The role of p16 / Ki-67 dual staining in HPV positive and negative women in the early diagnosis of cervical precancerous lesions: Cytology, colposcopy and conization protocol

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Summary

Aim: The aim of this study was to evaluate the relationship between p16 / Ki-67 dual staining used for the definition of precancerous cervical lesions with histological results and HPV positivity.

Materials and Method: This study is a cross-sectional study of 468 patients who were followed up in our center with the diagnosis of cervical intraepithelial neoplasia between 2016 and 2019 using the cytology, colposcopic biopsy and conization results, HPV test and p16 / Ki-67 dual staining results. SPSS 22 program was used in the analysis of the data. In the analysis of qualitative data, chi-square test and binary logistic regression analysis were used. The compatibility of both models with bilateral logistic regression test was good (omnibus test \( < 0.001 \)). The correct estimate percentage of the model is 71.4% and 80.3%. \( p < 0.05 \) is considered important.

Results: In the binary logistic regression test established between HPV types and p16 / Ki-67 dual staining positivity, other high risk HPV types, HPV 16, 18 and 16-18, increased p16 / Ki-67 positivity ratio in this order. In the binary logistic regression test established between abnormal cytology and p16 / Ki-67 dual staining positivity in the colposcopy results, there was p16 / Ki-67 positivity increasing in proportion with the degree of HGSIL lesions.

Conclusion: This study created with cytology / colposcopy and LEEP conization protocol shows that; in effective screening for early diagnosis and treatment in cervical cancer, p16 / Ki-67 biomarkers can be used effectively.

Key words: p16/Ki-67 dual staining; HGSIL; cytology; colposcopy; LEEP conization; HPV positive

Introduction

Cervical cancer is the fourth most common cancer seen in women after breast cancer, colorectal cancer and lung cancer. The incidence for cervical cancer is 13.1 in 100,000 women worldwide [1]. Cytology (Pap test) screening has been accepted to reduce the incidence and mortality of cervical cancer [2]. However, the rate of false positivity is also high in early stage changes such as atypical cells (ASCUS) and cervical intraepithelial neoplasia (CIN) grade I [3]. Therefore, pap test alone is considered to be insufficient in detecting cervical cancer.

Human Papilloma Virus (HPV) is a sexually transmitted virus. It is known that persistent cervical infection with high risk HPV genotypes causes the development of CIN II, a precursor lesion for the development of cervical cancer [2]. HPV 16 is the most carcinogenic HPV genotype and is positive in about 55-60% of all cervical cancers. HPV 18 is the next most carcinogenic HPV genotype and constitutes 10-15% of cervical cancer, other high risk HPV types, HPV 16, 18 and 16-18, increased p16 / Ki-67 positivity ratio in this order. Those with persistent HPV infection (especially with HPV 16 type) have an increased risk of developing CIN 3 lesions between two and five years [5]. Based on these data, it is recommended to use pap test and Food And Drug Administration (FDA) approved HPV tests together in screening to determine the frequency of precancerous lesions [2].

Proving that HPV is an oncogenic virus has made it necessary to investigate the pathophysiological mechanisms that cause it. In researches, oncogenic E6 and E7 proteins in the structure of HPV affect p53 and Retinoblastoma (Rb) proteins located in the apoptotic pathway that prevent tumor formation, respectively. p16 is a cyclin-dependent kinase (CDK) inhibitor involved in this pathway. It affects CDK 4 and CDK 6 enzymes and prevents the abnormal activity of Rb. In this way, it prevents abnormal tumoral proliferation. However, the fact that the E7 protein directly activates the RB pathway causes this mechanism to fail and p16 to accumulate in the environment. Thus, p16 values reach measurable levels [6]. Ki-67; it is a nuclear antigen showing cellular proliferation, expressed in all cell cycle phases except G0 [6]. In normal cells, p16 and Ki-67 markers are not found at the same time and work in opposite mechanisms. In the presence of CIN developing due to HPV, both increase and become positive [7].

It is very important to determine the screening intervals together with HPV test and pap test or to apply advanced diagnosis methods on time. Most women with HPV positivity do not have a precancerous lesion at diagnosis or have a low degree [8]. For this reason, it is not appropriate to decide colposcopy with positivity only in HPV types.

Biomarkers that can fully predict the outcome of the dis-
Table 1. — Demographic characteristics and evaluation results of patients

|                                 | P16/Ki-67 positive | P16/Ki-67 negative |
|---------------------------------|--------------------|--------------------|
| **Age**                         | 41.35 ± 10.33 (min:28-max:57) | 40.08 ± 5.01 (min:22-max:47) |
| **Educational Status**          |                    |                    |
| Middle School and Below         | 24 / 14.6          | 47 / 15.5          |
| Middle School or Above          | 37 / 22.4          | 89 / 29.4          |
| License                         | 60 / 36.4          | 139 / 45.9         |
| Master                          | 44 / 26.6          | 28 / 9.2           |
| **Menopausal status**           |                    |                    |
| Premenopausal                   | 11 / 6.6           | 75 / 24.8          |
| Perimenopausal                  | 12 / 7.3           | 72 / 23.8          |
| Postmenopausal                  | 53 / 32.1          | 0 / 0.0            |
| **Cervical Cytology**           |                    |                    |
| Negative                        | 17 / 19.1          | 72 / 80.9          |
| ASCUS                           | 41 / 21.2          | 152 / 78.8         |
| ASC-H                           | 23 / 88.5          | 3 / 11.5           |
| LGSIL                           | 74 / 51.4          | 70 / 48.6          |
| HGSIL CIN II-III                | 4 / 40.0           | 6 / 60.0           |
| HGSIL-CIN II                    | 6 / 100.0          | 0 / 0.0            |
| **Colposcopic Biopsy Results**  |                    |                    |
| ASCUS                           | 0 / 0.0            | 6 / 100.0          |
| CIN I                           | 51 / 16.8          | 252 / 83.2         |
| CIN II+III                      | 9 / 69.2           | 4 / 30.8           |
| CIN II                          | 60 / 66.7          | 30 / 33.3          |
| CIN III                         | 43 / 89.6          | 5 / 10.4           |
| **Conization Biopsy Results**   |                    |                    |
| Untreated                       | 34 / 11.9          | 251 / 88.1         |
| ASCUS                           | 0 / 0.0            | 2 / 100.0          |
| CIN I                           | 36 / 46.8          | 41 / 53.2          |
| CIN II+III                      | 6 / 100.0          | 0 / 0.0            |
| CIN II                          | 47 / 88.7          | 6 / 11.3           |
| CIN III                         | 40 / 97.6          | 1 / 2.4            |
| Negative                        | 1 / 20             | 4 / 80             |
| HPV                             |                    |                    |
| HPV Negative                    | 14 / 10.0          | 126 / 90.0         |
| HPV 16                          | 69 / 55.6          | 55 / 44.4          |
| HPV 18                          | 18 / 38.3          | 29 / 61.7          |
| HPV 16-18                       | 24 / 77.4          | 7 / 22.6           |
| **OTHER HPV**                   | 40 / 31.7          | 86 / 68.3          |
| **BMI**                         |                    |                    |
| <25                             | 22 / 13.3          | 87 / 28.7          |
| 25-29,99                        | 101 / 61.2         | 179 / 59.1         |
| >30                             | 42 / 23.5          | 37 / 12.2          |
| **Smoking**                     |                    |                    |
| Positive                        | 48 / 29.1          | 39 / 12.9          |
| Negative                        | 117 / 70.9         | 264 / 87.1         |

Atypical Squamous Cells Of Undetermined (ASCUS); Low Grade Squamous Intraepithelial Lesion (LGSIL); High Grade Squamous Intraepithelial Lesion Cervical Intraepithelial Neoplasia grade II (HGSIL CIN II); High Grade Squamous Intraepithelial Lesion Cervical Intraepithelial Neoplasia grade III (HGSIL CIN III); High Grade Squamous Intraepithelial Lesion Cervical Intraepithelial Neoplasia grade II-III (HGSIL CIN II-III); Atypical Aquamous Cells (which cannot exclude HSIL) (ASC-H); Human Papilloma Virus (HPV); NEGATIVE; there is no pathologic result this patient; n= patient number; OTHER HPV; patient group infected with other high-risk HPV types.

ease allow clinicians to make an accurate decision about management for a particular patient. Biomarkers can assist in screening, detecting, diagnosing the disease, and evaluating the prognosis. In the previous studies; in cytolog-
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Figure 1. — Cytological screening test results, patients with HPV positivity, HPV positive and negative patients, HPV types and p16 / Ki-67 dual staining results are shown in

Figure 2. — Colposcopic biopsy results, patients with HPV positivity, HPV positive and negative patients, HPV types and p16 / Ki-67 dual staining results are shown in

The aim of this study was to evaluate the relationship between p16 / Ki-67 dual staining used for the definition of precancerous cervical lesions with histological results and HPV positivity.

Material and method

Study Population:
There were 154912 patients who were admitted to our outpatient clinic between 2016 and 2019, and who were administered pap / HPV test protocol. 923 patients with abnormal results and / or positive HPV test were detected in the Pap test. 143 of these patients were excluded from the study because they did not follow-up and 107 were negative pap test results after antibiotherapy. In addition, 205 patients who did not come to the study despite colposcopy
Figure 3. — The patients with HPV positivity, HPV positive and negative patients, HPV types and p16 / Ki-67 dual staining in 183 patients with conization.

Table 2. — Comparison of cytology results and p16 / Ki-67 results

| Cytology Result | P16/Ki-67 (n/%) |              |              |
|-----------------|----------------|--------------|--------------|
|                 | Positive       | Negative     | p            |
| NEGATIVE        | 17/19.1        | 72/80.9      |              |
| ASCUS           | 41/21.2        | 152/78.8     |              |
| ASC-H           | 23/88.5        | 3/11.5       | < 0.001      |
| LGSIL           | 74/51.4        | 70/48.6      |              |
| HGSIL CIN II-III| 4/40.0         | 6/60.0       |              |
| HGSIL-CINII     | 6/100.0        | 0/0.0        |              |

Table 3. — Comparison of colposcopy results and p16 / Ki-67 results

| COLPOSCOPY      | P16/Ki-67 (n/%) |              |              |
|-----------------|----------------|--------------|--------------|
|                 | Positive       | Negative     | p            |
| ASCUS           | 0/0.0          | 6/100.0      |              |
| LGSIL           | 51/16.8        | 252/83.2     |              |
| HGSIL CIN II-III| 9/69.2         | 4/30.8       | < 0.001      |
| HGSIL-CINII     | 60/66.7        | 30/33.3      |              |
| HGSIL-CINIII    | 43/89.6        | 5/10.4       |              |
| NEGATIVE        | 2/25.0         | 6/75.0       |              |

Table 4. — Comparison of conization results and P16-Ki-67 results

| CONIZATION      | P16/Ki-67 (n/%) |              |              |
|-----------------|----------------|--------------|--------------|
|                 | Positive       | Negative     | p            |
| Untreated       | 34/11.9        | 251/88.1     |              |
| ASCUS           | 0/0.0          | 2/100.0      |              |
| LGSIL           | 36/46.8        | 41/53.2      |              |
| HGSIL (CIN II+III)| 6/100.0       | 0/0.0        | < 0.001      |
| HGSIL-CINII     | 47/88.7        | 6/11.3       |              |
| HGSIL-CINIII    | 40/97.6        | 1/2.4        |              |
| NEGATIVE        | 1/20           | 4/80         |              |

Table 5. — Comparison of conization results and P16-Ki-67 results

| HPV TYPE        | POSITIVE | NEGATIVE | p            |
|-----------------|----------|----------|--------------|
| HPV NEGATIVE    | 14/10.0  | 126/90.0 |              |
| HPV 16          | 69/55.6  | 55/44.4  |              |
| HPV 18          | 18/38.3  | 29/61.7  | < 0.001      |
| HPV 16-18       | 24/77.4  | 7/22.6   |              |
| OTHER HPV       | 40/31.7  | 86/68.3  |              |

appointment due to abnormal cytology and / or HPV positivity could not be included in the study. This study is a cross-sectional study involving 468 patients who underwent colposcopic biopsy due to abnormal cervical cytology result and / or HPV positivity, and the history, examination, cervical cytology results, HPV DNA test results, colposcopic biopsy results and conization results, if any.

Being under the age of 18, previously receiving treatment for cervical disease (including loop electrosurgical excision procedure (LEEP), cold knife conization, cryotherapy, Light Amplification by Stimulated Emission of Radiation (LASER) treatment or hysterectomy), previously received a diagnosis of cervical neoplasia and radiotherapy and / or chemotherapy, having an autoimmune disease history, immunosuppressive drug use history, pregnancy were exclusion criteria for the study. In patients who were di-
Table 6. — HPV type and p16 / Ki-67 Binary logistic regression results

| HPV TYPE      | β    | p       | O.R. | %95 C.I. for O.R |
|---------------|------|---------|------|-----------------|
| HPV 16        | 2.42 | < 0.001 | 11.29| 5.85 - 21.75    |
| HPV 18        | 1.72 | < 0.001 | 5.58 | 2.49 - 12.51    |
| HPV 16-18     | 3.42 | < 0.001 | 30.85| 11.27 - 84.45   |
| OTHER HPV     | 1.63 | 0.002   | 5.14 | 1.83 - 14.39    |

Table 7. — Colposcopic biopsy result and p16 / Ki-67 logistic regression results

| COLPOSCOPY       | β    | p       | O.R. | 95% C.I.for O.R |
|------------------|------|---------|------|-----------------|
| ASCUS            | -20.1| 0.999   | 0    | 0               |
| LGSIL            | -0.49| 0.548   | 0.6  | 0.11 - 3.09     |
| HGSIL CINII+III  | 1.91 | 0.06    | 6.75 | 0.92 - 49.23    |
| HGSIL-CINII      | 1.79 | 0.034   | 6    | 1.14 - 31.53    |
| HGSIL-CINIII     | 3.25 | 0.001   | 25.8 | 4.06 - 163.91   |

Table 8. — Sensitivity and specificity values and confidence intervals of p16 / Ki-67 dual staining positivity test

|               | %95 Confidence Interval |
|---------------|------------------------|
| Sensitivity   | 35,9                   |
| Specificity   | 85,71                  |
| Positive Likelihood | 0.69 - 9,11  |
| Negative Likelihood | 0.6-0.94    |
| Prevalence    | 98,79                  |
| Positive Predictive Value | 95,74 - 99,66 |
| Negative Predictive Value | 3,96            |
| Accuracy      | 37,39                  |

Cytology, colposcopic biopsy and conization results, HPV test and p16 / Ki-67 dual staining results were noted. The diagnosis of CIN was investigated in histopathological evaluations. In cases with more than one cytology result, the result of cytological evaluation before colposcopic biopsy was noted. The treatment protocol of the patients whose first cytology results were ASCUS was determined according to the post-treatment cytology results and these data were included in the study. In the histological examination of colposcopic biopsy and conization, the highest grade cervical intraepithelial neoplasia specified in the diags was included in the data. The patient group, which was equally distributed in the diags, was evaluated separately from the other groups.

Statistical analysis:

Statistical analysis was performed with IBM® SPSS® Statistics 22 (International Business Machines Corp., Armonk, New York) for Windows software. In the analysis of qualitative data, Chi-square test and binary logistic regression analysis were used. In logistic regression analysis, dependent variable was determined as reference category (0): P16-Ki67 negative, risk group (1): P16/ Ki-67 positive, and independent variable as HPV subtypes and colposcopy results. The compatibility of both models was found to be good (omnibus test < 0.001). The correct estimation percentage of the model is 71.4% and 80.3%. Exact and benforin corrections were made in chi-square tests. p < 0.05 is considered important. Sensitivity, specificity, positive and negative predictive values and disease prevalence are expressed in percentages. When calculating confidence intervals for sensitivity, specificity and accuracy, Clopper-Pearson confidence intervals were used. While calculating the confidence intervals of likelihood ratios, the 'log method' method and the confidence intervals of the predictive values were taken as the basis for standard logit confidence intervals.
Results

Age, education, menopausal status, smoking, body mass index (BMI), cytology, colposcopic biopsy, conization results and HPV types of patients are shown in Table 1.

Cytological screening test results, patients with HPV positivity, HPV positive and negative patients, HPV types and p16 / Ki-67 dual staining results are shown in Figure 1.

Colposcopic biopsy results, HPV positive and negative patients, HPV types and p16 / Ki-67 dual staining results are shown in Figure 2.

Figure 3 shows the HPV positive and negative patients, HPV types and p16 / Ki-67 dual staining in 183 patients with conization.

When the relationship between cytology results and p16 / Ki-67 dual staining results was examined, it was found that there was a statistically significant relationship. In patients with negative cytology results or ASCUS, the p16 / Ki-67 dual staining test was often negative. P16 / Ki-67 dual staining test positivity was common in patients with high-grade squamous intraepithelial lesion (ASC-H), low-grade squamous intraepithelial lesion (LGSIL) and high grade squamous intraepithelial lesion (HGSIL) as a result of colposcopy (p < 0.001) (eg see also Table 2).

When the results of colposcopy and p16 / Ki-67 were examined, a statistically significant relationship was observed. The negative p16 / Ki-67 results were more frequent in the LGSIL group, while the positive p16 / Ki-67 results were common in the HGSIL-CIN II, HGSIL-CIN III and HGSIL-CIN II-III groups (p < 0.001) (eg see also Table 3).

Considering p16 / Ki-67 results in 183 patients with conization, a statistically significant relationship was found. While negative p16 / Ki-67 dual staining test results were common in patients without conization and LGSIL as a result of conization, HGSIL-CIN II, HGSIL-CIN III and HGSIL-CIN II-III groups had significantly higher positive p16 / Ki-67 results (p < 0.001), (eg see also Table 4).

When the HPV type and p16 / Ki-67 results were analyzed, it was seen that there was a statistically significant relationship. Compared to the group of patients with positive HPV 18 and OHr-HPV type, p16 / Ki-67 dual staining test negativity was common in the HPV negative patient group. In contrast, in the patient group where HPV 16 and HPV 16-18 were positive together, p16 / Ki-67 dual staining positivity was common (p < 0.001), (Table e.g. see also 5).

According to the logistic regression test results established to determine the relationship between HPV types and p16 / Ki-67 dual staining in colposcopy results; the risk of positive p16 / Ki-67 results in patients with HPV 16 positive is 11 times; 5.5 times in patients with HPV 18 positive; 30 times in patients with HPV 16-18 positive and 5.1 times in patients with other HPV positivity (Table e.g. see also 6).

In colposcopic biopsy results, the probability of p16 / Ki-67 double staining positivity in the HGSIL-CINIII patient group increased six-fold and twenty-five fold in the HGSIL-CINIII patient group (e.g. see also Table 7).

The sensitivity of p16 / Ki-67 dual staining positivity was 35% and the specificity was 85%. The positive predictive value of the test is 98% and the negative predictive value is 3% (e.g. see also Table 8). The p16 / Ki-67 dual staining test has low sensitivity to detect the presence of CIN compared to histopathological examination with colposcopic biopsy. Only 35% of cases with p16 / Ki-67 dual staining positivity have CIN. However, only 15% of women with negative p16 / Ki-67 dual staining tests were found to have CIN, and the specificity of the test was high. That is, the negativity of the p16 / Ki-67 dual staining test can rule out the presence of CIN.

Discussion

As far as the author knows; this work; this is the most comprehensive and current study investigating the role of p16 / Ki-67 dual staining test in Turkish women with HPV positivity in cytology.

In the patient population included in this study, p16 / Ki-67 double staining test increased positivity rates in proportion to the degree of lesion, and this finding supports previous studies [10, 14, 15].

It is known that persistent infection with high-risk HPV subtypes causes proliferative and tumoral effects on cervical epithelial cells by affecting cytokines, chemokines, free oxygen radicals, apoptotic pathways and specific miRNA species, causing long-term chronic inflammation [14]. HPV 16 and 18 are more oncogenic than OHr-HPV types [15-16]. In patients with HPV 16, 18 and HPV 16-18 positivity, this finding supports up to thirty fold increase in p16 / Ki-67 positivity compared to OHr-HPV types. Also in this study; the positivity of OHr-HPV types has been shown to be associated with p16 / Ki-67 positivity. In China, Yu et al. showed a strong correlation between OHr-HPV types and p16 / Ki-67 dual staining positivity [15]. These studies show that it is not surprising to see p16 / Ki-67 double staining positivity in HPV-infected cervical epithelial cells, similar to our study.

It has also recently emerged that miRNAs are the main epigenetic regulators in controlling various vital processes such as growth, differentiation, angiogenesis and development. It has been stated that deregulation of these molecules in HPV-infected cells may be related to the initial stages of cervical cancer [17]. In a recent study, curcumin has been shown to act on miRNA pathways, preventing abnormal proliferation and invasion of cervical cancer cells [18]. Moreover, it has been shown that melatonin, which induces apoptosis and has an antioxidant effect, may be effective on cervical cancer treatment [19]. These studies show that; it is very important to know the pathological pathways in the diagnosis and treatment of CIN lesions.

In this study, all patients diagnosed as normal cytology, ASCUS and LGSIL in cytological evaluation were evaluated together with colposcopic biopsy results. In this way,
we were able to analyze the role of p16 / Ki-67 dual staining positivity in cytological evaluation by comparing it with colposcopy. In the cytological evaluation, it was statistically significant to obtain a negative result in the p16 / Ki-67 dual staining test in the patient groups diagnosed with ASC-US, whereas p16 / Ki-67 dual staining positivity was significant in LGSIL lesions. Wentzensen et al. also reported that dual staining had lower positivity and higher sensitivity and specificity in detecting precancerous lesions compared to the cytology obtained as a result of ASCUS [20].

Considering the results of colposcopic biopsy in our study, p16 / Ki-67 negativity was found significant in patients evaluated as LGSIL. Similarly, one study showed that p16 / Ki-67 positivity was more common in the patient group whose cytological result was LGSIL compared to the group whose cytological result was negative or ASC-US [21]. Moreover, it has been shown in another study that the presence of p16 / Ki-67 positivity in minor atypical changes such as ASCUS and LGSIL in the cytology results may predict HSIL in biopsy results [22]. Although it has been reported in previous retrospective studies, colposcopic biopsies can be reduced by p16 / Ki-67 dual staining in patients with ASCUS and LGSIL cytological evaluation results [23]; according to this study, the choice of colposcopic biopsy should be prioritized in approaching LGSIL lesions.

In the cervical cancer screening, it is stated in the USA guideline that HPV positive patients should not be lead directly to colposcopy. Instead, it has been reported that HPV type should be determined first and colposcopy may be recommended if HPV 16 and / or HPV 18 types are positive. No evidence has yet been reported for biomarkers [2]. Again, Yu et al. in a retrospective study; the sensitivity of the p16 / Ki-67 test was found to be higher for CIN II lesions than HPV genotyping, but its specificity was low [24]. In a retrospective study of 86 diseases with LEEP conization, it was stated that p16 / Ki-67 positivity could only be a marker for recurrence in HSIL lesions [23]. Unlike those mentioned in this study; the results of conization in patients with indication were also analyzed. p16 / Ki-67 dual staining positivity was found statistically high in patients whose conization results were evaluated as HSIL (CIN II, CIN II-III, CIN III) and p16 / Ki-67 dual staining negativity was shown in patients evaluated as LGSIL. This supported other studies in which colposcopic biopsy results were reported [15, 18]. Moreover, p16 / Ki-67 double staining negativity in conization may shed light on contradictions for colposcopy triage of LGSIL patients.

This study has some limitations. Most importantly, this study evaluates the performance of p16 / Ki-67 dual staining in cytology in cross-section. It does not allow evaluation of HSIL lesions for a long time. However, this limitation was tried to be eliminated by including the results of conization in patients with HSIL. Another one is that our study contains single center data. However, since it is a single center, pathological evaluations and HPV tests of all patients were performed with the same methods and devices and technical heterogeneity was excluded. These limitations should be considered before generalizing to society.

As a result; this study created with cytology / colposcopy and LEEP conization protocol shows that; in effective screening for early diagnosis and treatment in cervical cancer, p16 / Ki-67 biomarkers can be used effectively. Prospective studies with larger groups are needed in the future to be included in the screening protocol to reduce colposcopy requirement.

Ethics Approval and Consent to Participate
All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Com mittee of Adiyaman Univercity (approval number: 2020/1-11).

Acknowledgments
I would like to thank Reşat Özercan, the Head of the Department of Pathology of our University, for his contribution to the data collection.

Conflict of interest
The author declare no competing interests.

Submitted: January 06, 2020
Accepted: May 14, 2020
Published: August 15, 2020

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