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The Lineage and Sublineage Investigation of Human Papillomavirus Type 16 in Tehran, Iran, During 2022–2023: A Cross-Sectional Study

Zohreh Khezeli[1] | Zabihollah Shoja[2] | Mehrnaz Kaffashian[1] | Rahim Soleimani-Jelodar[1] | Somayeh Jalilvand[1]

[1]Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran | [2]Department of Virology, Pasteur Institute of Iran, Tehran, Iran

Correspondence: Somayeh Jalilvand (sjalilvand@tums.ac.ir)

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Keywords: cervical cancer | human papillomavirus type 16 | lineage | sublineage

ABSTRACT

Background and Aims: Diverse HPV 16 variants have been known to vary in geographic distribution and oncogenic potential. On this point, the nucleotide changes of the E6 gene were studied to find lineages of HPV 16 in normal, premalignant, and malignant stages of uterine cervical samples.

Methods: In this study, 120 HPV 16-infected samples were investigated using PCR and sequencing.

Results: In our samples, three lineages A, C, and D were found and lineage D was predominant (79.2%) followed by lineages A (20%) and C (0.8%). Concerning the association between histopathological stages and lineages, no statistically important differences were found.

Conclusion: Our finding revealed that two lineages A and D were circulating, with the dominancy of lineage, in Tehran, Iran. This finding emphasizes the geographic diversity of distinct HPV 16 (sub)lineages is different in the world.

1 | Introduction

Globally, cervical cancer represents the fourth most frequent cancer in women. It was estimated that 604,127 new cases and 342,000 new deaths would occur in 2020 [1]. Cervical cancer is reported as the 7th cancer in Iran with an age-standardized rate of 6.5 [2]. It is well-known that human papillomavirus (HPV) is the etiological agent of this cancer [3, 4].

HPV infection is the most prevalent viral sexually transmitted infection in the world [3, 5]. Nevertheless, a large proportion of HPV infections are removed by the immune system within 6–24 months. In a small proportion, it can persist and progress towards cervical intraepithelial neoplasia I–III (CIN I–III) or invasive cervical cancer [6, 7]. Fourteen high-risk (HR)-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 are considered etiological agents for almost all cervical cancer [8–12], among which HPV16 is the leading cause and accounting for nearly 61.7% of cervical cancer [5]. Nevertheless, numerous studies showed that a few of women infected with HPV 16 advanced to cervical cancer [6, 7], suggesting that other viral or host risk factors are needed for the development of cervical cancer. It has been proposed that distinct lineages of HPV 16 could have a higher chance for persistent infection and, ultimately, the development of cervical cancer [8].

The lineages and sublineages of HPVs were defined as 1-10% and 0.5-1% variability in the nucleic acid sequence of the complete HPV genome, respectively. In this respect, four distinct lineages (A–D) and 16 different sublineages (A1–4, B1–4, C1–4, and D1–4) were

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defined for HPV 16 [9]. It is recommended that distinctive lineages of HPV 16 can influence persistency and subsequently progression towards cervical cancer [9–13].

While it is known that HPV 16 is a prominent HPV type among Iranian women with cervical cancer, less is known regarding the circulation of distinct HPV 16 lineages in this population [14–16]. Hence, this study was intended to characterize the lineages and sublineages of HPV16 in Iran. Collecting more data in this regard can provide a useful means for epidemiological, phylogenetic, and evolutionary of HPV 16 [17].

2 | Materials and Methods

2.1 | Study Population

A total of 120 HPV 16-infected uterine cervical tissue biopsies from Tehran were included in this study. Concerning histopathological stages, 66 normal, 19 CIN I–II, 11 CIN III, and 24 cervical cancer samples were investigated. The samples were collected from patients who signed the informed consent that was approved by the ethical committee of the Tehran University of Medical Sciences (TUMS) (IR.TUMS.SPH.REC.1402.126). The age and histopathological diagnosis data were collected from patients' medical records.

2.2 | The Investigation of Lineage and Sublineage of HPV 16 Based on the E6 Gene

The extraction of DNA was performed by Exgene Tissue SV Plus (GeneAll, Seoul, Korea) from uterine cervical biopsy specimens according to the manufacturer's instructions. Since the length of the E6 gene was longer than the E7 gene and the number of mutations was greater, this gene was selected for analysis. The nucleotide variations of HPV 16 E6 (nucleotide 83–559) were investigated using PCR with the following primer pair: 5′-CCGAAACCGGTTAGTATAAAAGCA-3′ and 5′-CAG TTGTCTCTGGTTGCAAATCT-3′ to amplify a 571 bp fragment. The PCR reactions and the thermal cycle conditions were done according to the previously described study [14].

To determine HPV 16 lineages/sublineages, the PCR amplicons were sequenced. All of the studied sequences were aligned against reference sequences (A1–4, B1–4, C1, C3, C4, and D1–4) with the following accession numbers K02718, AF536179, HQ644236, AF534061, AF536180, HQ644298, KU053915, KU053914, AF472509, KU053920, KU053925, HQ644257, AY686579, AF402678, and KU053931 [18]. To draw the phylogenetic tree of the E6 gene, the maximum likelihood method was applied in Mega software version 11. The reliability of the drawn tree was done using the bootstrap method with 1000 replicates. Our sequences were deposited in Nucleotide—NCBI (nih.gov) with the following accession numbers: PP974705–PP974824.

2.3 | Analysis of Data

Data analysis was accomplished using the Mantel-Haenszel test for estimating a common two-factor association parameter or Fisher's exact test when the sample size was small (Epi Info 7.2.6.0, Statistical Analysis System Software). When the two-sided p-value was less than 0.05, it was considered statistically significant.

3 | Results

The whole sequence of the HPV 16 E6 gene (position 83–559 of the HPV 16 genome) was investigated in 120 HPV 16-infected samples. All studied samples were aligned against the E6 of the reference sequence (GeneBank KO2718) to find nucleotide changes. In our samples, three lineages A, C, and D were identified as lineage D was predominant (79.2%) followed by lineages A (20%) and C (0.8%) (Table 1 and Figure 1). Sublineage analysis was shown that A1, A2, C1, and D1/4 sublineages presented in 15.8%, 4.2%, 0.8%, and 79.2% of our samples, respectively. Concerning the E6 gene, the differentiation between sublineages of D1 and D4 was not possible.

As indicated in Table 1, the analysis of 120 obtained sequences of this study presented that 10 nucleic acid substitutions occurred in the E6 gene, of which 5 nucleotide substitutions were transition and 5 were transversion mutations. Among these, 4 changes at sites of A131G, G145T, C335T, and T350G were identified to be missense mutations that lead to amino acid substitutions at positions of R10G/I, Q14H/D, H78Y, and L83V, respectively. The remaining 6 changes (T190C, G132T, C143G, T286A, A289G, and A403G) were considered silent substitutions. The amino acid changes were found in 101 of 120 samples (84.2%). The most frequent amino acid substitution was L83V, which was observed in 100 samples (83.3%). Other amino acid substitutions included Q14H/D and H78Y in 96 samples (80.0%) and R10G in 4 samples (3.3%) (Table 2). From the four amino acid changes observed in this study, Q14H was specific to lineage D and was detected in 95 samples (79.25%). H78Y substitution was specific to the B, C, and D lineages, which can differentiate these lineages from the A lineage. L83V change was found in the A2 and B4 sublineages and D lineages. R10G substitution was specific to the A2, B2, B3, and B4 sublineages that were seen in 3 samples of this study that belonged to the A2 sublineage.

Concerning the HPV 16 lineages identified in the present study, the following patterns of amino acid changes were observed: Pattern 1 with no changes (15.8%); Pattern 2 with L83V change (1.7%), Pattern 3 with R10G/L83V substitutions (2.5%), Pattern 4 with R10G/Q14D/H78Y changes (0.8), and pattern 5 with Q14H/H78Y/L83V substitutions (79.2%) (Table 1).

As shown in Table 1, in the normal group, lineage A showed 25.8% and lineage D in 74.2% of cases. Among CIN I-II samples, lineages A, C, and D were identified in 21%, 5.2%, and 73.8%, respectively. In the cancer group, lineage A was found in 8.6% and lineage D in 91.4%. Concerning histopathological stages, although lineage D was more prevalent among women with CIN III/ICC than normal/CIN I–II (91.4% vs. 75%), a statistically significant difference was not found in this regard ($p = 0.064$) (Table 3). As shown in Table 3, the distribution of HPV 16 lineages based on HPV infection types did not show any significant differences. Lineage A was identified in 18.6% and

TABLE 1 | HPV 16 sublineages identified based on the E6 gene in normal, premalignant, and invasive cervical cancer samples of Iranian women.

			Nucleotide changes of the E6 gene														
Reference ID	**Lineages**	**Sublineages**	**109**	**131**	**132**	**143**	**145**	**178**	**276**	**286**	**289**	**295**	**335**	**350**	**403**	**433**	**532**
K02718	**A**	**A1**	T	A	G	C	G	T	A	T	A	T	C	T	A	G	A
A-F536179	**A**	**A2**	—	G	—	—	—	—	—	—	—	—	—	G	—	—	—
H-Q644236	**A**	**A3**	—	—	—	—	—	—	G	—	—	—	—	—	—	—	—
A-F534061	**A**	**A4**	—	—	—	—	—	G	—	—	—	—	—	—	—	—	—
A-F536180	**B**	**B1**	—	—	C	G	T	—	—	A	G	—	T	—	—	—	—
H-Q644298	**B**	**B2**	—	G	—	G	T	—	—	A	G	—	T	—	—	—	—
K-U053915	**B**	**B3**	—	G	—	G	T	—	—	A	G	—	T	—	—	—	—
K-U053914	**B**	**B4**	—	G	—	G	T	—	—	A	G	G	T	G	—	—	—
A-F472509	**C**	**C1**	C	—	T	G	T	—	—	A	G	—	T	—	G	—	—
K-U053920	**C**	**C3**	—	—	—	G	T	—	—	A	G	—	T	—	—	—	—
K-U053925	**C**	**C4**	—	—	—	G	T	—	—	A	G	—	T	—	—	—	—
H-Q644257	**D**	**D1**	—	—	—	—	T	—	—	A	G	—	T	G	—	—	—
A-Y686579	**D**	**D2**	—	—	—	—	T	—	—	A	G	—	T	G	—	—	G
A-F402678	**D**	**D3**	—	—	—	—	T	—	—	A	G	—	T	G	—	A	G
K-U053931	**D**	**D4**	—	—	—	—	T	—	—	A	G	—	T	G	—	—	—

																	Studied groups			
Studied samples																	**Normal *N* = 66**	**CIN I–II *N* = 19**	**CIN III/ICC *N* = 35**	**Total *N* = 120**
Pattern 1 (No change)	A	A1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13 (19.8)	3 (15.8)	3 (8.6)	19 (15.8)

(Continues)

TABLE 1 | (Continued)

Reference ID	Nucleotide changes of the E6 gene																				
	Lineages	Sublineages	109	131	132	143	145	178	276	286	289	295	335	350	403	433	532				
Pattern 2 (T350G)	A	A2	—	—	—	—	—	—	—	—	—	—	—	G	—	—	—	2 (3.0)	—	—	2 (1.7)
Pattern 3 (A131-G/ T350G)	A	A2	—	G	—	—	—	—	—	—	—	—	—	G	—	—	—	2 (3.0)	1 (5.2)	—	3 (2.5)
Pattern 4 (T109C/ G132T/ C143G/ G145T/ T286A/ A289G/ C335T/ A404G)	C	C1	C	—	T	G	T	—	—	A	G	—	T	—	G	—	—	—	1 (5.2)	—	1 (0.8)
Pattern 5 (G145T/ T286A/ A289G/ C335T/ T350G)	D	D1/D4	—	—	—	—	T	—	—	A	G	—	T	G	—	—	—	49 (74.2)	14 (73.8)	32 (91.4)	95 (79.2)
			Amino acid substitutions																		
			—	R1-0G	R1-0T/I	Q14-D	Q14-H	D3-2E	N6-5S	—	—	D71E	H78Y	L83V	—	—	—				

Abbreviations: CIN, cervical intraepithelial neoplasia; ICC, invasive cervical cancer.

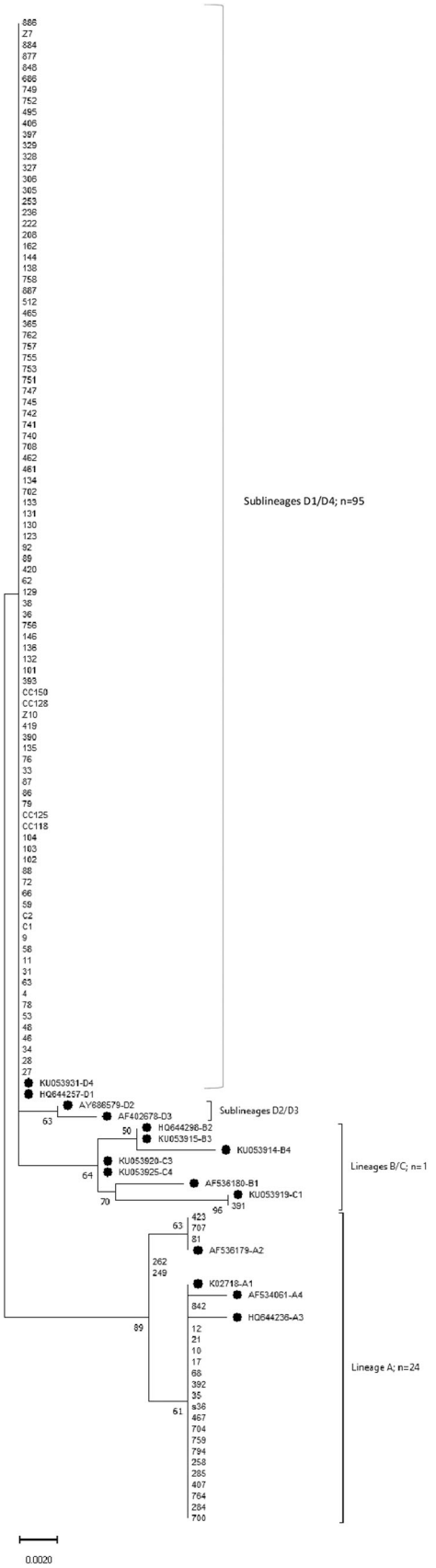

FIGURE 1 | The Phylogenetic tree of the HPV 16 E6 gene was conducted in MEGA11 by the Maximum Likelihood method based on the Tamura 3-parameter model. The reference sequences including accession numbers K02718, AF536179, HQ644236, AF534061, AF536180, HQ644298, KU053915, KU053914, AF472509, KU053920, KU053925, HQ644257, AY686579, AF402678, and KU053931 were indicated by black circles. Concerning the E6 gene, the differentiation between sublineages of D1 and D4 was not possible.

22.5% of single and multiple HPV infections, respectively. Lineage D was found in 81.4% of single infections and 77.5% of multiple infections. As shown in Table 4, multiple HPV infections were found in 66.7%, 26.3%, and 2.9% of normal, CIN I–II, and CIN III/ICC samples, respectively. A statistically significant difference was found in this regard ($p = 0.00000001$). Our results showed that 20% and 80% of samples were co-infected with low-risk and high-risk HPV types, respectively.

4 | Discussion

The results of this study indicate that lineages A and D are circulating in Tehran, Iran, and lineage C is detected sporadically. These results are according to previous studies in different provinces of Iran that reported the high prevalence of lineage D followed by A in those regions [14, 16, 19] (Figure 2A). However, our results are inconsistent with the data from the world. In a study that sampled 52 countries in 2019, it was found that lineage A was the most common, with a prevalence of 78.6%. The remaining samples belonged to lineages D, C, and B with the following frequencies of 9.2%, 6.4%, and 8.5%, respectively [20]. The distribution of HPV 16 lineages in different geographical regions of the world [20] was shown in Figure 2B. As indicated in Figure 2B, lineage D was not dominant anywhere in the world.

Sublineage analysis showed that the sublineages D1/4 were dominant (79.2%), followed by A1 (15.8%), A2 (4.2%), and C1 (0.8%) sublineages, respectively. According to the spreading of the HPV 16 sublineages, A1 and A2 have the highest prevalence in Europe, South/Central America, North America, South Asia, and the Pacific Ocean, while A3 and A4 were more common in East Asia [20]. Concerning D lineage, D1 sublineage is common in South Asia and North Africa, D2 and D3 are more frequent in North and South/Central America, and D4 is dominant in North Africa. Also, distinctive sublineages B and C were found only in Africa [20].

It is found that distinctive variants of HPV 16 have diverse chances of persisting in a population. It is shown that A1–3 have more opportunity to establish persistent infection in Caucasians [21, 22], A4 in East-Asian [21, 23, 24], B and C in Africans [21, 22], and D in Iran and North African populations [14–16, 21, 25, 26]. These results revealed that the host genetic variations might play an important role in the population-based carcinogenesis of HPV 16 variants. Indeed, some studies have suggested the difference of HPV 16 variants in coordination with HLA can be considered an effective factor in the difference of variants circulating in different regions in the world [27, 28]. For example, it has been reported that some HLA class I and II alleles in concert with distinct HPV16 (sub)lineages compared to the prototype can have a protective or predisposable effect on the development of cervical cancer [29] and regulate the proliferative response during HPV infection.

TABLE 2 | Amino acid substitutions in the E6 gene among normal, premalignant, and invasive cervical cancer samples of Iranian women.

Amino acid substitutions	Studies groups				*p*-value
	Normal($n = 66$)	CIN I–II($n = 19$)	CIN III/ICC($n = 35$)	Total($n = 120$)	
R10G/I[a]	3 (4.5)	1 (5.3)	—	4 (3.3)	0.68
Q14H/D[b]	49 (74.2)	15 (78.9)	32 (91.4)	96 (80.0)	0.17
H78Y	49 (74.2)	15 (78.9)	32 (91.4)	96 (80.0)	0.17
L83V	53 (80.3)	15 (78.9)	32 (91.4)	100 (83.3)	0.44

Abbreviations: CIN, cervical intraepithelial neoplasia; ICC, invasive cervical cancer.
[a]R10G and R10I amino acid changes were specific to the A2 and C1 sublineages, respectively.
[b]Q14H and Q14D amino acid changes were specific to the D and B/C lineages.

TABLE 3 | The frequency of HPV 16 lineages stratified by histopatology or HPV infection types in cervical samples of Iranian women.

Variables	HPV 16 lineages			*p*-value
	Lineage A*N* (%)	Lineage D*N* (%)	Total*N* (%)	
Histopatology				
Normal & CIN I–II	21 (25.0)	63 (75.0)	84 (100)	0.064[a]
CIN III & ICC	3 (8.6)	32 (91.4)	35 (100)	
Total	24 (20.2)	95 (79.8)	119 (100)	
HPV infection types				
Single type	13 (18.6)	57 (81.4)	70 (100)	0.605
Multiple types	11 (22.5)	38 (77.5)	49 (100)	
Total	24 (20.2)	95 (79.8)	119 (100)	

Abbreviations: CIN, cervical intraepithelial neoplasia; ICC, invasive cervical cancer.
[a]Fisher exact test.

TABLE 4 | The frequency of HPV infection types and co-infected types stratified by histopatology in cervical samples of Iranian women.

	Histopatology				*p*-value
	Normal*N* (%)	CIN I–II*N* (%)	CIN III–ICC*N* (%)	Total*N* (%)	
HPV infection types					
Single type	22 (33.3)	14 (73.7)	34 (97.1)	70 (58.3)	
Multiple types	44 (66.7)	5 (26.3)	1 (2.9)	50 (46.7)	0.00000001
Total	66 (100)	19 (100)	35 (100)	120 (100)	
Co-infected HPV types					
Low-risk	7 (15.9)	2 (40.0)	1 (100)	10 (20.0)	
High-risk	37 (84.1)	3 (60.0)	0 (0.0)	40 (80.0)	0.057
Total	44 (100)	5 (100)	1 (100)	50 (100)	

From an evolutionary perspective, HPV 16 A lineage split from their last common ancestor (BCD) 500,000 years ago, approximately at the same time as the split of archaic and modern humans occurred. Consequently, the HPV 16 A lineage was present in the Archaic ancestral population out of Africa and HPV 16 BCD lineages remained in the modern human population in Africa. After the last human migration out of Africa about 60,000–120,000 years ago, the ancestral populations of modern humans that moved out of Africa carried HPV 16 D and HPV 16 BC remained in Africa. Finally, a host shift occurred due to sexual transmission of the virus from Archaic to modern humans over genetic mixing in the last 80,000 years, whereby HPV 16 A was transferred from the Archaic population to the ancestors of modern humans. Lineage A then spread rapidly in modern human populations and eventually became the predominant lineage in the Eurasian and American regions [30, 31].

Evidence suggests that 2%–4% of the modern human genome outside of Africa (Eurasia) has originated from Archaic Neanderthals. Interestingly, these genes were not randomly transferred and most of the genes involved in the differentiation of keratinocytes and innate immunity (Toll-like receptor, HLA, and APOBEC3A genes) are enriched in the modern human population. Probably, this genetic change has affected the interaction between HPV 16 and humans. Therefore, it has been

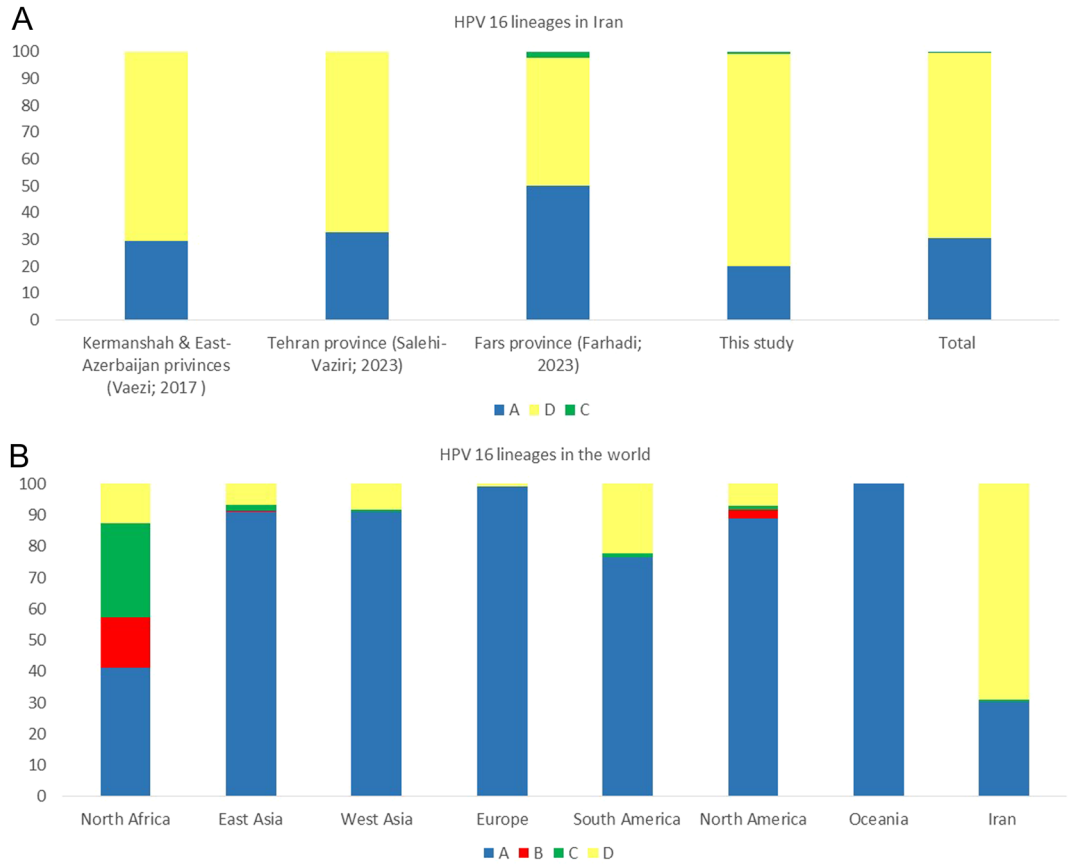

FIGURE 2 | The distribution of HPV 16 lineages in some provinces of Iran [14, 16, 19] (A) and different geographical regions of the world [20] (B).

assumed that this genetic change in the human genome has caused a change in the ecological niche of HPV 16 so that HPV 16 A has adapted to its new host and expanded in it [31].

Although a statistically significant difference was not observed between histopathological status and HPV 16 lineages, the D lineage was more prevalent in the cancer group than the normal group. It is possible that the low sample size in the CIN II–III/ICC group does not allow us to find a significant difference. Various studies conducted in the world have shown the difference in pathogenicity by different lineage/sublineages of HPV 16, which indicates that these variations can adjust cell signaling pathways that finally direct to cervical cancer. Indeed, several studies show that A4, B, C, and D sub/lineages of HPV 16 have a greater risk of carcinogenesis in comparison to A1–A3 [9, 10, 32, 33].

Remarkably, epidemiological studies found that the incidence of cervical cancer is more frequent in areas where the lineages B, C, and D are more prevalent [21, 32]. This fact suggests that distinctive HPV 16 lineages show dissimilar biological activities. In point of fact, it was observed that women with (sub)lineages A4/B/C/D of HPV16 were 4.5-fold more at risk of CIN 3.2 compared to women with A1–3 sublineages [34]. In another study, it was shown that lineage D had an eightfold higher risk of cervical cancer development than A1–3 variants [25]. Distinctive variants can affect carcinogenesis by different mechanisms, such as diverse E6 and E7 activities, increased viral replication, and/or transcription. It has been shown that in tumors containing lineage D, transcript levels E6/E7 are 2.1-fold more than in samples with the A1–3 variants [35].

In this study, the amino acid substitutions of Q14H, H78Y, and L83V were found in the most samples. Becoming increasingly evident is that lineage D with Q14H/H78Y/L83V amino acid alterations can be related to an increased chance of transforming human keratinocytes [36] and a greater activity of the P_{97} promoter [37]. It has also been proposed that lineage D has a higher propensity to integration in comparison to lineage A, which results in the higher expression of E6 and E7 oncogenes [38].

Several studies have pointed to which amino acid substitution of L83V (observed in A2 and D1–4 sublineages) could increase their oncogenic potential. Indeed, this change upregulates the hTERT expression and downregulates E-cadherin than the prototype [39]. Moreover, Insulin-like growth factor receptor 1 (IGF1R) is overexpressed in variants that harbor L83V substitution, radio-resistance in cervical cancer was observed [40].

The biggest limitation of this study was that all samples were obtained from Tehran. It is highly recommended that in future studies, samples be collected from different parts of Iran. Also, the low sample size in the studied groups is the other limitation of this study.

In conclusion, our results revealed that two lineages A and D were predominant in Iranian women. Interestingly, lineage D was frequent in each of the three groups. Our findings

also highlight that a particular HPV 16 lineage was dominant, which may be linked with geographic variety in the world. Additional studies are mandatory to investigate the whole genome sequencing of HPV 16 by high-throughput sequencing to clarify the distribution of HPV 16 (sub)lineages in different geographical parts of Iran. Also, the association between HLA and HPV 16 lineages of studied samples will be analyzed by HLA sequencing to elucidate a relationship between HPV 16 (sub)lineages and the genetic pool.

Author Contributions

Zohreh Khezeli: investigation, methodology, validation. **Zabihollah Shoja:** visualization, writing – review and editing. **Mehrnaz Kaffashian:** data curation. **Rahim Soleimani-Jelodar:** software, formal analysis. **Somayeh Jalilvand:** conceptualization, supervision, funding acquisition, writing – original draft, project administration, resources.

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Ethics Statement

Our research was conducted ethically by the World Medical Association Declaration of Helsinki. The study was approved by the local ethical committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1402.126).

Consent

Informed consent was obtained from all study subjects.

Conflicts of Interest

The authors declare no conflicts of Interest.

Data Availability Statement

The data that support the findings of this study are available in GeneBank at https://www.ncbi.nlm.nih.gov/nucleotide/. Our sequences were deposited in Nucleotide—NCBI (nih.gov) with the following accession numbers: PP974705–PP974824.

Transparency Statement

The lead author Somayeh Jalilvand affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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