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Original Article

Human Leucocytes Antigen and Cervical Precancerous Lesions of Women Attending the Gynecology Unite of the Yaoundé University Teaching Hospital

Antigènes des leucocytes humains et lésions précancéreuses cervicales chez les femmes fréquentant l'unité de gynécologie du Centre Hospitalier Universitaire de Yaoundé

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Mots clés : HLA-DQB1 ; lésion cytologique cervicale; HPV

ABSTRACT

Background. The Human Leucocyte Antigen (HLA) genes are polymorphic and genetically predisposed. Our study aimed at evaluating the genetic predisposition and the association between HLA-DQB1 genes and cervical precancerous lesions in patients referred to Yaoundé Teaching Hospital. **Materials and method.** A case-control study was carried out (from 2000 to 2019). Enrolled in this study were women with cervical cytological lesions from specialized reference center involved in the treatment and management of women with cervical cancers. **Results.** Cervical lesions found were ASCUS (8.9%), L-SIL (48.9%) and le H-SIL (42.2%). HR-HPV types identified included HPV 16, HPV 18, HPV 33, HPV 39 and HPV 45. It was found that HLA-DQB1*0302 (OR= 0.3; CI: 0.01-0.35; P=0.001) and HLA-DQB1*0501(OR = 0.18; CI: 0.05-0.64; P=0.01) were significantly low in the group with cervical cytological lesions compared to controls. A multi-variable analysis was performed and found that HLA-DQB1*0302 were significantly decreased in cases compared to controls (adjusted OR=0.052; CI: 0.005- 0.501; P= 0.01). **Conclusion.** We observed a variability of HPV subtypes amongst our study population. This information will help to upscale HPV vaccines coverage in the country We also identified that HLA-DQB1*0302, HLA-DQB1*0501 may be protector factors against cervical cytological abnormalities and could be an added information on the pathogenesis of HPV.

RÉSUMÉ

Introduction. Les gènes de l'antigène des leucocytes humains (HLA) sont polymorphes et génétiquement prédisposés. Notre étude visait à évaluer la prédisposition génétique et l'association entre les gènes HLA-DQB1 et les lésions précancéreuses cervicales chez des patients référés au Centre Hospitalier Universitaire de Yaoundé. **Matériels et méthodes.** Nous avons réalisé une étude cas-témoin (de 2000 à 2019). Les femmes enrôlées dans cette étude étaient atteintes de lésions cytologiques cervicales et provenaient d'un centre de référence spécialisé impliqué dans le traitement et la prise en charge des femmes atteintes de cancers du col de l'utérus. **Résultats.** Les lésions cervicales retrouvées étaient ASCUS (8,9 %), L-SIL (48,9 %) et le H-SIL (42,2 %). Les types HR-HPV identifiés comprenaient le HPV 16, le HPV 18, le HPV 33, le HPV 39 et le HPV 45. 0501 (OR = 0,18 ; IC : 0,05-0,64 ; P = 0,01) étaient significativement faibles dans le groupe présentant des lésions cytologiques cervicales par rapport aux témoins. Une analyse multivariée a été réalisée et a révélé que seul le HLA-DQB1*0302 était significativement diminué chez les cas par rapport aux témoins (OR ajusté = 0,052 ; IC : 0,005-0,501 ; P = 0,01). **Conclusion.** Nous avons observé une variabilité des sous-types de HPV au sein de notre population d'étude. Ces informations aideront à étendre la couverture vaccinale contre le HPV dans le pays. Nous avons également identifié que HLA-DQB1*0302, HLA-DQB1*0501 peuvent être des facteurs protecteurs contre les anomalies cytologiques cervicales et pourraient constituer une information supplémentaire sur la pathogenèse du HPV.

HIGHLIGHTS

What this study adds to our knowledge

1. The incidence of invasive cervical cancer is low as compared to the prevalence of HPV infection among women
2. . Immune system also plays a role in the evolution and the prognostic of the disease.
3. HLA-DQB1*0302 are significantly decreased in cases compared to controls.

How this is relevant to practice, policy or further HLA-DQB1*0302, HLA-DQB1*0501 may be protector factors against cervical cytological abnormalities and could be an added information on the pathogenesis of HPV.

INTRODUCTION

Cervical cancer is the most commonly diagnosed type of cancer and the leading cause of mortality in sub-saharan Africa. In 2020, global estimated new cases and death of cancer were 19 million and 10 million respectively. Of the new cases 604 127 and 341 831 among the deaths were cervical cancer cases [1]. It is estimated that these figures are going to double in 20 years due to the aging population of sub-Saharan Africa. Studies found that Human Papilloma Virus (HPV) plays an important role in the development of cervical cancer [2, 3]. The incidence of invasive cervical cancer is low as compared to the prevalence of HPV infection among women. This may suggest that there are others factors that intervene in the pathogenesis of the disease. Colonization of epithelial cell of the cervix by high oncogenic rate HPV (HR-HPV) is needed for the development of cancer. Immune system also plays a role in the evolution and the prognostic of the disease. Human Leucocyte Antigen (HLA) system is one of the factors that influences the immune response against infection by HPV [4]. Class II HLA is necessary for the antigen cell presenting and are important for the host immune response against viruses and others pathogens. Class II HLA genes influences the bond and presentation of antigens to T-cells [5]. Given that the host immune response is a determining factor of the persistence of the HPV infection, the evolution of the infection to high grade and in cancer, HLA ethnical variation can influence the pathogenesis of cervical cancer through the immune response [6]. Previous studies found an association between the class II HLA allelic polymorphism, infection by HPV and the onset of invasive cervical cancer [7-9]. However, this relationship is obscured, as the degree of association between HPV and specific genes of the major histo-compatibility complex region varies considerably from one population to another. Because of these variations between HPV infection and HLA in different setting, the study of HLA alleles as a prognostic factor have to be done in every population.

In our context, studies carried out were on the risk factors associated to the prevalence of HPV infections and cervical neoplasia in women. These studies found that the risks were higher in youth, unmarried individuals and housewives. The genotypes 16/18 were found in cancer in Cameroon [10]. Our study is aimed at evaluating the association between HLA-DQB1 genes and cervical precancerous lesions in women in Yaoundé, Cameroun.

MATERIALS AND METHODS

Study design

A case-control study was carried out in February 2020 in Yaoundé University Teaching Hospital for data collection. We included patients with pre-cancerous lesions of the cervix followed up in this hospital and the control population was selected randomly from an opportunistic screening program in the same hospital. Cervical smear cytology detection confirmed negative findings of intraepithelial lesion or malignancy. Clinical and para-clinical data were collected from their medical files and reported on an anonym inquiry form for case group after obtaining their informed consent. Socio-demographic data of the control group were also collected on an anonymous inquiry form.

Study subjects

We registered 116 files of patients with cervical precancerous lesions from 2000 to 2019. Twenty-seven files were incomplete, 12 patients were dead, 22 were not available, 10 refused to participate and 45 patients were therefore included in the study. Patient's samples and data were collected from patients diagnosed for any confirmed case of cytological abnormalities of the cervix and have received no prior treatment for that. Population included women aged 24 to 66 years. The control population was randomly selected from a routine screening program in the same hospitals with negative cervical smear confirmed results. The population included 15 women aged 24 to 66 years also. All patients were recruited in the Yaoundé University Teaching Hospital. Peripheral blood samples and cervical swabs were collected from all participants with written informed consent, used as HLA typing and DNA for HPV respectively.

Cytology Technique

The participants underwent a gynaecological evaluation (pelvic examination and cervical visual appearance) using a non-lubricated clean and single-use speculum (Hybribio Biochemical Company Limited China). The materials for oncotic cytological examination were collected using Ayre's spatula and the specimen was rolled onto a previously identified slide and immediately fixed in 95% ethanol and allowed to air-dry for subsequent staining using the Papanicolaou method. The slides were evaluated in the cytopathology laboratory of the two reference hospitals by certified cytopathologists who were blinded to all other study results. The cytology results were classified according to the 2001 Bethesda classification [11]. Pap smears were considered to be abnormal if they contained ASCUS, low-grade or high-grade squamous intraepithelial lesions (SILs).

An endocervical brush ((cytobrush®)) was used for cervical sample collection and the collected cervical material was distributed by shaking the brush in a previously identified commercial aqueous buffered specimen collection and transport media (STM) (Roche Diagnostic Systems, Meylan, France). Cervical samples were processed at the Centre for the study and control of

communicable diseases (CSCCD) of Faculty of Medicine and Biomedical Sciences, Yaoundé1.

HPV Genotyping

HPV DNA Extraction: HPV DNA was isolated from cervical exfoliated cells using the AmpiLute Liquide Media Extraction Kit, as described in previous studies which yields HPV target DNA and human HPV suitable for PCR amplification. Briefly, 200µl of samples was added to 200µl of lysis solution and 20µl of proteinase K solution. DNA was eluted in 100µl of elution buffer [12]. Nucleic acid concentration and purity were measured using the NanoDrop UV spectrophotometer NanoDrop-8000 (Thermo Scientific, Wilmington, DE, USA). Besides the concentration, A260:230 and A260:280 ratios were determined, which were used as indicators of the purity of the samples.

Polymerase Chain Reaction (PCR): Double-stranded DNA concentration were specifically quantified on the Rotor Gene 6000 PCR machine, (Applied Biosystems (USA) Gold-plated 96-Well) according to the manufacturer's instructions. The Master Mix reagent contained a set of primers: a generic set (GP5_/GP6) widely used to detect all HPV subtypes (F:5'-TTTGTTACTGTGGTAGATACTAC-3', R:5'GAAAAATAAACTGTAAATCATATTC-3'). Reactions containing 5µl of 5x Amplitaq Go flexi PCR buffer, 2µl of 25mM magnesium chloride, 0.5 µmol/L of each primer, 0.5 mmol/L deoxynucleotide triphosphates (dNTPs), 0.25µl of Amplitaq Gold DNA polymerase (Life Technologies), and 2µl of DNA was prepared in a volume of 25µL. PCR conditions were: 2 minutes at 50°C, 9 minutes at 93°C; 40 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 60 seconds; then 72°C for 45 minutes. PCR products were visualized on 1% agarose gel and scored for the presence or absence of bands.

HPV DNA amplification and detection using the Linear Array HPV Genotyping Test kit: HPV genotyping was performed using the Roche Linear Array HPV Genotyping Test kit (Roche Diagnostics, USA) on all positive cases.

DNA amplification: The master mix (MMX) was prepared by adding 125 µl of HPV Mg2+ to the entire vial of MMX. The total volume of the vial was 705 µl. The necessary calculations were done to determine the number of MMX vials needed for the number of samples to be processed. The MMX reagent contain two sets of primers: a generic set (GP5/GP6) widely used to detect all HPV subtypes (F:5'-TTTGTTACTGTGGTAGATACTAC-3', R: 5'-GAAAAATAAACTGTAAATCATATTC-3') and an HPV16-specific set (F:5'-TCAAAAGCCACTGTGTCCTG-3', R: 5' CGTGTTCTTGATGATCTGCA-3'). A control PCR of a 79-bp fragment of β_globin (HBB) were used (F:5'-GGATCCTTCTCTTGTGTTGG-3', R: 5'-CTACCATTGGAAAAGCAACC-3'). Fifty microliters (50 µl) of the MMX were added to the amplification tubes and 50µl of each processed specimen and control added to the appropriate labelled amplification tubes containing working MMX. PCR conditions were: 9 minutes at 93°C; 40 cycles of 94°C for 20 seconds, 53°C for 20 seconds, and 72°C for 40 seconds; then 72°C for 7 minutes.

Detection of amplicons: Genotyping was performed by hybridization of the amplified DNA. Test strip results were read independently by two molecular scientists. Samples that were ß-globulin negative were excluded from the analysis. The HPV subtypes detected by the Roche array were classified as HR-HPV or LR-HPV. A study subject was considered as HPV positive if the specific HPV genotype was detected on a cervical sample.

Detection of the 6 alleles of HLA-DQB1

DNA was extracted from blood samples using DNeasy Qiagen Kit, South Africa [13]. HLA-DQB1*0201, HLA-DQB1*0301, HLA-DQB1*0302, HLA-DQB1*0402, HLA-DQB1*0501, HLA-DQB1*0602 alleles were identified using sequence-specific-primers based on the PCR-SSP method [14]. The primers used for HLA-DQB1*0201 were as followed: forward, 5'-GGTTGCTGGAAAGATGCATCT-3' and reverse, 5'-CCGCTGCACTGTGAAGCTCT-3'. The primers used for HLA-DQB1*0301 were as followed: forward, 5'-TACTTCCATAACCAGGAGGAGA-3' and reverse, 5'-TGCAGTAGTTGTCCACCCG-3'. The primers used for HLA-DQB1*0302 were as followed: forward, 5'-GTTTCTTGGAGCAGGTTAAACA-3' and reverse, 5'-CTGCACTGTGAAGCTCTAC-3'. The primers used for HLA-DQB1*0402 were as followed: forward, 5'-AGTTCCTGGAAAGACTCTTCT-3' and reverse, 5'-CCGCTGCACTGTGAAGCTCT-3'. The primers used for HLA-DQB1*0501 were as followed: forward, 5'-ACGTTTCTTGGAGTACTCTACG-3' and reverse, 5'-CCGCTGCACTGTGAAGCTCT-3'. The primers used for HLA-DQB1*0602 were as followed: forward, 5'-CGTGCGTCTTCTGACCAGAT-3' and reverse, 5'-GCTGTTCCAGTACTCGGCAT-3'. The primers used were synthesis in Inqaba Biotec in South Africa. PCR reactions were performed as follow: pre-degeneration at 95 °c for 5minutes, denaturation at 95°c for 20 seconds, annealing at 65°c for 50 seconds and an extension at 72°c for 20 seconds, for a total of 30 cycles. PCR were electrophoresed in 2% agarose gel, which was then imaged under ultraviolet light and photograph (Figure 1).

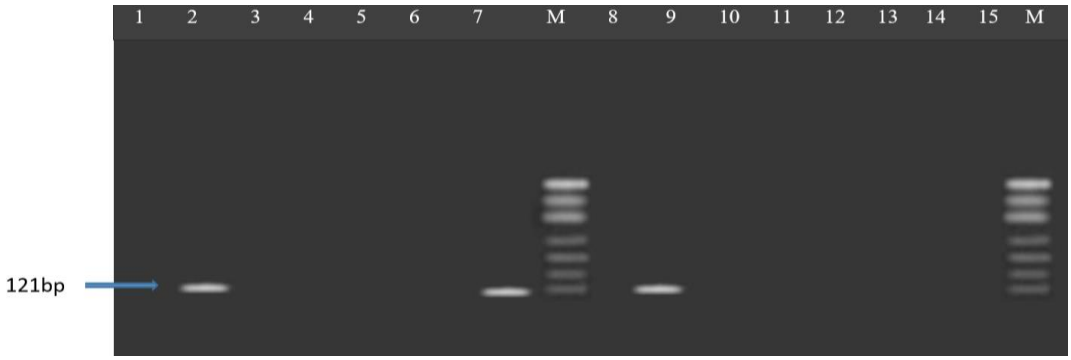

Figure 1 : HLA DQB1*0602 PCR gel electrophoresis results

M: Molecular Weight Marker; 1-7 and 8-15samples, 2,7 and 9 are positive samples (121 bp)

Quality control

DNA preparation, PCR and PCR product detection were performed in different rooms. DNA quality was confirmed using a NanoDrop spectrophotometer and DNA with sufficient quality was chosen for PCR.

Statistical analysis

All statistical analysis was done using SPSS version 20.0. The qualitative variables were analyzed using Fischer exact test and X^2 test. Proportions of HLA alleles were compared using X^2 test. Multivarial analysis was done on alleles with different proportions between case and controls. Logistic regression was used to identify relationship between HLA alleles and the severity of pre-cancerous lesions. Odds ratios (OR) and 95% CI were calculated using logistic regression and considered P values less than 0.05 significant.

Ethics consideration

This study received the ethical clearance of the Institutional Ethics Committee of Yaoundé Gynaeco-Obstetric and Pediatric Hospital and Yaoundé University Teaching Hospital, with reference number 703/CIERSH/DM/2018. Written informed consent was obtained from all participants and the study was conducted in accordance with the principles of the Declaration of Helsinki.

RESULTS

Samples with cervical abnormalities were specifically amplified with a pair of generic primers and HPV genotyping was performed using the Roche Linear Array HPV Genotyping Test kit. From the samples, were HPV positive with one or more HPV types.

The different HPV types identified were HR-HPV and LR-HPV. The HR-HPV types identified included HPV 16 HPV 18 HPV 33 HPV 39 and HPV 45 as presented in Figure 2

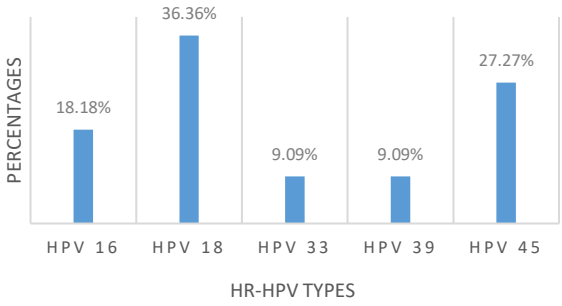

Figure 2: HR-HPV types identified in Cervical Swabs

The case and control groups had similar age ranges and DNA spectrophotometer showed that all subjects have sufficient quality for HLA analyses.

The fractions of cervical lesions found in case group were ASCUS (8. 9%), L-SIL (48. 9%) and H-SIL (42. 2%).

The distribution the 6 alleles of HLA-DQB1 in the case and control groups is shown in the Table 1. Frequencies of HLA-DQB1*0302 in women with cervical cytological abnormalities and in those women without abnormalities were respectively 31.1% and 89.7%. This frequency was significantly low in the case group (OR= 0.3; CI: 0.01-0.35; P=0.001). Similar result was found with HLA-DQB1*0501 with frequencies of 26.7% in case group and 66.7% in control group (OR = 0.18; CI: 0.05-0.64; P=0.01). After a multi-variable analysis was carried we found that only HLA-DQB1*0302 were significantly decreased in case group compared to the control group (adjusted OR=0.052; CI: 0.005- 0.501; P= 0

Table 1: Distribution of HLA-DQB1 in case and control group

HLA-DQB1 alleles	Case (N=45)		Controls (N=15)		P-Value
	Yes	**No**	**Yes**	**No**	
HLA-DQB1*0602	25 (55,6%)	20 (44,4%)	11 (73,3%)	4 (26,7%)	0,224
HLA-DQB1*0301	14 (31,1%)	31 (68,9%)	8 (53,3%)	7 (46,7%)	0,122
HLA-DQB1*0302	14 (31,1%)	31(68,9%)	13 (86,7%)	2 (13,3%)	**0,0001**
HLA-DQB1*0501	13 (28,9%)	32 (71,1%)	10 (66,7%)	5 (33,3%)	**0,009**
HLA-DQB1*0201	24 (53,3%)	21 (46,7%)	11 (73,3%)	4 (26,7%)	0,174
HLA-DQB1*0402	32 (71,91%)	13 (28,9%)	13 (86,7%)	2 (13,3%)	0,228

With the difference of expression between HLA-DQB1*0302 and HLA-DQB1*0501 in case and controls groups, we found an association with pre-cancerous lesions. Table 2 shows no relationship between the alleles of HLA-DQB1 studied and the severity of cervical cytological abnormalities.

Table 2: Multivarial analysis of HLA-DQB1 associated to pre-cancerous lesions

Cervical cytological abnormalities		OR (IC 95%)	p-value
Controls without lesions	HLA-DQB1-0302	0,350 (0,019 - 6,389)	0,479
	HLA-DQB1-0501	2,031 (0,137 - 30,081)	0,606
Cases with H-SIL	HLA-DQB1-0302	2,74 (0,184 - 40,894)	0,465
	HLA-DQB1-0501	10,73(0,684 - 168,454)	0,091
Cases with L-SIL	HLA-DQB1-0302	6,485 (0,456 - 92,253)	0,168
	HLA-DQB1-0501	3,131 (0,221 - 44,441)	0,399

DISCUSSION

Cervical cancer is one of the most frequent cancers of the women in Africa. Studies done have shown that HPV plays a major role in the development of cervical cancer of the cervix. Although infection and colonization by high risk oncogenic HPV are necessary for the development of cancer, the immune system plays a role in the evolution and the prognostic of the disease. HLA system is one of factor of the immune system that influences cellular mediation of the immune response against HPV infection and cancer. Many studies found and association between class II HLA allelic polymorphism, infection by HPV and the occurrence of cervical invasive cancer [7-9]. However, this relationship is obscured, as the degree of association between HPV and specific genes of the major histo-compatibility complex region varies considerably from one population to another.

For this study we performed a case-control study. Our study size may be explain by the high number of death that occurs the years within diagnosis. For more than half of the patients diagnosed for cervical cancer dies the year within the diagnostic this because of the diagnostic at late stages in development countries. The diagnosis of the cervical cytological abnormalities was performed by qualified cytopathologists by Papanicolaou colorations. We used the classification of Bethesda to classify the different abnormalities found [15]. HLA-DQB1 detection were realized by *Polymerase Chain Reaction-Sequence Specific Primers* like in many others studies done on this subject. The 6 alleles of HLA-DQB1 we did were the most frequent done in studies published on the subject so as to be able to compare our result with other studies in different population.

In our study the mean age was 41±9 years for the patients with cervical cytological abnormalities this result are similar to those found in Cameroon in 2015 by Rosa Catarina et al. [12] and may explain the mean age at diagnostic of cervical cancer of the cervix of 52.43±3.82 years found by Sando et al. in Cameroon in 2014 [16].

The mean age at diagnosis was 38 ± 10 years this is near the age of 39 ± 5 years shown by the study done by Nyengidiki et al. in 2015 in Nigeria [17].

Cervical cytological abnormalities found was ASCUS (8.9%), L-SIL (48.9%) and H-SIL (42.2%) but in 2014 Angelo Meloni et al. found in Italy in their study ASCUS (46.9), L-SIL (41.2%) and H-SIL (11.9%) [18]. We found a bigger number of H-SIL this shows that there are many under diagnosed cases of precancerous lesions, which can lead to cancer and can explain the diagnosis at late stage of evolution of the disease in development countries like us.

We found that the frequencies of HLA-DQB1*0302 and of HLA-DQB1*0501 were significantly decreased in the group of patients with cytological abnormalities than of those without abnormalities. In 2014 Jian et al. in China found that HLA-DRB1*1501 and HLA-DQB1*0602 were also significantly decrease in patients carriers of invasive cervical carcinoma and infected with HPV [8]. An association between HLA-DRB1*1501 and persistent infection of HPV 16 was found in the Swedish population, in United Kingdom no association was found between persistent infection of HPV and HLA-DRB1*1501; HLA-DQB1*0602 [19,20]. A study done in Tunisia found that HLA-DRB1*15, DRB1*13, DQB1*06, DQB1*03 where associated with cervical cancer whereas HLA-DRB1*13-DQB1*06 where protector [21]. These differences between the relationship of HLA and cervical cancer may show the large genetic diversity of ethnics groups, may also be explain by the used of different techniques for HLA typing and searching of cervical cytological abnormalities. These associations between HLA-DQB1*0302 and HLA-DQB1*0501 may suggest that the oncogenecity of this disease can be due of default in immune-regulation.

Limitations of the study

The main limits of this study is the small sample size but it can be a pilote study of a study on a larger sample including many others regions of the country. By lack of financial means we did type all the alleles of HLA-DQBB1

CONCLUSION

In this study, the cervical cytological abnormalities found on the cervix were: ASCUS, L-SIL and H-SIL. We observe a variability of HPV subtypes amongst our study population. This information will help upscale HPV vaccines coverage in this region HLA-DQB1*0302, HLA-DQB1*0501 may be protector factors against cervical cytological abnormalities.

Author's contributions

Study concept and design: G. M. Nangué-Tabekou, G. Mondinde;
Data acquisition and analysis: G. M. Nangué-Tabekou;
Draft of the manuscript: G. M. Nangué-Tabekou, A. Tabekou;
Critical revision of the manuscript: E. Lyonga, M. Mesembe,; C. H. Mbakam, E. Guidem, G. Doh; G. Mondinde; G. J. Essomba Zanga
Statistical analysis: G. M. Nangué-Tabekou, E. Guiedem;
Administrative, technical and material support: G. M. Nangué-Tabekou, C. H. Mbakam ; C. Bomki,
Study supervision: G. Mondinde, M. C. Okomo,
All authors discussed the results and contributed to the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest

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