Original Article

Detection of CDR3s diversity and its prediction of persistent high-risk HPV infection and cervical intraepithelial neoplasia risk: a prospective study

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Background: We aimed to determine the potential role of complementarity-determining region 3s (CDR3s) in prognosis of high-risk human papillomavirus (hr-HPV) infections and cervical intraepithelial neoplasia (CIN) in a prospective study for 12 months.

Methods: Twenty-six women aged 30–64 years were recruited using cytology and HPV DNA test in China. After obtaining written informed consent, our team utilized amplicon rescued multiplex polymerase chain reaction (ARM-PCR) and second-generation high throughput sequencing to detect the diversity 50 (D50) value of CDR3s diversity among the groups of cancer (n=5), CIN 2/3 (n=4), CIN 1 (n=6), hr-HPV positive (n=8) and normal control (n=3) at the baseline year. Additionally, cytology and HPV DNA test adopted to the groups of CIN 1 and hr-HPV found the status of cervical lesions and hr-HPV infected persistence between CIN 1 (n=6) and hr-HPV (n=7) groups.

Results: The prevalence of CDR3s diversity staining was 9.2±7.9, 5.7±5.6, 4.0±6.0, 13.6±7.7, 8.0±7.6 among women with normal, hr-HPV positive, CIN 1, CIN 2/3 and cancer biopsies. Decreased CDR3s diversity were not significantly associated with disease progression (P=0.093). There is no significant difference between CDR3s diversity and HPV clearance (P=0.173). All CIN1 cases regressed.

Conclusions: CDR3s might be a biomarker to predict HPV-positive outcomes. The detection of CDR3s may assist in the clinical management of CIN 1. Women with CIN 1 and decrease of CDR3s diversity may benefit from closer follow-up at frequently intervals. (The trial registration number in Chinese Clinical Trial Registry: ChiCTR2000038164 and date of registration: September 11, 2020).

Keywords: Diversity 50 (D50); complementarity-determining region 3s (CDR3s); T cell receptor; high-risk human papillomavirus (hr-HPV); cervical intraepithelial neoplasia (CIN)

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Introduction

The incidence and prevalence of cervical cancer have been decreasing year by year with the maturity of three-stage cervical cancer screening and the concept of screening and treatment (1) and follow-up (2). Advances in cervical cancer screening and vaccination have exacerbated geographic differences, including in the United States and China (3,4). In 2016, the American College of Obstetricians and Gynecologists still recommended immediate surgical treatment for cervical intraepithelial neoplasia (CIN) grade 2 (CIN 2) at ≥25 years old, and all CIN 3 or more severe (≥ CIN 3) (5). However, previous studies have confirmed that most CIN 2 will naturally return to normal, not all CIN 3 will progress to cervical cancer, and the probability that untreated CIN 3 progressing to cervical cancer in 30 years is still less than 50% (6,7). In addition, HPV mRNA and DNA (Hybrid Capture 2, HC2) assays are highly sensitive for CIN 2 or more severe (≥ CIN 2) and much more sensitive than cytology, while HPV mRNA is more specific than HC2 and similar to cytology (8). Therefore, current screening guidelines inevitably lead to overtreatment. More importantly, excessive examination and treatment bring extravagant psychological burden (9) and economic pressure to patients.

Sustained infection of HPV will lead to precancerous lesions, and the precancerous lesions progressing to invasive cancer is relevant to cell immunity of B cells and T cells (10-14). To a large extent, complementarity-determining region 3s (CDR3s) diversity of T cell receptor (TCR) repertoire is able to represent the capacity of T cell immune diversity (15,16). Previous studies have found that TCR CDR3s immune diversity may be an effective indicator for predicting persistent high-risk HPV (hr-HPV) infection and CIN risk (17,18).

We performed this prospective study to simulate the natural pathogenesis of cervical cancer, and used the quantitative index of immune diversity, namely diversity 50 (D50), to determine the potential role of CDR3s. According to follow up, the relationship between CDR3s immune diversity and natural clearance or persistent infection of HPV was reported. At the same time, the information of low-grade CIN degradation, persistence and progression was collected to seek the relationship between CDR3s immune diversity and CIN 1 risk. We present the following article in accordance with the TREND reporting checklist (available at https://gpm.amegroups.com/article/view/10.21037/gpm-21-48/rc).

Methods

Study population and procedure

This single center study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of West China Second University Hospital, Sichuan University, China (No. 2013-036) and informed consent was taken from all individual participants. The subjects were recruited from October 2016 to December 2017. The exclusion criteria: (I) women during pregnancy; (II) history of cervical surgery; (III) congenital or primary immunodeficiency disease; (IV) long-term medication history of glucocorticoids, immunosuppressants, immunomodulators and so on; (V) history of mental illness; (VI) history of other malignant diseases, radiotherapy and chemotherapy; (VII) anemia, malnutrition; (VIII) history of bone marrow, thymus, thyroid and other diseases; (IX) history of diabetes, or kidney transplantation; (X) smokers, or drinkers. The inclusion criteria were: (I) females aged 30–64 years; (II) being sexually active but non-pregnant; (III) no history of cervical surgery; (IV) having civil decision-making authority and signed informed consent. Finally, 3 normal controls, 8 with hr-HPV infections, 6 with CIN 1, 4 with CIN 2/3, and 5 with cervical cancer were included. In this prospective single-center study, peripheral blood, cervical exfoliated cells and frozen cervical tissues samples were collected. *Figure 1* shows the procedure flowchart for our study.

T cell separation

Ten ml of peripheral blood was put into the tube with heparin and T cell isolation was performed by magnetic activated cell sorting (Miltenyi Biotec, Germany). $CD8^+$ T cells were first screened negatively by anti-CD4 magnetic beads, and then separated by anti-CD8 magnetic beads for positive screening. $CD4^+$ T cells were isolated by positive screening using anti-CD4 magnetic beads. $CD4^+$ $CD25^+$ Tr cells were positively screened by anti-CD25 magnetic beads. All extracted cells were immediately stored in RNA preservation solution (Qiagen, Germany).

Amplicon rescued multiplex polymerase chain reaction (ARM-PCR)

RNA was extracted by using the RNeasy Mini Kit (Qiagen, Germany), and the complementary DNA (Toyobo, Japan) was subsequently used to perform real-time PCR with

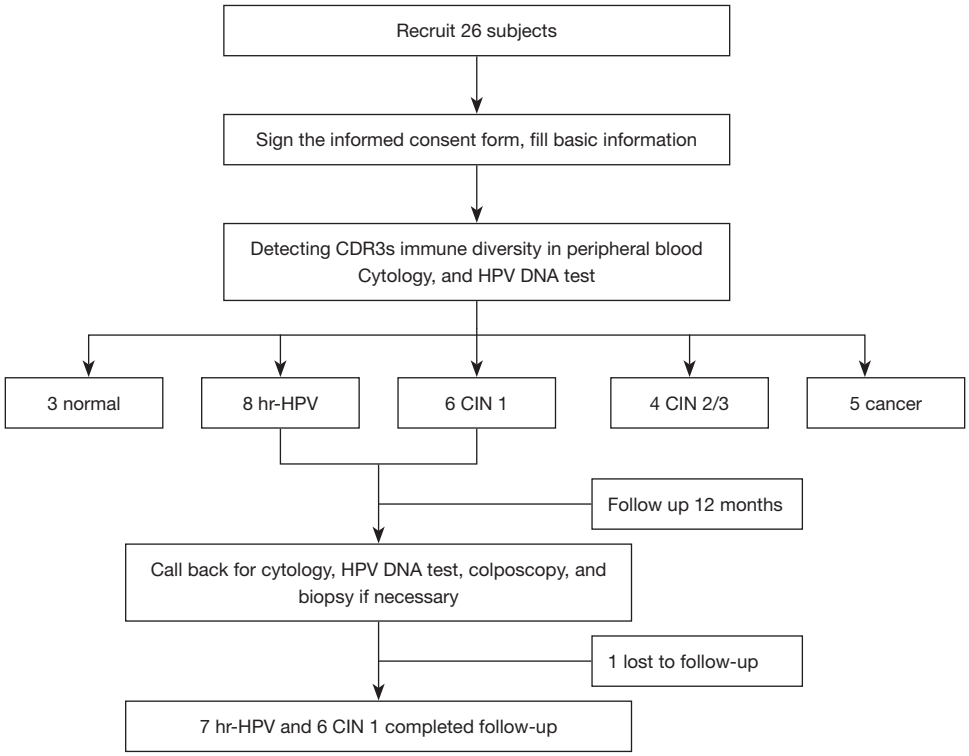

Figure 1 Flow diagram showing procedures involved in every step of the study. CDR3s, complementarity-determining region 3s; hr-HPV, high-risk human papillomavirus; CIN, cervical intraepithelial neoplasia.

SYBR™ chemistry (Takara, China) using gene-specific primers (forward 5'-GGCAGAGGAGAGGAGTACCA-3'; reverse 5'-AGCCATAGCCACCTACTCCA-3'). The amplified products from different samples were mixed and loaded into 2% agarose. By electrophoresis, DNA fragment from agarose gel with purified for a 250–500 bp DNA fragments.

Second generation high throughput sequencing (HTS)

Titanium reagent and 454 GS FLX system were used to sequence DNA (SeqWright, USA). The sequencing results of each test sample result were classified according to the coding tags. The reference sequences of cell lines V and J were downloaded from the IMGT database and read by Irmap. CDR3s border area was read through the surveying and mapping information mapping sequence. CDR3s confined region was isolated and transformed into amino acid sequence.

The diversity index is defined as 100 area under the curve between percentage of total reads and percentage of unique CDR3s, when the frequencies of unique CDRs are accumulated from most frequent to least frequent. Importantly, the D50 value is used to evaluate the diversity of immune database, and it explains at least 50% of CDR3s with a minimum proportion of differential CDR3s (19,20). D50 detection abandons the complex model fitting and only focuses on richer subgroups, calibrated by the area of the whole population (21). D50 value of a specific repertoire is positively related to CDR3s sequences diversity, whereas negatively the cumulative frequency difference between each CDR3s clone (21,22).

HPV DNA test

All the cervical exfoliated cells samples were detected by LUMIX system (Tellgen, China), which can qualitatively distinguish the 9 genotypes of hr-HPV (31, 35, 39, 45, 51, 56, 59, 66 and 68), and specificity detect hr-HPV subtype of 16, 18, 33, 52 and 58 closely associated with cervical cancer.

ThinPrep cytology test (TCT)

The cervical exfoliated cells samples were tested by the

Table 1 The subjects' demographic and clinical characteristics of the basic year

Variables	Total (n=26)	Normal (n=3)	hr-HPV (n=8)	CIN 1 (n=6)	CIN 2/3 (n=4)	Cancer (n=5)	P
Age (year)	45.7±9.7	38±5.3	51.0±8.6	47.8±7.5	36.5±7.9	46.8±11.6	0.069
Menarche (year)	14.6±2.0	15.3±0.6	15.4±2.4	14.5±1.2	14.3±3.3	13.3±1.0	0.510
Age at sex debut (year)	21.9±2.6	24.7±1.5	20.8±1.7	20.2±1.2	23.0±3.0	26.0±2.8	0.002
Menstrual conditions							0.129
Premenopausal	18 (69.2)	3 (100.0)	5 (62.5)	2 (33.3)	4 (100.0)	4 (80.0)	
Postmenopausal	8 (30.8)	0 (0.0)	3 (37.5)	4 (66.7)	0 (0.0)	1 (20.0)	
Contraceptives							0.173
No	11 (44.0)	2 (66.7)	2 (25.0)	5 (83.3)	1 (25.0)	1 (25.0)	
Yes	14 (56.0)	1 (33.3)	6 (75.0)	1 (16.7)	3 (75.0)	3 (75.0)	
Pregnant frequency (times)							0.279
0–1	6 (23.1)	1 (33.3)	1 (12.5)	0 (0.0)	2 (50.0)	2 (40.0)	
2–3	10 (38.5)	1 (33.3)	2 (25.0)	5 (83.3)	1 (25.0)	1 (20.0)	
4–5	10 (38.5)	1 (33.3)	5 (62.5)	1 (16.7)	1 (25.0)	2 (40.0)	
Parturition frequency							0.345
0–1	18 (69.2)	3 (100.0)	4 (50.0)	4 (66.7)	4 (100.0)	3 (60.0)	
≥2	8 (30.8)	0 (0.0)	4 (50.0)	2 (33.3)	0 (0.0)	2 (40.0)	

Data are shown as mean ± standard deviation or number (percentage). hr-HPV, high-risk human papillomavirus; CIN, cervical intraepithelial neoplasia.

method ThinPrep (Hologiccytyc, USA). Cytological diagnosis was precisely made by two cytologists accordance with the term of the Bethesda system.

Colposcopy, cervical biopsy and pathological diagnosis

Colposcopy was performed by a professional gynecologist and the cervical biopsy was taken if necessary. All specimens were routinely embedded in paraffin and sectioned in the pathology department of West China Second Hospital. First, the two histopathologists read the slides independently. When the results were inconsistent, the third pathologist read the slides again. The final diagnosis was made by majority rule. Surgical treatment was indicated for women with histological diagnosis of ≥ CIN 2.

Follow-up

The subjects of the hr-HPV group and the CIN 1 group were respectively followed up to confirm the HPV infection, cytology and the status of cervix in the 12th month.

Statistical analysis

These analyses and charting were performed using GraphPad Prism software (version 7.00) and SPSS (version 24.0). All experiments were strictly carried out with the instructions and repeated at least three times. For quantitative data, *t*-test/variance analysis/nonparametric test were used for comparison; for qualitative data, chi square test was used. $P<0.05$ was statistically significant.

Results

The description of patients' characteristics at the baseline year

The grouping depended on the results of colposcopy-directed biopsy, which was only but one diagnosed chronic inflammation in the hr-HPV group. The characteristics of these groups were presented in *Table 1*. There was no significant difference of the age, menarche, menstrual conditions, contraceptives, pregnant frequency, parturition frequency and D50 value among the five groups. All the subjects had no smoking and drinking history. More importantly, there was significant among age at sex debut

Table 2 The CDR3s diversity among different cervical lesions

Group	D50
Control (n=3)	9.2±7.9
hr-HPV (n=8)	5.7±5.6
CIN 1 (n=6)	4.0±6.0
CIN 2/3 (n=4)	13.6±7.7
Cancer (n=5)	8.0±7.6
P	0.252

Data are shown as mean ± standard deviation. CDR3s, complementarity-determining region 3s; D50, diversity 50; hr-HPV, high-risk human papillomavirus; CIN, cervical intraepithelial neoplasia.

Table 3 The relationship between CDR3s diversity and cervical diseases

Cervical diseases	D50
< CIN 2 (n=14)	5.7±6.0
≥ CIN 2 (n=9)	10.5±7.7
P	0.093

Data are shown as mean ± standard deviation. CDR3s, complementarity-determining region 3s; D50, diversity 50; CIN, cervical intraepithelial neoplasia.

Table 4 The relationship between CDR3s diversity and HPV infection

HPV infection	D50
HPV negative (n=4)	8.6±6.6
HPV positive (n=22)	7.1±7.1
P	0.709

Data are shown as mean ± standard deviation. CDR3s, complementarity-determining region 3s; HPV, human papillomavirus; D50, diversity 50.

(P=0.002). In the cancer group, the main clinic manifestations were neoplasm and bleeding with all HPV positive results.

At baseline year, CDR3s diversity was not associated with cervical lesions

The prevalence of CDR3s diversity staining was 9.2±7.9, 5.7±5.6, 4.0±6.0, 13.6±7.7, 8.0±7.6 among women with normal, hr-HPV positive, CIN 1, CIN 2/3 and cancer biopsies. However, it was no statistically significant difference (P=0.252) (*Table 2* and Table S1).

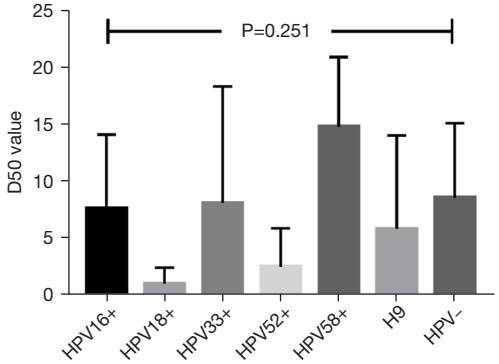

Figure 2 The relationship between CDR3s diversity and HPV subtypes. H9 means the 9 subtypes of hr-HPV (31, 35, 39, 45, 51, 56, 59, 66 and 68) positive infection. +, positive results; –, negative. CDR3s, complementarity-determining region 3s; hr-HPV, high-risk human papillomavirus.

Based on the risk of cervical diseases, subjects were divided into the CIN less severe than CIN 2 (< CIN 2) group (normal, hr-HPV and CIN 1, n=17) and the ≥ CIN 2 group (CIN 2/3 and cancer, n=9). The CDR3s diversity between the two groups was not significant (P=0.093) (*Table 3*).

At baseline screening, CDR3s diversity was not also associated with hr-HPV infection

The D50 value was not associated with the HPV infected results (P=0.709) (*Table 4*). Further analysis of HPV subtypes also showed no statistical differences (P=0.251) (*Figure 2*).

The CDR3s was unsignificant with the CIN1 regression and stabilization after 12 months

One patient in the hr-HPV group was lost to follow up. All results of visual inspection of the cervix were normal, including one Nessler's cyst and one mild erosion. Based on the examination results of the baseline year and following up 12 months, the subjects were divided into the stabilization group and the degradation group.

According to the 12th-month HPV results, there was not apparent significance of the D50 value in the degradation group and the stabilization group (P=0.173) (*Table 5*). Only 1 case of atypical squamous cells of undetermined significance (ASC-US) was found and her biopsy result was chronic cervical and endocervical inflammation with squamous

Table 5 The CDR3s prediction capacity according to the 12th-month HPV results

hr-HPV	D50
Stabilization (n=4)	2.5±3.4
Degradation (n=3)	8.8±7.1
P	0.173

Data are shown as mean ± standard deviation. CDR3s, complementarity-determining region 3s; hr-HPV, high-risk human papillomavirus.

Table 6 The CDR3s prediction capacity according to the 12th-month TCT

CIN 1	D50
Stabilization (n=1)	0.84
Degradation (n=5)	4.7±6.5
P	–

Data are shown as mean ± standard deviation. CDR3s, complementarity-determining region 3s; TCT, ThinPrep cytology test; CIN, cervical intraepithelial neoplasia; D50, diversity 50.

metaplasia. The rest results were normal in the CIN 1 group. It made statistical analysis impossible between the degradation group and the stabilization group in the CIN 1 group because of the too few cases (*Table 6*). Colposcopy examination was normal in patients with abnormal results from TCT or HPV test, according to cervical cancer screening guidelines.

Discussion

In this study, we used D50 to detect CDR3s for the first time, and explored the clinical value of CDR3s may work as a biomarker to predict the risk of persistent hr-HPV infection and CIN 1. We hypothesized that the higher the D50 value of CDR3s immune diversity detections, the greater the possibility of HPV natural clearance and CIN conversion to normal. In order to increase the external validity of the results, 5 groups of research subjects were selected to simulate the natural pathogenesis of cervical cancer. To minimize the bias of different diagnostic classifications and reduce misdiagnosis, pathologists independently finished the diagnosis of cytology and histopathology.

The results of the baseline study suggested that the differences of CDR3s between the < CIN 2 group and the ≥ CIN 2 group were not statistically significant. Previous studies have found that patients with $CD4^+$ and $CD8^+$ T cells in CIN 2/3 have a higher CIN clearance rate, especially eliminating HPV infected cervical epithelial cells (23-27). The diversity of TCR is mainly determined by CDR3s (15,16,21). But our results don't support the unique study reporting the diversity of TCR CDR3s decreased during the cervix carcinogenesis and progression (17).

Therefore, the population in this prospective study included hr-HPV infected and CIN 1 women. The diagnosis of CIN 1 was determined after a thorough discussion by two or three histopathologists. The follow-up personnel did not know the CDR3s test results of HPV-positive and CIN 1 populations at baseline screening. In order to avoid destroying the natural course of cervical diseases, our team followed up on the target population using TCT and HPV test after 12 months.

For the CIN 1 group, all follow-ups were completed. Follow-up results revealed that 5 had normal cytology, and 1 had ASC-US without abnormalities of a referral to colposcopy. All the subjects did not find CIN 1 or more severe lesions. However, high TCR diversity may not degrade CIN 1 to normal, because only one patient with low D50 level persisted CIN 1, failing to statistical analysis. While for the CIN 1 persisted or progressed, Woo *et al.* reported that the infiltrating $CD8^+$ T cells decrease activity (28). These findings suggest that CIN 1 women with low CDR3s diversity should receive close follow-up every 6 months or annually.

In the hr-HPV group, seven patients were followed up, of which 4 continued to be infected with HPV, and 3 became negative. The statistical results of only a few enrolled subjects suggested that there was no statistical difference of CDR3s between the two groups, though D50 in the hr-HPV degradation group was higher than that in the hr-HPV stabilization group. CDR3s cannot yet be considered as a biomarker to predict HPV-positive outcomes.

Our study has several limitations. First of all, this disease usually takes years for HPV infection to induce abnormal cell transformation and then for the lesion to appear (29). Considering with our 12-month follow-up, expanding the sample size of enrolled subjects and extending the follow-up time are inevitable choices to more effectively determine the predictive value of CDR3s in future studies. Secondly, some of the subjects' CIN 1 lesions may be small at baseline screening, or all of the CIN 1 lesions' tissues may have been removed at biopsy, which may also interfere with the follow-up results. In addition, the confounding factors may affect the outcome of D50 and the cervical lesions,

including HLA alleles and haplotypes (30). For example, there was only one statistically significant result of age at sex debut but it is unclear whether this is clinically significant there were low n values and no apparent correlation with level of disease. It's necessary to amplify samples number.

Due to the limitation of research funding, it is too relatively small of the sample size to find out the difference of CDR3s. Thus, CDR3s is not yet considered as a biomarker to predict HPV-positive outcomes. The detection of CDR3s may assist in the clinical management of CIN 1. Women with CIN 1 and decrease of CDR3s diversity may benefit from closer follow-up at frequently intervals.

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Footnote

Reporting Checklist: The authors have completed the TREND reporting checklist. Available at https://gpm.amegroups.com/article/view/10.21037/gpm-21-48/rc

Data Sharing Statement: Available at https://gpm.amegroups.com/article/view/10.21037/gpm-21-48/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://gpm.amegroups.com/article/view/10.21037/gpm-21-48/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of West China Second University Hospital (No. 2013-036) and informed consent was taken from all individual participants.

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Supplementary

Table S1 The original data of CDR3s diversity and HPV subtypes at the baseline year

Case	Group	D50	hr-HPV subtype result
1	Normal	12.84	–
2	Normal	14.7	–
3	Normal	0.2	–
1	hr-HPV	2.02	18+
2	hr-HPV	16.15	16+
3	hr-HPV	2.26	H9+
4	hr-HPV	0.07	52+
5	hr-HPV	8.12	58+
6	hr-HPV	0.2	52+
7	hr-HPV	9.08	16+
8	hr-HPV	7.31	52+
1	CIN 1	0.01	16+, H9
2	CIN 1	0.84	33+
3	CIN 1	0.78	16+
4	CIN 1	6.6	–
5	CIN 1	15.29	16+, H9
6	CIN 1	0.58	16+
1	CIN 2/3	2.5	52+
2	CIN 2/3	15.39	33+
3	CIN 2/3	20.14	58+
4	CIN 2/3	16.29	58+
1	Cancer	8.56	16+
2	Cancer	0.02	18+
3	Cancer	10.62	16+
4	Cancer	1.78	+
5	Cancer	19.14	+

H9 means the 9 subtypes of hr-HPV (31, 35, 39, 45, 51, 56, 59, 66 and 68) positive infection. +, positive results; –, negative. CDR3s, complementarity-determining region 3s; hr-HPV, high-risk human papillomavirus; CIN, cervical intraepithelial neoplasia.

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