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HPV Types and Variants Among Cervical Cancer Tumors in Three Regions of Tunisia

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Cervical cancer is the second most common cancer among Tunisian women, and the incidence rates vary by region. Three Tunisian registries report age-standardized rates of 6.3/10^5 in the central region, 5.4/10^5 in the north, and 2.7/10^5 in the south. High-risk human papillomavirus (HPV) types and their variants differ in carcinogenic potential and geographic distribution. The HPV type and variant distribution could be a factor in the differing rates between regions of Tunisia. Tumor tissue was collected from 142 Tunisian cervical cancer patients. Demographic and reproductive characteristics of the patients were abstracted from cancer registry and hospital records. HPV type and variant analyses were performed using PCR-based Luminex and dot-blot hybridization assays. Eighty-three percent of tumors were infected with at least one HPV type. European variants of HPV16/18 were the most prevalent in tumors from all three regions, with all HPV18 infections and 64% of HPV16 infections being of European lineage. A higher frequency of HPV16 was present in Northern Tunisia (80%) than in Central (68%) or Southern Tunisia (50%) ($P = 0.02$). HPV18/45 was significantly more common in adenocarcinomas (50%) than in squamous cell carcinomas (11%) ($P = 0.004$). Frequent infection with European HPV variants most likely reflects the history of European migration to Tunisia. In addition to the importance of understanding the variants of HPV in Tunisia, behavioral and cultural attitudes towards screening and age-specific infection rates should be investigated to aid the development of future vaccination and HPV screening programs and policies. ***J. Med. Virol. 83:651–657, 2011.*** © 2011 Wiley-Liss, Inc.

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INTRODUCTION

Cervical cancer is the second most common cancer among women worldwide, with 80% of disease burden occurring within developing countries [WHO/ICO, 2010]. Cervical cancer is estimated to be responsible for 493,243 incident cases and 273,505 deaths annually, amounting to 8% of the total burden of cancer among women worldwide [Castellsague et al., 2007; WHO/ICO, 2010]. Starting in the 1950s and 1960s, women in developed countries have benefited immensely from organized cytological screening programs, with mortality rates falling to between 3/10^5 and 8/10^5 [Boyle and Levin, 2008]. In developing countries where screening

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is less prevalent, mortality rates remain between $10/10^5$ and $25/10^5$, and incidence rates have been recorded to exceed $25/10^5$ [Boyle and Levin, 2008]. Cervical cancer incidence in North Africa, where generally conservative Muslim culture forbids sexual intercourse before marriage, is considerably lower than not only other regions of the world, but also other regions of Africa [Castellsague et al., 2007]. The lower cervical cancer incidence in North Africa is similar to that seen in developed countries, despite much lower screening coverage.

Tunisia has an especially low cervical cancer rate within North Africa. At an age-standardized rate (ASR) of $6.8/10^5$, Tunisia's cervical cancer rate is nearly half of what is reported in Morocco (14.1) and Algeria (10.4) [Ferlay et al., 2008; WHO/ICO, 2010]. Tunisia also has geographical variation of cervical cancer rates between its three cancer registry regions, with rates of $5.4/10^5$ and $6.3/10^5$ in the northern and central registries, respectively [Korbi et al., 2008; Ben Abdallah et al., 2009]. The southern registry, however, reports an ASR of only $2.7/10^5$, excluding cervical cancer from the top ten most common incident female cancers in that region [Sellami et al., 2002]. Tunisia's prohibition of marriage until age 17, the prohibition of polygamy, and cultural stigma associated with sexual intercourse before marriage most likely contribute to its low rates of cervical cancer [Ben Abdallah, 1997; Maalej et al., 2004]. However, Morocco and Algeria also both have a minimum marriage age of 18 for women [An-Na'im, 2010] and similar cultural stigmas associated with premarital sex.

Development of cervical neoplasia is known to be causally associated with infection by high-risk types of genital human papillomaviruses (HPV), members of the genus *Alphapapillomavirus* of the family *Papillomaviridae* [Bernard et al., 2010]. Of the over 120 HPV types that have been identified, approximately 40 (mucosal) types of HPV have been shown to infect the anogenital tract of which approximately 13 can lead to cancer, with HPV types 16 and 18 accounting for 2/3 of cervical cancer cases worldwide [Schiffman et al., 2009; Li et al., 2011].

HPV classification is based on sequence similarity of the viral capsid (L1) gene. Types and variants are defined as those strains with at least 90% similarity and generally greater than 95% similarity, respectively [Bernard et al., 2010]. Intra-typic variants of HPVs 16 and 18 have been the most well-studied and characterized to date. Four phylogenetic variant branches of HPV16 have been identified, labeled as European (including an Asian clade), Asian/American, African-1 and African-2 as they correspond to their most commonly occupied geographic regions [Chen et al., 2005]. African and non-African variant lineages have also been described for HPV18 [Chen et al., 2009]. These variants are geographically distributed and suggest HPV has coevolved with human population migration [Ho et al., 1993]. Differences in carcinogenic potential have also been shown for different HPV16 variants, with non-European variants being found more frequently in aggressive and high-grade cervical cancer lesions [Schiffman et al., 2010]. Studies in India, Thailand, Brazil, Italy, Ecuador, Portugal, and Mongolia have shown minor differences in the frequency of European and non-European variants of HPV16 and 18 [Tornesello et al., 2004, 2008; Pista et al., 2007; Chimeddorj et al., 2008; Junes-Gill et al., 2008; Pande et al., 2008; Chopjitt et al., 2009; Pillai et al., 2009].

To date, little HPV variant research has been performed in Tunisia or North Africa. Tunisia presents an interesting population in which to study HPV variants due to the historical record of population movements through North Africa, as well as North Africa's striking intra- and inter-country incidence differences. One would expect higher rates of European HPV16 due to historical and continued travel both to and from Tunisia and Europe. Thus, the aim of this study was to investigate the types and variants of HPV among Tunisian cervical cancer patients. An additional aim was to study the relationship between HPV variants and Tunisia's intra-country variation in cervical cancer rates.

MATERIALS AND METHODS

Sample Collection and Data Abstraction

The study was conducted at the Institut Salah Azaiez (ISA) in Tunis, and the Hôpital Farhat Hached (HFH) in Sousse, between May 2009 and April 2010.

Inclusion criteria for cases from both centers included cancers that were primary, had a histopathologically confirmed diagnosis of invasive cervical cancer and had a paraffin-embedded cervical cancer tissue sample available for sectioning. Cases for which cervical cancer was a secondary cancer to another diagnosis, or had a previous history of any cancer type were excluded. Cases were also excluded if the medical or registry record could not be located.

ISA is the primary breast and cervical cancer referral center in Tunisia, and is home to the population-based Northern Tunisian Cancer Registry as well as a hospital-based registry [Ben Abdallah, 1997; Ben Abdallah et al., 2009]. The pathology database of the Anatomy-Pathology department of ISA was searched for all cervical cancer diagnoses (excluding CIS) for which a tissue sample was retrieved and analyzed at the institute in 2007 and 2008. Of the 262 cases identified through this method, 27 had an inadequate tissue sample, 23 medical records could not be found, and 85 were diagnosed outside of ISA and thus did not have a tissue sample available for sectioning. Of the remaining 127 medical records that were abstracted, 32 were excluded due to clinical exclusion criteria, 1 paraffin block could not be located, and 8 cases had come from Sousse governorates. These eight were excluded due to the possibility of being doubly included when later abstraction occurred at the Central Tunisian Cancer Registry in Sousse. A total of 86 samples were cut into 10 μm sections and

transported back to the United States for HPV testing. Of these 86 cases, 13 were from governorates included in the Southern Tunisia Cancer Registry.

The HFH Anatomy-Pathology department houses the Central Tunisian Cancer Registry. Registry records were used to extract the same reproductive and demographic information that was taken from ISA. Registry data were examined for all 204 cervical cancer diagnoses included in the registry between 2002 and 2007. Of these, 35 had been diagnosed outside the hospital and thus had no pathology report associated with them. Another 15 had no pathology record for unknown reasons, 18 had no medical record information in the registry, and 2 were from southern governorates that had no way of being matched to the southern cases already abstracted from ISA and thus were excluded. Cases recorded in the ISA hospital registry and pathology database do not overlap with cases in the HFH registry. To ensure no overlap occurred, the governorate of each case from ISA was checked to ensure that its origin was in the northern registry region, and the same was done for HFH cases and the central registry region. Of the remaining 134 cases, 56 had an existing paraffin block sample that could be sectioned for analysis. Ten-micron sections were cut and transported to the United States for HPV testing.

HPV Type and Variant Analyses

A total of 142 samples were transported to the Albert Einstein College of Medicine. After DNA extraction and PCR amplification of target sequences, Luminex and dot-blot hybridization assays were used to identify HPV types and variants. Probes for sequences within E1, E2, E6, E7, and L2 were used to identify variants for HPV types 16 and 18. HPV16 and HPV18 variants were classified as either European or non-European, as previously described [Chen et al., 2005, 2009]. Probes were also used to detect HPV types 16, 18, 26, 30, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82, 85, and 97. Beta-globin amplification was included as a measure of sample adequacy for PCR. Samples neither PCR positive for HPV nor beta-globin were considered inadequate for HPV testing. One case was excluded due to suspected contamination during the cutting process.

The study was approved by the Institutional Review Boards of the University of Michigan and the Albert Einstein College of Medicine. All statistical analyses were performed using SAS version 9.2. Associations were tested using standard chi-square, Student's *t*-test, and odds ratio tests.

RESULTS

Of the 142 cases collected, 73 were from the northern registry catchment area, 56 from the central registry catchment area, and 13 from the southern registry catchment area. The average age at cancer diagnosis for all cases was 55 years, and differed significantly between regions, reaching 58 years in Northern Tunisia, 51 years in Central Tunisia, and 54 years in Southern Tunisia (Table I). The average age at menstruation and marriage also differed significantly between regions, with the highest average age at menstruation (15 years) being seen in Southern Tunisia, while the highest age at marriage (20 years) was seen in Northern Tunisia (Table I). There were no significant regional differences in the age at first pregnancy, menopause, nor in a variety of reproductive variables. Interestingly, the average duration of symptoms before seeking treatment was 6 months for all 142 cases, and reached a high of 36 months in one case, although no difference was seen between regions (Table I).

The majority of cases were diagnosed at stages II and III, representing 41%, and 33% of cases, respectively (Table II). There were no differences seen between regions in the frequencies of stage at diagnosis (Table II). There was a statistically significant difference in histopathological status between regions, with the highest rate of squamous cell carcinoma being found in Central Tunisia (98%), followed by Northern Tunisia (82%), and Southern Tunisia (69%) ($P = 0.01$) (Table II).

One case was excluded from HPV testing due to suspicions of contamination during the sectioning process. Of the remaining 141 cases, 117 (83%) were infected with at least one HPV type (Table III). Among all cases, the frequency of infection with HPV16 and/or HPV18 was 70% (Table III). HPV16 and HPV18 were present in 73% and 10% of cases infected with a single HPV type (n = 105) respectively (Table III). The frequency of single infections with either HPV16 or HPV18 differed significantly between the three registry regions, with a higher frequency (80%) of HPV16 present in Northern Tunisia than in Central Tunisia (67.6%) or Southern Tunisia (50%) ($P = 0.02$, Table III). HPV types 30, 31, 35, 39, 45, 56, 58, 66, 68, and 73 were also identified (Table III). Among squamous cell carcinomas infected with one HPV type (n = 84), 76% were infected with HPV16, and 5.9% were infected with HPV18 (data not shown). Among adenocarcinomas infected with one HPV type (n = 13), 46% were infected with HPV16, and 38% were infected with HPV18 (data not shown). When restricted to only those cases singly infected with HPV16 or HPV18 and/or 45, HPV18/45 was significantly more common in adenocarcinomas than squamous cell carcinomas (50% vs. 11%, $P = 0.004$, data not shown). Multiple infections were identified in 12 cases (Table III).

All of the HPV18 and 64% of HPV16 positive cases were of the European variant lineage (Table III). The distribution of European variants among HPV16 single infections was 66% in Northern Tunisia, 61% in Central Tunisia, and 67% in Southern Tunisia (Table III). Cases infected with a non-European variant of HPV16 had an earlier average age at diagnosis than European variant infections (53 years vs. 56 years, respectively) although this difference was not significant. There was a higher proportion of non-European HPV16 variants in Stage III cancers (50%) than Stage I cancers (38%), although this difference was not significant and did not significantly follow a linear trend.

TABLE I. Demographic and Reproductive Characteristics Among Abstracted Cervical Cancer Cases, Tunisia, 2002–2008[a]

Variable	All cases			Northern Tunisia			Central Tunisia			Southern Tunisia			P
	Mean (SD)	Min	Max	Mean (SD)	Min	Max	Mean (SD)	Min	Max	Mean (SD)	Min	Max	
Age at diagnosis (141, 73, 55, 13)[b]	55 (14)	28	95	58 (14)	30	95	51 (14)	28	82	54 (9)	40	71	0.02*
Age at menstruation (111, 60, 38, 13)[b]	13 (2)	9	20	13 (2)	9	20	13 (2)	10	16	15 (1)	12	17	0.01*
Age at marriage (82, 66, 3, 13)[b]	20 (5)	12	37	20 (5)	12	37	14 (2)	13	17	18 (4)	12	26	0.03*
Age at first pregnancy (69, 55, 2, 12)[b]	21 (4)	13	31	21 (4)	13	31	23 (4)	20	25	19 (4)	15	27	0.5
Age at menopause (60, 35, 18, 7)[b]	48 (6)	31	59	49 (6)	31	59	46 (7)	33	57	48 (5)	41	55	0.3
Duration of symptoms (months) (101, 47, 44, 10)[b]	6 (8)	0.1	36	5 (7)	0.1	36	7 (9)	0.3	36	8 (9)	1	24	0.3
Number of pregnancies (128, 62, 54, 12)[b]	6 (3)	0	13	6 (3)	0	13	6 (3)	0	13	7 (2)	3	10	0.5
Number of births (135, 68, 54, 13)[b]	5 (3)	5	13	5 (3)	0	12	5 (3)	0	13	6 (2)	2	10	0.2
Number of miscarriages (94, 55, 27, 12)[b]	0.5 (1)	0	7	0.7 (1)	0	7	0.2 (0.8)	0	4	0.4 (0.7)	0	2	0.2
Number of induced abortions (93, 54, 27, 12)[b]	0.4 (1)	0	5	0.6 (1)	0	5	0.1 (0.3)	0	1	0.3 (0.5)	0	1	0.1
Number of terminated pregnancies (123, 57, 54, 12)[b]	1 (1)	0	7	1 (1)	0	7	1 (1)	0	4	0.7 (0.7)	0	2	0.1

*Statistically significant difference between regions at $P = 0.05$.
[a]Includes HPV-negative, untyped, and multipally infected cases.
[b]Values in parentheses represent n for all cases, Northern Tunisia, Central Tunisia, and Southern Tunisia, respectively.

DISCUSSION

This is the first study to report on the distribution of HPV16 and HPV18 variants in North Africa from all three cancer registry regions of Tunisia. Eighty-three percent of cases included in the study were infected with at least one HPV type, and 70% of all cases were infected with either HPV16 or 18, including one case that was infected with both. There was a slightly higher prevalence of non-European HPV16 in the Central Tunisia region, where the incidence rate is highest, although this difference was not significant. European variants of HPV16 were found to be more prevalent than non-European variants. It should be noted that this prevalence of HPV positivity among sampled cases is likely to be an under-estimate of the actual prevalence of HPV positivity. The process of preserving tissue in formalin-fixed paraffin embedded blocks can result in a loss of DNA integrity over time.

This study's finding of HPV16/18 present in 70% of Tunisian cases is comparable to the North African HPV16/18 prevalence of 72.5% estimated by the HPV Information Centre of the World Health Organization (WHO) [WHO/ICO, 2010]. HPV16 was found to be significantly more frequent in Northern Tunisia than in the other two regions. This could possibly be due to the low rate of included adenocarcinomas from the Central Tunisia region. Due to data storage differences between registry regions, few adenocarcinomas were collected from the Central Tunisia region. This is the most likely reason for the observed significant difference in the proportion of adenocarcinomas between regions shown in Table I. The highest proportion of squamous cell carcinoma was found in Central Tunisia (98%), followed by Northern Tunisia (82%), and Southern Tunisia (69%). These proportions differ slightly from the histopathological frequencies reported in the registries, which report squamous cell carcinoma frequencies of 78.2% in Central Tunisia, 75.9% in Northern Tunisia, and 92.3% in Southern Tunisia [Korbi and Descoteaux-Chatti, 1995; Sellami et al., 2002; Ben Abdallah et al., 2009]. The overall frequency of squamous cell carcinoma in all included cases in this study (86%) is not significantly different from a Tunisian study of all cervical cancer cases diagnosed in 1994 (91.23% squamous cell carcinoma) [Maalej et al., 2004]. However, when data from this study were restricted to only those cases singly infected with HPV16 or HPV18/45, HPV18/45 was significantly more common in adenocarcinomas (50%) than in squamous cell carcinomas (11%, $P = 0.004$), a commonly found association [Altekruse et al., 2003].

There have been very few HPV typing studies performed among Tunisian women. A study in legal prostitutes working in Tunis, found an HPV16 prevalence of 38%, an HPV58 prevalence of 27%, an HPV82 prevalence of 15%, and a lack of any HPV18-infected cases [De Marco et al., 2006]. An additional study from Sousse, Tunisia, reported an HPV16 prevalence of 37% [Hassen et al., 2003]. However, both of these studies had limited sample sizes (64 and 51, respectively), and

TABLE II. Histopathology, Stage, and Menopausal Characteristics of Abstracted Cervical Cancer Cases, Tunisia, 2002–2008[a]

Variable	All cases	Northern Tunisia	Central Tunisia	Southern Tunisia	P^*
Histopathological type (n = 130)[b]					
Squamous cell carcinoma	112 (86%)	59 (82%)	44 (98%)	9 (69%)	0.01**
Adenocarcinoma	18 (14%)	13 (18%)	1 (2%)	4 (30%)	
Stage at diagnosis (n = 124)[c]					
I	21 (17%)	11 (16%)	9 (20%)	1 (8%)	0.51
II	51 (41%)	30 (45%)	14 (32%)	7 (54%)	
III	41 (33%)	21 (31%)	15 (34%)	5 (38%)	
IV	11 (9%)	5 (8%)	6 (14%)	0	
Menopausal status (n = 94)					
Pre-menopausal	13 (14%)	8 (15%)	4 (12%)	1 (12%)	0.95
Post-menopausal	81 (86%)	46 (85%)	28 (88%)	7 (88%)	

*Represents chi-squared test for independence between the three registry regions.
**Significant due to abstraction differences between the two data collection sites.
[a]Includes HPV-negative, untyped, and multipally infected cases.
[b]Does not include CIS, CNOS.
[c]Does not include stage = 0 (CIS).

were sampling only legal prostitutes. This study is the first published to date to report HPV type prevalence among diagnosed cervical cancer cases in Tunisian women.

The results of this study show a slightly higher frequency of HPV16 non-European variants present in Central Tunisia than in the other two regions. As non-European HPV16 variants are more often associated with more aggressive cancers diagnosed at later stages [Sichero et al., 2007], it is possible that cervical cancers caused by non-European HPV16 cause more women in Central Tunisia to progress to cancers

TABLE III. HPV Type and Variant Frequencies Among HPV-Positive Cervical Cancer Cases, Tunisia, 2002–2008

HPV type	All cases	Northern Tunisia	Central Tunisia	Southern Tunisia	P
All infections					
16	85 (60%)	52 (91%)	27 (84%)	6 (67%)	—
18	12 (9%)	5 (9)	4 (13%)	3 (33%)	
16/18	1 (1%)	0	1 (3%)	0	
Total	98 (70%)	57	32	9	
Single infections[a] (n = 105)					
16 (all variants)	77 (73%)	48 (80%)	25 (68%)	4 (50%)	0.02*
16E[b]	41 (64%)	25 (66%)	14 (61%)	2 (67%)	0.92[c]
16NE[b]	23 (36%)	13 (34%)	9 (40%)	1 (33%)	
18E	10 (10%)	4 (7%)	3 (8%)	3 (38%)	
30	1 (1%)	1 (2%)	0	0	
31	2 (2%)	1 (2%)	1 (3%)	0	
35	1 (1%)	0	1 (3%)	0	
39	1 (1%)	1 (2%)	0	0	
45	4 (4%)	0	3 (8%)	1 (12%)	
56	2 (2%)	1 (2%)	1 (3%)	0	
58	1 (1%)	0	1 (3%)	0	
66	3 (3%)	2 (3%)	1 (3%)	0	
68	2 (2%)	1 (2%)	1 (3%)	0	
73	1 (1%)	1 (2%)	0	0	
Multiple infections					
16/18	1	0	1	0	
16/35	1	0	0	1	
16/45	1	0	0	1	
16/45/66	1	1	0	0	
16/56	1	1	0	0	
16/58	1	1	0	0	
16/66	1	0	1	0	
16/73	2	1	1	0	
18/35	1	0	1	0	
18/45	1	1	0	0	
35/70	1	1	0	0	

*Significant at $P = 0.05$ using chi-squared test for independence between the three regions, restricted to cases positive for HPV16 and HPV18.
[a]Percentages will not add up to 100 due to inclusion of HPV16 variants; see footnote below.
[b]Percentage represents percentage of each variant among cases positive for HPV16.
[c]Chi-squared test for independence between the three regions, restricted to cases positive for HPV16E and HPV16NE.

necessitating treatment. The frequencies of Stage III and Stage IV cancers were slightly higher in Central Tunisia than Northern Tunisia, although staging differences between regions were not statistically significant. It should also be noted that cases infected with non-European variants of HPV16 had a slightly younger age at diagnosis.

The primary strength of this study is that it is the first to date to investigate HPV variants in North Africa or Tunisia. Over 40 HPV types including all of the "high risk" types were tested. Cervical cancer cases from nearly all 27 governorates of Tunisia were represented, giving as many opportunities as possible to detect differences in HPV16 and 18 variant frequencies between regions. Unfortunately, the collection of only 13 samples from the Southern Tunisian Cancer Registry catchment area limits any conclusions about the lower cervical cancer rate seen in that part of the country.

This study also has a few limitations. Only confirmed cervical cancer cases were included, and comparable variants studies done in other countries typically include a higher proportion of in situ and control cases. Other limitations of the present study include a limited sample size. Of the 262 identified cases at ISA and the 204 identified at HFH, only 86 (33%) and 56 (42%) could be sampled for HPV testing, respectively. Sampled cases as a percentage of the total number of women with cervical cancer between 2002 and 2008 would be even lower due to the fact that not all women suffering from cervical cancer seek treatment at a health facility. However, both of these centers are major referral centers for cervical cancer cases in their respective regions, and it can be expected that they receive a high percentage of the women who do decide to seek care.

The paradox of Tunisia's low cervical cancer rate has not gone unnoticed, however, little research has been done to investigate the phenomenon [Ben Abdallah, 1997; Hassen et al., 2003; Maalej et al., 2004]. Previous cervical cancer research in Tunisia has largely been limited to behavioral studies related to screening practices and access to care. Studies investigating screening practices in Tunisia show a high acceptance of cytological screening, but a very low frequency of performance by physicians and midwives, and a low frequency of uptake among women [Njah et al., 1994; Hsairi et al., 2003]. The high prevalence of European HPV variants most likely reflects the history of European migration to Tunisia. In addition to the importance of understanding the variants of HPV in Tunisia, behavioral and cultural attitudes about screening and age-specific infection rates should be investigated to aid the development of future vaccination and HPV screening programs and policies.

The presence of high-risk HPV types in Tunisian cervical cancer patients and the low uptake of regular cervical cancer screening could make Tunisia a good candidate country for one-time HPV testing implementation instead of cytological screening programs. A 2009 study in India by Sankaranarayanan et al. [2009] showed one-time HPV testing to be more effective at reducing cervical cancer mortality and lowering the rates of late-stage cervical cancer diagnosis than cytological smears or visual inspection with acetic acid. As the majority of cervical cancer cases in this study were diagnosed at Stage II or later, it appears that the Tunisian population could benefit from screening using HPV DNA testing. Additionally, the implementation of HPV vaccination against HPV16/18 in Tunisia could potentially eliminate 2/3 of the country's cervical cancer cases.

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