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Human papillomavirus prevalence and type in liquid-based cervical samples from Turkish women in a selected risk group

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Aim: To detect the prevalence and type distribution of human papillomavirus (HPV) in different cytological diagnostic categories.

Materials and methods: Between 2007 and 2010, a total of 1014 liquid-based thin preparations of cervical smears were selected and classified according to cytology results. HPV DNA polymerase chain reaction (PCR) was also performed using these samples. HPV DNA-positive samples were genotyped by DNA sequencing.

Results: Of those enrolled in the study, 45.3% were negative cytologically, 36.4% had atypical squamous cells of undetermined significance, 0.3% had atypical squamous cells preventing the exclusion of a high-grade squamous intraepithelial lesion, 16.8% showed a low-grade squamous intraepithelial lesion, and 1.3% had an high-grade squamous intraepithelial lesion.

Conclusion: PCR assays showed HPV positivity in 63.0% of cytologically negative and 90.8% of cytologically positive samples. The most common types of HPV detected were 16, 6, 18, 31, 66, 56, 53, 81, 45, and 62. Of HPV DNA-positive samples, 47.7% were high, 4.7% were intermediate, and 17.9% were low risk. The high-risk types of HPV detected were 16, 18, 31, 56, 53, 45, 62, 58, 59, 67, 51, 35, 73, 52, 33, 39, 68, and 82.

Key words: Cytology, human papillomavirus DNA, genotype, polymerase chain reaction

1. Introduction

Infections caused by certain viruses have a role in the pathogenesis of some cancers in humans (1). Cervical cancer is among the most important causes of cancer-related death among women worldwide; 500,000 cases occur annually with a mortality rate of 50%. A strong causal relationship has been established between infection with human papillomavirus (HPV) and cervical cancer, with an estimated 99.7% prevalence of HPV in patients with cervical cancer (2,3). The prevalence of HPV infection is 2%–44% worldwide, and there are differences in prevalence between different societies (4). Generally, HPV infection prevalence is high among young women and becomes lower with age (5). Approximately 15 oncogenic HPV genotypes are generally accepted to be the cause of all cervical cancer (6). Cytological examination results also have great importance in HPV infections. In a multicenter study in Turkey involving 33 collaborating healthcare centers, cervical epithelial abnormalities were detected in 1.8% of samples, and the prevalence rates of atypical squamous cells of undetermined significance, atypical squamous cells of high significance, low-grade squamous intraepithelial lesions, and high-grade squamous intraepithelial lesions were 1.07%, 0.07%, 0.3%, and 0.17%, respectively (5). These rates are lower than those reported in Europe and North America, which may be due to sociocultural differences and the lack of a national screening program in Turkey (5). Although the conventional Papanicolaou (Pap) test complements HPV DNA testing, some data suggest that a single baseline HPV DNA test is more sensitive than a single conventional Pap test (7–10). HPV are typed according to their L1 capsid gene sequence differences (11). More than 150 HPV types known to occur have been categorized (12). Most of the HPV infections (80%) are temporary and are generally cleared by the immune system, without any clinical signs, within 6–12 months (5). Persistence of a high-risk infection is a risk factor for the development of cervical cancer (13). The intermediate types generally infect the skin or genital region (11,14). Benign condylomata are not precursors of malignant carcinomas (15). The high-risk group includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73,

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and 82; the intermediate group includes types 26 and 66; and the low-risk group includes types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and 83 (11,14).

Because HPV genotype information is useful clinically for follow-up and prognosis, the detection of oncogenic HPV genotypes is generally proposed (16). Because HPV 16 causes approximately 50%–60% of all cervical cancers and HPV 18 causes 15%–20%, they should be determined in women infected with oncogenic HPV to identify those at greater risk of developing cervical cancer (6,17–22). The prevalence of HPV infection and high-risk HPV types varies among different populations. The HPV test is not used for cervical cancer screening in Turkey, and there is limited information about HPV prevalence and the prevalence of HPV genotypes. Few data are available regarding HPV prevalence in Turkey, which is 2%–6% in the female population (23–26). The frequency of HPV DNA positivity according to age group was striking; those between 17 and 30 years showed a prevalence of 38%, and this rate lowered to 5.1% in the over-55-year-old female population in Turkey (27).

The aim of this study was to evaluate the prevalence of HPV infection in women between 15 and 68 years of age in categorized age groups in Turkey to determine the prevalence of particular HPV genotypes and to examine whether there was a correlation between molecular and cytological results.

2. Materials and methods

2.1. Study design

In this study cervical samples were collected according to liquid-based cytology technology. In this method, the cervical cells are immersed in a conserving liquid before being fixed on the slide, which prevents desiccation and reduces the quantity of obscuring material (28). A total of 35,685 liquid-based samples collected from women attending the gynecological outpatient clinics of 6 hospitals for regular gynecological monitoring between 2007 and 2010 were analyzed. The samples collected between 2007 and 2010 from 1014 patients who were positive cytologically and who had a family history of cervical cancer were included in this retrospective analysis. The women were between 15 and 68 years of age. A nested multiplex PCR assay using GP5/GP6 primers was used. No personal information of the patients was included in the study; therefore, informed consent was not needed. Our university's ethics committee approved this study (no. 2010/84; 22.07.2010).

2.2. Sample preparation

The samples were prepared using liquid-based cytology techniques (ThinPrep; Cytyc Corporation, Boxborough, MA, USA) and the Liqui-PREP^{T} system (LGM International Inc., Melbourne, FL, USA), and the slides were stained with Pap. The Bethesda System (TBS) 2001 was used for cytological classification (29). The following terminology was used in this study: "cytologically negative" was used in place of "negative for intraepithelial lesion or malignancy". "Cytologically positive" was used for "squamous cell abnormalities", including "atypical squamous cells of undetermined significance", atypical squamous cells, cannot exclude "high-grade squamous intraepithelial lesion"; "low-grade squamous intraepithelial lesion"; and "high-grade squamous intraepithelial lesion". After the preparation of standard slides, residual samples were placed in tubes with 0.9% saline, stored at –20 °C, and delivered to the molecular microbiology laboratory.

2.3. HPV DNA detection and genotyping

Detection and genotyping of HPV was performed using PCR and DNA sequence analysis with a sequence analyzer, which is a fluorescence-based capillary electrophoresis system.

2.3.1. DNA preparation

Liquid-based cervical samples (5 mL) were collected in Falcon tubes. They were centrifuged at 4500 rpm for 5 min. The 200-µL pellet was added to a 1.5-mL tube and extracted by QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.3.2. PCR procedures

PCR cycles were performed in a final volume of 50 µL. Each PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 6 mM $MgCl_2$, a 200 µM concentration of each dNTP, 5 U (instead of 7–10 U) of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Weiterstadt, Germany), 50 pmol of primers MY09 and MY11, 5 pmol of primer HMBB01, and 5 pmol of primers PC04 and GH20 for the simultaneous amplification of a 248-bp product of the human β-globin housekeeping gene. Amplifications were performed with AmpliTaq Gold activation. PCR cycles with primers GP5+ and GP6+ were performed as described in the original publication with 2 minor modifications, which are indicated parenthetically below (10). To increase the sensitivity of HPV detection, nested PCR cycles were performed using MY09-MY11 as outer and GP5+-GP6+ as inner primers. Two microliters of the MY09-MY11 PCR product was used as a template for the nested PCR amplification with GP5+-GP6+ primers. Two microliters of the PCR product served as a template for the nested PCR cycles. Ten microliters of the amplification products were analyzed by electrophoresis on 2% agarose gels and SYBR green nucleic acid gel stain.

2.3.3. Sequencing of PCR products

PCR products were sequenced by the MegaBACE 750 (Leipzig, Germany) using 5 pmol of either forward or back primers.

The primers used in this study are listed in Table 1 (30).

Table 1. Primers used in the study.

Type	Primers	
Outer primers	MY09 and MY11 primer cocktail is used [28]	
Inner and sequence primers	GP5+	5′-TTT GTT ACT GTG GTA GAT ACT AC-3′
	GP6+	5′-GAA AAA TAA ACT GTA AAT CAT ATT C-3′
Human β-globin housekeeping gene (248-bp)	HMBB01	5′-GCG ACC CAA TGC AAA TTG GT-3′
	PC04	5′-GAA GAG CCA AGG ACA GGT AC-3′
	GH20	5′-CAA CTT CAT CCA CGT TCA CC-3′

3. Results

Of 1014 examined samples, cytological diagnosis was negative in 459 (45.3%), atypical squamous cells of undetermined significance were found in 369 (36.4%), atypical squamous cells of high significance in 3 (0.3%), low-grade squamous intraepithelial lesions in 170 (16.8%), and high-grade squamous intraepithelial lesions in 13 (1.3%). A total of 706/1014 (69.6%) samples were HPV DNA-positive and were typed by DNA sequencing. The prevalence and distribution of HPV types according to cytological diagnostic categories are given in Table 2.

The most common types of HPV detected were 16, 6, 18, 31, 66, 56, 53, 81, 45, and 62 with prevalences of 14.2%, 8.8%, 5.5%, 4.5%, 3.8%, 3.3%, 2.6%, 2.3%, 2.2%, and 2.1%, respectively.

High-risk HPV types were detected in 484 (47.7%) samples, and the 5 most common ones were HPV 16 (14%), 18 (5.5%), 31 (4.5%), 56 (3.3%), and 53 (2.6%).

A total of 181 samples contained low-risk HPV types; the 5 most common ones were HPV 6 (8.8%), 81 (2.3%), 11 (1.7%), 90 (1.0%), and 83 (0.9%). Finally, 38 samples were classified into the intermediate risk group, and there was only 1 type detected in this group, type 66 (3.8%).

HPV DNA was detected in 63.0% of cytologically negative and 75.1% of cytologically positive samples.

To demonstrate the discrepancy between cytologically negative and positive groups, the McNemar paired proportion test was used. The difference between the 459 cytologically negative and 555 cytologically positive samples was 17.6% (95% confidence interval, 13.7%–21.2%), and it was statistically significant ($P < 0.0001$). HPV DNA type analysis according to cytologic diagnostic category is given in Table 3.

The prevalence of high-risk HPV types according to age was also examined in this study. Of the 484 detected HPV cases of any type, 57% were in women aged 25–34 years and 31% were in women 35–44 years old.

The types of high-risk HPV found in the 25–34 age group was, in order of prevalence, types 16, 18, 31, 56, 53, 45, 58, 62, 59, 67, 51, 35, 73, 39, 33, 68, 82, 52, and 86. The types of high-risk HPV in the 35–44 age group was, in order of prevalence, types 16, 18, 31, 56, 51, 53, 59, 62, 52, 45, 58, 67, 73, 39, 33, 35, and 68. The prevalence of high-risk types according to age is shown in Table 4.

4. Discussion

While HPV screening is not routinely practiced in Turkey, the findings of this study do not reflect HPV screening results in Turkey, but rather show the results of a selected risk group (patients with positive cytology, family history, and physician order) that underwent HPV testing. In this study 2 methods were used in combination: cytological examination and HPV DNA detection by PCR, followed by DNA sequence analysis. The main cytological classification was performed according to TBS 2001. In total, 1014 samples were examined, of which 170 (16.8%) were both cytologically and HPV DNA PCR-negative. Of 459 cytologically negative and 555 cytologically positive samples, 289 (63.0%) and 417 (75.1%) were HPV DNA-positive, respectively. HPV positivity was most common in the high-risk group (47.7%), followed by the low (17.9%), and intermediate (3.8%) risk groups, according to HPV type classification. Of the 1014 samples evaluated in this study, 484 were infected with HPV. The 3 most common HPV types detected were types 16 and 18 (high risk group) and type 6 (low risk group).

The most prevalent high-risk HPV types were 16 (14.2%), 18 (5.5%), 31 (4.5%), 56 (3.3%), 53 (2.6%), 45 (2.2%), 62 (2.1%), 58 (2.0%), 59 (1.8%), 67 (1.6%), 51 (1.6%), 35 (1.2%), 73 (1.2%), 52 (1.1%), 33 (0.9%), 39 (0.9%), 68 (0.7%), 82 (0.4%), and 86 (0.2%).

In another study in Turkey in which the presence of HPV-DNA was analyzed, 356 cervical smear samples were examined by 2 different methods, MY09/11 consensus real-time polymerase chain reaction (RT-PCR) and type-specific RT-PCR. Frequencies of detection of high-risk HPV types in the HPV-positive samples were as follows: HPV-16, 32 (33.7%); HPV-52, 12 (12.6%); HPV-58, 11

Table 2. The prevalence and distribution of HPV according to risk group in different cytological diagnostic categories.

	Cytologically negative	ASC-US	ASC-H	LSIL	HSIL	Total
HPV type (high-risk)						
Type 16	47	48	1	41	7	144 (14.2%)
Type 31	16	20	-	10	-	46 (4.5%)
Type 18	28	17	-	10	1	56 (5.5%)
Type 56	16	7	-	10	-	33 (3.3%)
Type 51	1	5	-	10	-	16 (1.6%)
Type 53	6	12	-	8	-	26 (2.6%)
Type 52	4	3	-	3	1	11 (1.1%)
Type 45	7	11	-	4	-	22 (2.2%)
Type 62	9	7	-	5	-	21 (2.1%)
Type 58	6	8	-	5	1	20 (2.0%)
Type 59	9	5	-	4	-	18 (1.8%)
Type 67	6	6	-	4	-	16 (1.6%)
Type 35	5	6	-	1	-	12 (1.2%)
Type 73	7	3	-	1	1	12 (1.2%)
Type 33	2	4	-	3	-	9 (0.9%)
Type 39	3	4	-	2	-	9 (0.9%)
Type 68	5	2	-	-	-	7 (0.7%)
Type 82	1	2	-	-	1	4 (0.4%)
Type 86	1	1	-	-	-	2 (0.2%)
Total sum of HPV type (high-risk)	179 (17.7%)	171 (16.9%)	1 (0.1%)	121 (11.9%)	12 (1.2%)	484 (47.7%)
Total sum of HPV type (intermediate-risk): type 66	13 (1.3%)	9 (0.9%)	-	16 (1.6%)	-	38 (3.8%)
HPV type (low-risk)						
Type 6	48	27	-	13	-	88 (8.8%)
Type 81	12	8	-	3	-	23 (2.3%)
Type 11	10	4	-	2	1	17 (1.7%)
Type 90	5	3	-	2	-	10 (1.0%)
Type 83	3	5	-	1	-	9 (0.9%)
Type 84	2	5	-	1	-	8 (0.8%)
Type 54	5	3	-	-	-	8 (0.8%)
Type 61	3	2	-	-	-	5 (0.5%)
Type 43	3		-	1	-	4 (0.4%)
Type 87	2		-	1	-	3 (0.3%)
Type 55	1	1	-	-	-	2 (0.2%)
Type 74	2		-	-	-	2 (0.2%)
Type 91		1	-	-	-	1 (0.1%)
Type 40	1	-	-	-	-	1 (0.1%)
Total sum of HPV type (low-risk)	97 (9.6%)	59 (5.8%)	0	24 (2.4%)	1 (0.1%)	181 (17.9%)
HPV-positive (undetermined-type)	-	3 (0.3%)	-	-	-	3 (0.3%)
Total sum of HPV-positive patients	289 (28.5%)	242 (23.9%)	1 (0.1%)	161 (15.9%)	13 (1.3%)	706 (69.6%)
Total sum of HPV-negative patients	170 (16.8%)	127 (12.5%)	2 (0.2%)	9 (0.9%)	0	308 (30.4%)
Total	459 (45.3%)	369 (36.4%)	3 (0.3%)	170 (16.8%)	13 (1.3%)	1014

ASC-US: atypical squamous cells of undetermined significance, HSIL: high-grade squamous intraepithelial lesion, ASC-H: atypical squamous cells of high significance, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion.

Table 3. HPV DNA type analysis according to cytologic diagnostic category.

HPV DNA type analysis	Cytologic diagnostic category		
	Negative	Positive	Total
Negative	170	138	308
Positive	289	417	706

Table 4. The prevalence of high-risk types according to age.

Type (high-risk)	Age					
	15–24 years	25–34 years	35–44 years	45–54 years	55–68 years	Total
16	8	84	46	5	1	144 (29.8%)
18	9	32	13	2	0	56 (11.6%)
31	4	27	13	1	1	46 (9.5%)
56	1	18	11	3	0	33 (6.8%)
53	0	15	8	3	0	26 (5.4%)
45	0	15	6	1	0	22 (4.5%)
62	0	11	7	3	0	21 (4.3%)
58	2	12	6	0	0	20 (4.1%)
59	0	10	8	0	0	18 (3.7%)
51	0	7	9	0	0	16 (3.3%)
67	1	8	5	2	0	16 (3.3%)
35	2	7	3	0	0	12 (2.5%)
73	0	7	5	0	0	12 (2.5%)
52	1	3	7	0	0	11 (2.3%)
39	0	5	4	0	0	9 (1.9%)
33	0	5	4	0	0	9 (1.9%)
68	0	5	2	0	0	7 (1.4%)
82	0	4	0	0	0	4 (0.8%)
86	0	2	0	0	0	2 (0.4%)
Total	28 (5.8%)	277 (57.2%)	157 (32.4%)	20 (4.1%)	2 (0.4%)	484

(11.6%); HPV-18, 7 (7.4%); HPV-31, 7 (7.4%); HPV-35, 7 (7.4%); HPV-68, 6 (6.3%); HPV-33, 4 (4.2%); HPV-82, 4 (4.2%); HPV-39, 3 (3.2%); and HPV-45, 2 (2.1%) (31). In this study, types 52 and 58 are more prevalent than type 18, which is different from the results of our study and the studies we compared our study with. This may be due to the different methods used, lower quantity of patients analyzed, and the period of analysis.

The prevalence of specific HPV types worldwide shows variation according to geographical differences. For example, in East Asia the most common HPV types in invasive cervical cancer were types 16, 18, 52, and 54, with prevalences of 57.1%, 17%, 11.4%, and 8.5%, respectively (32). In addition, according to a study in China, the most common and persistent high-risk HPV types were 16 (18.21%), 58 (13.2%), 18 (8.66%), 52 (7.06%), and 33

(6.78%). Their results showed that 45.6% of women were infected with HPV in China (33).

Additionally, HPV infection in Spain was detected in 43.2% of women between 15 and 75 years (34).

In Italy, it was determined that 35.9% of women between the ages of 15 and 54 were HPV-positive (35). In Turkey, the HPV infection rate is 2%–6%. As in this study, global results demonstrated that HPV 16 was the most prevalent infection (28%), whereas HPV 18 was found more rarely (34). For example, in Venezuela HPV 16 was the most common type (60%), followed by HPV 18 (20%), HPV 6 (10%), and HPV 58 (10%) (36). With the exception of East Africa, China, Japan, and Taiwan, HPV 16 is the most prevalent type in all parts of the world (34). Interestingly, HPV type 18 is detected at the same frequency all over the world. In the Chinese study mentioned above, type 18 (8.6%) was detected most frequently after type 16 (33). Type 18 was detected at a rate of 8% in the Madrid study mentioned above (34). In Turkey, according to our study, type 18 was detected at a rate of 5.5%. In our study, type 31 was the third most common genotype (4.5%), which was similar to rates determined in a previous Italian study (35). On the contrary, in China this type was detected in less than 6% of HPV infections, and type 58 (13.2%) was the second most frequent type (33). However, in our study type 58 was detected at a rate of 2.0% in contrast to the commonly detected types; certain HPV types are more frequently observed in some parts of the world than in others. HPV type 35 is detected at a rate of 1.2% in our study, but, interestingly, it is not even mentioned in the studies from other Mediterranean regions, such as Madrid or North Sardinia (34,35). In some parts of Italy and Spain, HPV type 53 was the second most common type; however, as in this study, in Mediterranean countries such as Turkey and Greece, it is not as common (2.6%) (33,35). In this study, as shown in Table 2, nearly a third (30%) of HPV-positive samples were found in patients with normal cytology. Although this rate is lower in some parts of Europe, a study from Madrid showed similar results (34).

This high prevalence of HPV in women with normal cytology might originate from women who had abnormal Pap tests attending a specialized gynecology unit for cervical pathology. Alternatively, such cytologically negative but HPV DNA-positive samples may indicate early-phase infection with no significant morphological change.

Detected HPV genotypes can be different according to cytologic diagnostic category. In normal cytology samples from our study, HPV 16 was the most common high-risk genotype (10.2%), followed by HPV 18 (6.1%). However, in atypical squamous cells of undetermined significance, although HPV type 16 was still the most common high-risk genotype (13.0%), type 31 was the second most common type, and type 18 was the third most common genotype (4.6%). In the high-grade squamous intraepithelial lesion category the most frequent type was type 16 (54%), followed by 18, 52, 58, and 73 (7.7%). In this group, type 16 was again the most common high-risk genotype. These results are different than the results found in Spain, another country in the Mediterranean region (34). In normal cytology samples in Spain, although HPV 16 was the most common high-risk genotype (21%), type 53 was the second (16%) most common type. In atypical squamous cells of undetermined significance, although HPV type 16 was still the most common high-risk genotype (30%), type 53 was the second most common type, and type 31 was the third most common genotype (11.3%). In the high-grade squamous intraepithelial lesion category the most frequent type was 16 (50.6%) followed by 52 (13.9%), 31 (11.4%), and 33 (10.1%). In this group, type 16 was again the most common high-risk genotype.

As shown in Table 3, of the 555 samples that were positive cytologically, 138 (24.9%) were HPV DNA-negative and 417 (75.1%) were positive for HPV DNA. This distribution may indicate early-phase infection, at which point no morphological change has occurred.

The HPV types were similar in the 25–34 and 35–44 age groups. In another study in Turkey, HPV DNA positivity was detected in 38% of the female population between 17 and 30 years and in 5.1% of women over 55 years old (28). HPV-positive women were commonly found to be sexually active and of childbearing age. As is shown in Table 4, in the early age group (15–24), as well as the 25–34 and 35–44 age groups, type 18 was the second most frequently detected type. However, in the 45–54 age group it was the fifth most frequent type. Thus, its frequency decreased with age. Another interesting aspect of HPV type 18 is that its presence was significantly associated with adenocarcinoma and lymphatic metastasis ($P < 0.05$). Furthermore, HPV 18 persistence was associated with a cervical cancer prognosis ($P < 0.0001$) (33).

Recently, multiple HPV infections have been examined because with the development of HPV vaccines that do not cover all genotypes the distribution of infection with types not covered by vaccines could be impacted; the elimination of one HPV type could affect the natural history of the remaining genotypes. Therefore, obtaining a solid knowledge of genotype HPV distribution is becoming increasingly critical (34). As expected, a proportion of the patients in this study probably had multiple infections. It was not possible to detect more than one infecting agent based on the methods used in this study. HPV DNA-positive patients were typed according to their DNA sequences. Therefore, only the most prevalent type was likely reported. The HPV prevalence data in this study

differed from other study results in Turkey, as well as results from other countries, likely because our patients were referred by their gynecologists due to suspicion of HPV infection upon physical examination. However, the HPV types we report here are generally similar to the worldwide distribution, although a few different types, such as 66, 56, and 53, were common in samples from this study. The McNemar paired proportion test results of this study indicated that the 2 methods used differed significantly ($P < 0.0001$), suggesting that they are complementary. This result supports the use of our protocol, suggesting that the combined use of cytological and molecular typing methods yields a more precise result. The conventional Pap smear is the most effective cervical cancer-screening test, and PCR is a sensitive method for detecting and genotyping HPV DNA in normal and abnormal ThinPrep samples. This technique is extremely useful for routine investigation and facilitates better patient clinical management. Combining molecular testing with morphology analysis for cervical screening increased the sensitivity and reliability of HPV detection and typing.

The importance of treatment and prophylaxis for HPV can clearly be recognized when the 493,000 new cases of cervical cancer and 274,000 deaths per year are taken into consideration. The main strategy should be to develop prophylactic and therapeutic vaccines (37).

In conclusion, this study confirmed the high prevalence of HPV infection in Turkey and highlighted regional differences according to risk genotypes. Moreover, this study provides an important database for future research studies due to its wide patient spectrum.

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