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HPV genotyping in cervical cancer in Northern Thailand Adapting the linear array HPV assay for use on paraffin-embedded tissue

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Abstract

Objectives. The aims of this study were to determine the prevalence of HPV infection and distribution of HPV genotypes in Northern Thai women and thereby estimate the benefit of administering the HPV vaccine in the population.

Methods. Formaldehyde-fixed, paraffin-embedded samples of invasive squamous cell carcinoma from 99 patients were tested for HPV genotypes using the Linear Array HPV Genotyping Test.

Results. HPV was detected in 96/99 (96.9%) cases. Seventy-five (78.1%) cases were single infections and 21 (21.9%) multiple. HPV16 and HPV18 were the most common subtypes, detected in 62/96 (64.4%) cases. HPV52 and HPV58 infections were found in 17/96 (17.7%) cases. Co-infection always involved HPV16. The most common co-infection was HPV16 and 52 (7 cases) but never HPV16 and 18.

Conclusions. Although the prevalence of HPV infection in cervical cancer of Northern Thai women is comparable to the other regions worldwide, the distribution of HPV subtypes differs with lower frequencies of HPV16 and 18, and higher frequencies of HPV52 and 58. Moreover, multiple infections are common. The vaccine against HPV16 and HPV18 can be estimated to prevent approximately two thirds of the cervical cancer cases in Northern Thailand. Although designed for use on unfixed tissue, this study shows that the Linear Array HPV Genotyping Test can be successfully used for HPV genotyping on paraffin-embedded archival tissue. This methodology also provides a means for retrospective studies on serial samples for a greater understanding of HPV genotypes, co-infections, and relationship to cervical cancer.

Keywords: Human papillomavirus (HPV); Uterine cervix; Squamous cell carcinoma; Genotyping; Linear array

Introduction

Although the incidence of and the mortality from cervical cancer have declined substantially over recent decades [1], cervical cancer is still one of the most common cancers in women worldwide [2]. Cervical cancer rates as well as mortality rates are lowest in industrialized nations and highest in less developed countries, including those of Latin America, sub-Saharan Africa, and Southeast Asia [3].

Cervical cancer is the most common cancer among Thai women with approximately 6300 new cases per year and an age-standardized incidence rate (ASR) per 100,000 of 19.5 [4]. The ASRs vary between the four geographical regions of the country. The highest incidence is in the north (Chiang Mai,

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ASR = 25.6), followed by the central region (Bangkok, ASR = 20.7), the south (Songkhla, ASR = 16.1), and the north-east (Khonkaen, ASR = 15.0).

Infection with human papillomavirus (HPV) is a necessary step in the development of cervical cancer [5]. Not all HPV genotypes are equally associated with cervical cancer; only a subset are considered to be high-risk, including subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, 73, and 82 [5]. This knowledge has an impact on both primary and secondary prevention of cervical cancer. For primary prevention, the approach taken was to develop an HPV vaccine and, recently, a quadrivalent HPV prophylactic vaccine (GARDASIL®, Merck & Co., USA) has become available in many countries including Thailand. This vaccine is type-specific (for HPV6, 11, 16 and 18), and protection against cancer is expected to be in the 65–75% range, depending on the distribution of HPV genotypes in the population [6]. For secondary prevention, type-specific HPV testing has been proposed as an additional biomarker to stratify women according to risk for precancerous lesions and cancer [7]. Detection of high-risk HPV genotypes can be used as an adjunct to routine cytologic examination in primary screening for cervical precancerous states, management of borderline abnormal cytology (ASCUS) and follow-up of women treated for high-grade squamous intraepithelial lesions (HSIL).

HPV subtyping can be done by molecular genetics or in situ hybridization. The latter is easier for most diagnostic laboratories since the techniques are very similar to immunoistochemistry and can be done on automated staining machines. As well, results can be directly correlated with histologic findings. The disadvantages are that each subtype or mixtures of subtypes have to be tested on separate tissue sections adding time and expense to the test. As well, this technique is not ideal for samples with limited material (cytology smears and small biopsies). Molecular genetic techniques are based on nucleic acid hybridization to pooled extracted DNA from patient samples. Two molecular diagnostic kits approved by the United States Food and Drug Administration (US FDA) are the Hybrid Capture Tube test and the Hybrid Capture 2 test (HC2), produced by the Digene Corporation (Gaithersburg, Maryland). The kits detect 13 high-risk types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). However, results are reported as positive or negative and are not type-specific.

The Linear Array HPV Genotyping Test (Roche Molecular System, Inc., Branchburg, NJ) is designed to identify up to 37 HPV genotypes including 14 high-risk HPV in a patient sample. The manufacturer recommends that this kit be applied to fresh cytologic specimens collected in PreservCyt® solution. Recently, this kit has been used successfully on archival frozen tissue and DNA samples [8].

Our objectives were to determine the prevalence of HPV infection in Northern Thai women, explore the distribution of HPV genotypes, and with these data, estimate the potential benefit of administrating HPV vaccine in this population. Since the material available for this study consisted of paraffin-embedded tissue samples of cervical cancer, we chose to adapt the Linear Array HPV Genotyping Test for use on this type of material. We believe this to be the first application of this test on paraffin-embedded tissue.

Materials and methods

Patients and tissue samples

Formaldehyde-fixed, paraffin-embedded tissues from cases of invasive squamous cell carcinoma of the cervix were collected for this study. The samples were obtained from 99 cases treated at Chiang Mai University Hospital during the period 2004–2006, including hysterectomy specimens in 96 cases and biopsy in 3. This study was approved by the institutional ethics committee.

DNA extraction

Twenty-five μm of material (5 × 5 μm sections) was cut from the paraffin block from each case (or representative block when more than one were available). After deparaffinization in xylene and rehydration in ethanol, DNA was extracted from the tissues using QIAamp Tissue kit (Qiagen) following the manufacturer's protocol. A final volume of 40 μl was stored prior to the PCR reaction.

HPV testing with L1-consensus primers

All samples were screened for the first PCR amplification with primers MY09/MY11 located within the HPV *L1* gene. The amplified DNA samples were re-amplified using nested PCR and primers GP5+ and GP6+ according to a previously published protocol [9]. For the first-step PCR, 3 μl of DNA solution was used with a final volume of 25 μl. DNA samples that were negative by MY09/MY11 were re-amplified using 3 μl of the first PCR product as a template for the nested PCR. To determine that all samples originally contained DNA of sufficient quality and quantity, samples were co-amplified for the presence of an internal standard, in this case, beta globin. The amplification reaction was performed with initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C (MY09/MY11, globin sense/antisense), 53 °C (GP5+/GP6+) for 1 min, and extension at 72 °C for 1 min, and with a final extension at 72 °C for 5 min. The amplified products were run on a 2% agarose gel and stained with ethidium bromide for size verification (Fig. 1). The primer sequences were as follows:

MY09: 5′-CGTCCMARRGGAWACTGATC-3′,
MY11: 5′-GCMCAGGGWCATAAYAATGG-3′
(M = A or C, R = A or G, W = A or T, Y = C or T),
GP5+: 5′-TTTGTTACTGTGGTAGATACTAC-3′,
GP6+: 5′-GAAAAATAAACTGTAAATCATATT-3′,
Globin sense: 5′-ACACAACTGTGTTCACTAGC-3′, and
Globin antisense: 5′-TTCTCTGTCTCCACATGCCC-3′.

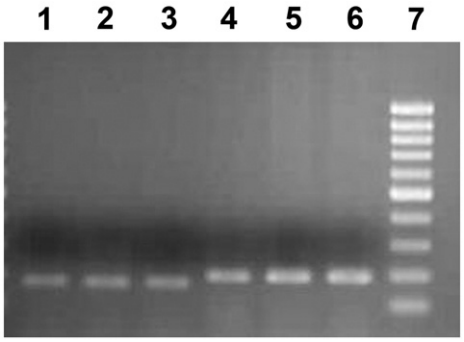

Fig. 1. PCR amplification of genomic DNA from cervical tissue. Lanes 1–3, nested PCR with GP5+/GP6+; lanes 4–6, PCR for control gene beta-globin; lane 7, 100-bp DNA ladder.

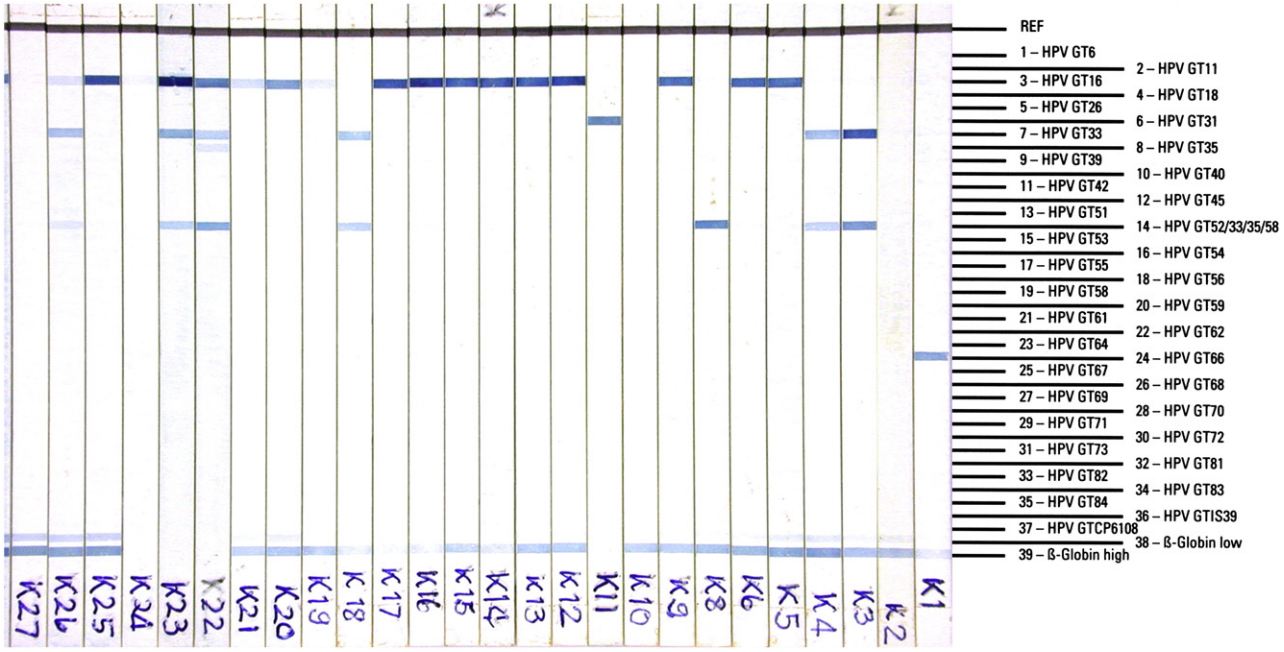

Fig. 2. Linear array results from 26 patients in the study showing positive hybridization signals for HPV subtypes.

HPV typing by Linear Array HPV Genotyping Test

Samples that were positive for HPV by the approach above were then analyzed by the Linear Array HPV Genotyping Test. This test as described by the manufacturer involves three steps: PCR amplification of target DNA, nucleic acid hybridization, and finally detection of up to 37 anogenital HPV genotypes. We modified the method to start with the PCR product obtained above rather than non-amplified genomic DNA extracted from fresh or frozen patient material. Briefly, PCR was performed in a total volume of 100 μl containing 50 μl of the manufacturer's master mix and 50 μl of patient DNA. Biotinylated primers were used for amplification. Amplification was performed in a Perkin-Elmer Gene Amp 9700. The program consisted of 2 min at 50 °C, and 9 min at 95 °C, followed by 40 cycles of 30 s at 50 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were denatured with denaturing solution and hybridized on to the strip containing specific probes for 37 HPV genotypes and beta globin reference lines. Detection was carried out using streptavidin–horseradish peroxidase and 0.1% 3,3′,5,5′-tetramethylbenzidine (TMB) as the chromagen. A positive reaction is visible as a blue line on the strip (Fig. 2).

Statistical analysis

The differences in frequencies of HPV subtypes between various institutes were tested by Chi-square test. Intercooled Stata version 8.0 was used for the data summarization and statistical analysis.

Results

For the 99 cases of invasive squamous cell carcinoma, the ages ranged from 27 to 69 years (mean 45.98±9.71 years). The distribution by FIGO stage was: stage IB1, 63 (63.6%); IB2, 21 (21.2%); IIA, 13(13.1%); IIB, 1(1.0%); and IIIB, 1(1.0%). Ninety-three specimens were obtained by radical hysterectomy, three from simple hysterectomy and three from biopsy.

In order to decrease the cost of the genotyping test, we found it economical to establish first that a specimen was positive for HPV. The majority of cases (~75%) were negative for HPV after the first round of amplification with the MY09 and MY11 primers but became positive after a second round of amplification with the GP5+ and GP6+ primers. Hence, we incorporated nested PCR as the first step of our method, and then proceeded to HPV genotyping using this PCR product as the starting material for the manufacturer's kit.

HPV DNA was detected in 96 of 99 cases (96.9%). Seventy-five cases (78.1%) were single infections and 21 cases (21.9%) were infected with 2 or 3 HPV types (Table 1). All three

Table 1
Distribution of HPV genotypes in invasive cervical cancer

HPV type	No. of cases (%)	No. of cases (%)
	As single infection (n=75)	In single and multiple infection (n=96)
16	56 (58.3)	77 (80.2)
18	6 (6.3)	8 (8.3)
31	1 (1.0)	3 (3.1)
33	4 (4.2)	8 (8.3)
45	2 (2.1)	2 (2.1)
52	1 (1.0)	10 (10.4)
58	3 (3.1)	7 (7.3)
66	1 (1.0)	1 (1.0)
11	1 (1.0)	1 (1.0)
	Multiple infections (n=21)	
16,52	7 (7.3)	
16,33	3 (3.1)	
16,58	3 (3.1)	
16,31	2 (2.1)	
16,35	2 (2.1)	
16,18,52	2 (2.1)	
16,33,35	1 (1.0)	
16,58,11	1 (1.0)	

negative cases gave a strong hybridization signal for the beta-globin control indicating the DNA had an adequate quality and these were true negative cases. Two of these cases are illustrated in Fig. 2 (lanes K2 and K10).

In the single infection group, eight high-risk HPV types, including 16, 18, 33, 58, 45, and 31, 52, 66 (in descending order of frequency), were identified. Interestingly, there was one case of a low-risk type (HPV11) infection that occurred in a case of "warty or condylomatous carcinoma" with deep stromal invasion and lymph node metastasis. HPV16 was present in 100% of the 21 cases of multiple infections, and all such cases had one or two other high-risk HPV types. Seventeen were double infections, and the most common were HPV types 16 with 52 (7 cases), followed by 16 with 33 and 16 with 58 (3 cases each). None of double infections involved HPV types 16 with 18. There were four triple infections which included one low-risk HPV type (HPV11) in one case.

Overall, there were 117 HPV infections in the 96 cases. The most common type was HPV16 (77/96, 80.2%), singly or in combination, followed by HPV52 (10/96, 10.4%), HPV18 and HPV33 (8/96, 8.3%, each), and HPV58 (7/96, 7.3%). Together, HPV16 and 18 were found in 85 cases (85/96, 88.5%) singly or in combination.

Discussion

The present study is the first to demonstrate the utility of paraffin-embedded tissue for HPV subtyping by the Linear Array HPV Genotyping Test. The Linear Array HPV Genotyping Test is currently the only available PCR-based kit that can qualitatively test for 37 HPV types in single detection reaction. However, the recommended instructions include many restrictions including the type of specimen (fresh cytologic specimen), collection medium (PreservCyt® solution), extraction kit (AmpliLute) and special equipment for hybridization/colorimetric steps (shaking water-bath incubator). Recently, there are several publications that have modified the Linear Array HPV Genotyping Test to make it more widely applicable in laboratories, including the use of archival frozen tissue and DNA samples, automated DNA extraction and a dry-air incubator [8,10,11]. We can now add the use of paraffin-embedded samples as an additional modification. In order to maximize the detection rate and subtyping from paraffin-embedded material, we found it was necessary to first perform nested PCR with MY09/11 and GP5+/6+ consensus primers [9,12] on the extracted DNA in order to amplify a low viral load or fragmented DNA sequences. The applicability of paraffin-embedded material now makes the Linear Array HPV Genotyping Test even more appealing to diagnostic laboratories, especially those where frozen material is not usually banked or readily available and will greatly facilitate studies using archival material.

Studies have compared the sensitivity and specificity of HPV typing by the Linear Array HPV Genotyping Test to other techniques including sequencing [8,21], Roche PGMY primer-based research prototype line blot assay [22], and SPF10-INNO LiPA HPV genotyping test [23]. All of these studies showed highly accurate results for HPV typing using the Linear Array system. Studies using Roche PGMY primer-based research prototype line blot assay [22] and SPF10-INNO LiPA HPV genotyping test [23] also confirmed the reproducibility in the detection of multiple infections by the Linear Array HPV Genotyping Test. In our study, 28 cases had been previously typed by a dot-blotting genotyping technique (results not shown). All cases showed the same HPV types using the Linear Array HPV Genotyping Test. Four of these cases showed double infections with other HPV subtypes by the Linear Array HPV Genotyping Test that were not detected by dot blotting. Thus, we believe the Linear Array system is an accurate form of testing with high sensitivity and specificity.

In our study, the detection rate of HPV DNA (96.9%) was comparable to the worldwide prevalence (99.7%) [13] but was greater than those from the previous studies in the same country, i.e., Southern Thailand (95.3%) [14], Central Thailand (68%, 82%, 86.3%) [15–17], and Northeast Thailand (86.7%) [18]. These differences in the detection rates are most likely related to differences in sample types and techniques used. We used nested PCR that can amplify HPV even in the cases of low viral load or fragmented DNA sequences, leading to a higher rate of detection.

In our study, HPV16 and HPV18 were the most common subtypes, occurring in 85/96 cases (88.5%). However, only 64.4% (62/96) of cases showed infection with HPV16 or HPV18 without other high-risk subtypes. Based on these results, together with our one case of HPV11, the approved quadrivalent HPV prophylactic vaccine might be able to protect only about two thirds of cervical cancer in Northern Thailand. This figure is comparable to the values of 67.7% obtained in the IRAC studies from nine countries (Morocco, Mali, Colombia, Brazil, Paraguay, Peru, Thailand, Philippines, Spain) [19] and 63.2% from Taiwan [12]. Of note, in the IARC study, there was also one HPV11-positive case and another HPV6/11-positive case. On the other hand, our figure is significantly lower than that published for the southern (241 of 322 cases, 74.8%, $p=0.048$) [14] and northeast (63 of 78 cases or 80.9%, $p=0.018$) [18] regions of Thailand (Table 2). Thus, there are important regional variations in HPV subtypes across Thailand that would influence the predicted efficacy of the vaccine that has become available.

The prevalence of HPV52 and HPV58 infection in this study was relatively high. HPV52 was the second most common subtype detected (10/96, 10.4%). Its frequency in cervical cancer of Northern Thai women was greater than that of Southern Thai women (9/322, 2.8%, $p=0.002$) [14]. In Northern Thailand, HPV52 and HPV58 infection accounted for 17.7% of cases (17/96). This number was comparable to that in the northeast (12/78, 15.3%, $p=0.683$), but significantly higher than that in the south (19/322, 5.9%, $p<0.001$). In the IARC study, the cases with HPV52 and HPV58 infection were not completely described, but the frequency calculated from the presented data was only 5–6% [19]. A more recent study on HPV type distribution in invasive cervical cancer confirms these results [20], showing that the proportion of HPV16 and HPV18 in Asia, Africa and South/Central America is lower (65–70%) than in Europe, North America and Oceania

Table 2
Comparison of HPV subtypes between different regions in Thailand, IARC study, and Taiwan

HPV type	Thailand				IARC [a] (%)	Taiwan (%) [12]
	North (%)	Northeast (%) [18]	South (%) [14]	Central (%) [16]		
16	56 (58.3)	46 (59.0)	186 (57.8)	30 (36.6)	950 (54.6)	70 (51.5)
18	6 (6.3)	15 (19.2)	52 (16.1)	12 (14.5)	192 (11.0)	9 (6.6)
16 with 18	0 (0)	2 (2.7)	3 (0.9)	5 (6.1)	36 (2.1)	7 (5.1)
16 or 18	62 (64.4)	63 (80.9)	241 (74.8)	47 (57.2)	1178 (67.7)	86 (63.2)
52	10 (10.4)	3 (3.8)	9 (2.8)	NA	NA	3 (2.2)
58	7 (7.3)	9 (11.5)	10 (3.1)	NA	NA	12 (8.8)
52 or 58	17 (17.7)	12 (15.3)	19 (5.9)	NA	NA	15 (11.0)
Unknown	0 (0)	2 (2.6)	31 (9.6)	32 (39.0)	111 (6.4)	3 (2.2)
Multiple	21 (21.9)	12 (15.5)	12 (3.7)	5 (6.1)	141 (8.1)	36 (26.5)

NA: data not available.
[a] International Agency for Research on Cancer (Morocco, Mali, Colombia, Brazil, Paraguay, Peru, Thailand, Philippines, Spain) [19].

(74–77%) whereas the prevalence of HPV52 and HPV58 is relatively high in Asia (9.4%). These figures together with ours indicate that there are regional differences in HPV infection. This concept deserves further study within Thailand, both for comparison to other countries as well as for future planning of prophylactic vaccines.

There have been only a few epidemiologic studies of HPV genotype distribution in Thailand (see Table 2). The samples used in the other studies included scraped cytology samples, fresh frozen tissue or paraffin-embedded tissues. All of these studies used a conventional PCR method and searched for HPV16, 18, and sometimes a few other types of high-risk HPV. As a result, some studies have a much higher proportion of untyped HPV, up to 32% of cases [14–16]. A more definitive epidemiologic study is now possible using the method presented in our paper. Such a study of the HPV genotype distribution is important. The data would provide the expected level of protection of an HPV vaccine in the population. The protective response is one of the key factors for determining whether to implement the vaccine, and for planning other health strategies in the country.

The prevalence of multiple infection in our study (21/96, 21.9%) was significantly higher than that from Southern Thailand (12/322, 3.7%, $p<0.001$) [14], and the IARC study (141/1739, 8.1%, $p<0.001$) [19]. The frequencies of multiple infection varied from 1.8% in Spain and Colombia to 26.5% in Taiwan [12]. The prevalence of multiple infections from all studies reported before the year 2000 was lower than 10% and used only single PCR reaction [14–16]. Some interesting results were obtained in our study in this regard. First, all multiple infections involved HPV16, suggesting infection with this genotype may facilitate infection by others. Second, although both HPV16 and HPV18 are recognized as high-risk subtypes, we did not find any case of co-infection. This is probably related to insufficient numbers of cases in our study, since other studies have documented co-infection of these two subtypes, ranging from 0.9 to 6.1% [12,14,16,18,19]. The ability of Linear Array HPV Genotyping Test to detect multiple HPV infections easily, together with the modifications that allow the use of paraffin-embedded tissue, will facilitate a study of the complex interaction of concomitant HPV infections in cervical carcinogenesis. In addition, since archival material can be used, sequential studies from multiple specimens on the same patient can be examined to study the time course of HPV infection with respect to specific subtypes, and how this correlates with the progression through precancerous states to invasive cancer.

In summary, we have developed a modification of the Linear Array HPV Genotyping Test that enables the use of paraffin-embedded tissue samples. This will greatly facilitate HPV subtyping in pathology laboratories and should result in more meaningful comparisons between different regions in a country and between different regions of the world. Using this approach, we found that the prevalence of HPV infection in cervical cancer of Northern Thai women is in keeping with the worldwide prevalence. However, the distribution of HPV subtypes is distinctive with a relatively low frequency of HPV16 and 18, and a relatively high frequency of HPV52 and 58. Moreover, multiple infections are quite common in this area. The vaccine against HPV16 and HPV18 is estimated to prevent approximately two thirds of the cervical cancer cases in Northern Thailand.

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Conflict of interest: The authors have no conflict of interest with any product used or referred to in this study.

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