder of the mutation lived in the Middle East because the prevalence of the mutation is higher in Arabs living in Israel, Jordan, Lebanon, and Syria than in most of the European populations.[4,5] In another study, a single FVL haplotype was confirmed in Mediterranean and Indian individuals, suggesting that both descended from the same ancestral population.[9] Moreover, Lucotte and Mercier[3] suggests an expansion in Europe during Neolithic period, possibly from an Anatolian center of origin in Turkey.

The fact that FVL on one hand increases the risk of thrombosis dramatically, and is, on the other hand, one of the commonest monogenic disorders, seems to be contradicting the principles of evolution. Surprisingly, scientific arena has limited information about why genetic diseases are present in a high frequency rather than not being eliminated by natural selection.[26] Most probably the answer lies in the survival advantage caused by the mutation reducing blood loss both for delivering mothers and for fighter ancestors living in a period when no satisfactory medical care was offered.[5,6,8] Even a small increase in the frequency of FVL for one generation would have yielded a significant difference through hundreds of generations.[6] In ancient periods, the disadvantageous impact of the mutation, resulting in a higher risk of thrombosis, might not have been as damaging because the risk factors associated with the lifestyle were rare.[4,5,25] A supporting result, came out of a contemporary study in which subjects were centenarians, suggested that FVL does not contradict extreme longevity and successful ageing.[8]

The retrospective studies about FVL made Anatolia an interesting geography for the purpose of looking into the mutation in ancient populations. Studies on the frequency of the mutation present in Turkish population reveal that the frequency ranges from 3.5% to 15% across the country.[10,11] Turkey, as a bridge connecting East and West, has played a major role in world history and has been the host to a significant number of earlier civilizations.[12] Because the origin of FV Leiden is believed to have emerged somewhere around Anatolia approximately 20 000 years ago, the existence of mutation in this geography is expected to be high.

Urartu, one of the ancient civilizations in Anatolia, was an Iron Age Kingdom, centered around Van lake in Eastern Turkey. Recent excavations of Urartian residential area shed lights on the unknown aspects of the civilization.[13] The archeological components gained from these excavations date back to 1000 BC.[14] Scientists carrying research on ancient times inquire various questions related to a specific period and its people through studies on ancient human remains as well as archeological materials. Mostly, teeth and bones are the subjects of such studies.[27] In this study, 60 teeth from the excavations of Van Yoncatepe fortress were subjected to DNA analysis, taking specific precautions against contamination in ancient DNA studies. Ancient DNA analyses render some specific difficulties stemming from the nature of the materials and analyzing process. Among these, contamination is the most important and challenging one.[27,28] Therefore, samples that will undergo DNA analyses must go through a careful decontamination process. In this context, teeth are more advantageous than the bone samples. As the contaminant DNA can come from individuals who excavate, study, and handle the remains, the contamination is concentrated mostly on the outer surface of the material. This is why careful decontamination procedures have to be applied. As the enamel is the hardest and most durable tissue in the body and is scarcely permeable, not only does it protect its content from outer contaminants but it also enables severe decontamination processes.[27,29] Studies comparing the efficiency of bone and teeth samples for DNA analyses reveal that although bone contains more cellular DNA, better amplification results were obtained in case of ancient teeth. This is most probably due to the less porous structure of teeth providing a better prevention from contaminants. In this study, besides the conventional precautions taken to prevent contamination, a technique that minimizes the contamination risk was used to get pulp samples (Alakoc YD, PhD and Aka PS, PhD (accepted, Forensic Science International, 2009)).

In ancient DNA studies, the size of the amplification product inversely correlates with the amplification efficiency.[30] An amplicon above 200 bp is unlikely to be of archeological origin and such an amplicon is considered to be the sign of contamination from a modern DNA source.[31] To evaluate our samples from this perspective, we performed a pilot study using a multiplex STR kit that consists of 13 different sized loci. The aim of this step is both to determine the gender of the samples and to detect the amplification performance of different base paired fragments. Out of 60 samples, 48 were amplified for 103 to 107 bp amelogenin locus used for gender identification. Only a few samples were amplified for a 123 to 170 bp D8S1179 locus. The results clearly indicated that elimination of modern DNA contamination for entire samples was successfully done. Studies on ancient DNA analysis suggest that the most important contamination risk is the contamination with PCR products of modern samples rather than cross sample contamination between the ancient remains, because only a minute amount of DNA is preserved in these remains.[28]

Having concluded that our ancient DNA samples are capable of amplifying approximately 200 bp fragments and preserved from modern DNA contamination, we performed a melting curve analysis using a lightcycler to determine the FV genotype of each sample. This system is advantageous for our samples, compared to other mutation detection techniques because it requires a little amount of DNA and it is more sensitive. Furthermore, as the amplification and mutation detection steps occur in 1 tube obviating the reopening of the tube, it minimizes the risk of PCR contamination.[32,33]

Application of melting curve analysis requires a positive control to indicate the system is working and a negative control to monitorize contamination. Out of the total 60 samples, 1 gave a positive amplification result for *FV* gene and was found to be heterozygous for FVL. The amplification curve was observed in the 34th cycle in a total of 45 cycles. The detention of the amplification can be explained by the low quantity and quality of the ancient DNA sample. The projection of the melting curve of positive control and the heterozygous sample fit properly and there was no amplification in the negative control. The heterozygous Urartian was found to be a male in the previous multiplex STR analysis. It is important to note that all steps of the laboratory procedures were performed by a FVL wild type female.

An important number of statistical studies were performed to evaluate the origin and prevalence of FVL. The fact that this is the first experiment carried out to determine the FV genotype in an ancient population makes this study considerably significant. Up to now, the studies showing that the mutation is approximately 21 000 to 34 000 years old were basically the calculations using haplotype frequencies. However, now the result of this study has revealed the fact that the mutation is seen in an ancient population of 3000 years.

Acknowledgment

We thank Prof Dr Mehmet Yaşar İşcan for his permission to work on the samples of his collection, Yasemin Aydemir and Ass Prof Hilal Özdağ for their guidance on the language of the paper.

Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

The authors received no financial support for the research and/or authorship of this article.

References

1. Perry DJ, Pasi KJ. Resistance to activated protein C and factor V Leiden. *QJM*. 1997;90(6):379-385.
2. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood*. 1995;85(6):1504-1508.
3. Lucotte G, Mercier G. Population genetics of factor V Leiden in Europe. *Blood Cells Mol Dis*. 2001;27(2):362-367.
4. Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet*. 1995;346(8983):1133-1134.
5. Zivelin A, Mor-Cohen R, Kovalsky V, Kornbrot N, Conard J, Peyvandi F, et al. Prothrombin 20210G> A is an ancestral prothrombotic mutation that occured in whites approximately 24,000 years ago. *Blood*. 2006;107(12):4666-4668.
6. Lindqvist PG, Dahlback B. Carriership of factor V leiden and evolutionary selection advantage. *Curr Med Chem*. 2008; 15(15):1541-1544.
7. Hartmut W, Bryce K, Lytle MC. Factor V leiden polymorphism modifies sepsis outcome: evidence from animal studies. *Crit Care Med*. 2004;32(5):3233-3238.
8. Mari D, Mannucci PM, Duca F, Bertolini S, Franceschi C. Mutant factor V (Arg506Gln) in healthy centenarians. *Lancet*. 1996; 347(9007):1044.
9. Castoldi E, Lunghi B, Mingozzi F. New coagulation factor V gene polymorphisms define a single and infrequent haplotype underlying the factor V Leiden mutation in Mediterranean populations and Indians. *Thromb Haemost*. 1997;78(3):1037-1041.
10. Akar N, Akar E, Dalgin G, Sözüöz A, Omürlü K, Cin S. Frequency of factor V (1691 G-A) mutation in Turkish population. *Thromb Haemost*. 1997;78(6):1527-1528.
11. Akar N. Factor V 1691 G-A mutation distribution in healthy Turkish Population. *Turk J Hematol* 2009; 26: 9-11.
12. Gökcümen O. *Ethnohistorical and Genetic Survey of Four Central Anatolian Settlements* [PhD Thesis]. Philadelphia, PA: University of Pennsylvania;. 2008:3-5.
13. Çilingiroğlu A. Urartu tarihi. Ege Universitesi Edebiyat Fakültesi Yayınları. Bornova, 1994:3-5.
14. O. Belli, M. Tozkoparan. 2004 Yılı Van-Yoncatepe Kalesi ve Nekropolü Kazısı. 27. Kazı Sonuçları Toplantısı 1. Cilt. T.C. Kültür ve Turizm Bakanlığı Yayınları. 2005:165-174.
15. Kemp BM, Smith DG. Use of bleach to eliminate contaminating DNA from the surface of bones and teeth. *Forensic Sci Int*. 2005; 154(1):53-61.
16. Angelina T, Bentley HA, Hawk AB, Manners RJ, Mokashi HA, Jeyaraj N, et al. Prevalence of factor V G1691A and factor II/prothrombin G20210A gene polymorphisms among Tamilians. *Exp Mol Pathol*. 2005;79(1):9-13.
17. Hong SH. Genotype distribution of the mutations in the coagulation factor V gene in Korean populations: absence of its association with coronary artery disease. *Korean J Biol Sci*. 2003;7(3):255-259.
18. Hermann FH, Koesling M, Schröder W, Altman R, Bonilla Jimenez R, Lopaciuk S, et al. Prevalence of factor V Leiden mutation in various populations. *Genet Epidemiol*. 1997;14(4): 403-411.
19. Yusoff NM, Abdullah WZ, Ghazali S, Othman MS, Baba AA, Abdullah N, et al. The absence of factor V Leiden mutation in Malays with recurrent spontaneous abortions. *Aust N Z J Obstet Gynaecol*. 2002;42(2):164-166.
20. Gregg JP, Yamane AJ, Grody WW. Prevalence of the factor V Leiden mutation in four distinct American ethnic populations. *Am J Med Genet*. 1997;73(3):334-336.
21. Hooper WC, Dilley A, Ribeiro MJA, Benson J, Austin H, Silva V, et al. A Racial difference in the prevalence of the Arg506-Gln mutation. *Thromb Res*. 1996;81(5):577-581.
22. Franco RF, Elion J, Santos SEB, Araujo AG, Tavella MH, Zago MA. Heterogeneous ethnic distribution of the factor V Leiden mutation. *Genet Mol Biol*. 1999;22(2):143-145.
23. Cox MJ, Rees DC, Martinson JJ, Clegg JB. Evidence for a single origin of factor V Leiden. *Br J Haematol*. 1996;92(4): 1022-1025.
24. Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, et al. A single genetic origin for a common Caucasian risk factor for venous thrombosis. *Blood*. 1997;89(2):397-402.

25. Bauder F, Lacombe D. Factor V Leiden, prothrombin 20210A methylenetetrahydrofolate reductase 677T, and population genetics. *Mol Genet Metab*. 2005;86(1-2):91-99.
26. Flint J, Harding RM, Clegg JB, Boyce AJ. Why are some genetic diseases common? Distinguishing selection from other processes by molecular analysis of globin gene variants. *Hum Genet*. 1993; 91(2):91-117.
27. Gilbert MTP, Rudbeck L, Willerslev E, Hansen AJ, Smith C, Penkman KEH, et al. Biochemical and physical correlates of DNA contamination in archeological human bones and teeth excavated at Matera, Italy. *J Archaeol Sci*. 2005;32(5):785-793.
28. Yang DY, Watt K. Contamination controls when preparing archaeological remains for ancient DNA analysis. *J Archaeol Sci*. 2005;32(3):331-336.
29. Ricaut FX, Keyser-Tracqui C, Crubezy E, Ludes B. STR-genotyping from medieval tooth and bone samples. *Forensic Sci Int*. 2005;151(1):31-35.
30. Meyer E, Wiese M, Bruchhaus H, Claussen M, Klein A. Extraction and amplification of authentic DNA from ancient human remains. *Forensic Sci Int*. 2000;113(1-3):87-90.
31. Burger J, Hummel S, Herrmann B, Henke W. DNA preservation: a microsatellite-DNA study on ancient skeletal remains. *Electrophoresis*. 1999;20(8):1722-1728.
32. Schlatterer K, Orth M, Fitzner R, Tauber R. Genotyping of factor V Leiden in research samples: a methodological comparison between allele-specific PCR (PCR-SSP) and lightcycler technology. *Biochemica*. 2000;1:31-34.
33. Von Ahsen N, Oellerich M, Armstrong VW, Schütz E. Application of a thermodynamic nearest neighbor model to estimate nucleic acid stability and optimize probe design: prediction of melting points of multiple mutations of apolipoprotein B-3500 and factor V with hybridization probe genotyping assay on the lightcycler. *Clin Chem*. 1999; 45(12):2094-2101.