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Systematic Review

The Role of p16/Ki-67 Immunostaining, hTERC Amplification and Fibronectin in Predicting Cervical Cancer Progression: A Systematic Review

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Simple Summary: Human papillomaviruses (HPV) are common sexually transmitted infections and they are responsible for cervical cancer (CC), as well as for several other anogenital cancers. CC is the fourth leading cause of death in women with cancer, although it could be preventable by enforcement of optimal screening programs. The Pap smear is the standard screening test for CC and precancerous lesions, and a combination of Pap smear and HPV testing is generally recommended as a triage step before colposcopy. However, these tests cannot predict lesion progression, which is why several adjunctive biomarkers have been studied. Our aim was to summarize current scientific data on the role of these biomarkers, with a view to determining which biomarkers could help to more accurately establish the need for colposcopy and at the same time, to limit the number of unnecessary colposcopy referrals.

Abstract: Human papillomaviruses (HPVs) are common sexually transmitted infectious agents responsible for several anogenital and head and neck cancers. Cervical cancer (CC) is the fourth leading cause of death in women with cancer. The progression of a persistent HPV infection to cancer takes 15–20 years and can be preventable through screening. Cervical cytology (Pap smear) is the standard screening test for CC and precancerous lesions. For ASC-US and ASC-H lesions, a combination of Pap smear and HR-HPV analysis is recommended as a triage step before colposcopy. However, these tests cannot predict progression to CC. For this purpose, we summarized current scientific data on the role of p16/Ki-67 immunohistostaining, telomerase and fibronectin in predicting progression to CC. p16 and p16/Ki-67 dual staining (DS) were more specific than HR-HPV DNA testing for the detection of CIN2+/CIN3+ in women with ASC-US and LSIL. Similarly, hTERC FISH analysis significantly improved the specificity and positive predictive value of HPV DNA testing in differentiating CIN2+ from CIN2 cytological samples. In conclusion, p16 IHC, p16/Ki-67 DS and hTERC FISH amplification are all valid adjunctive biomarkers which significantly increase the sensitivity and specificity of cervical dysplasia diagnosis, especially when combined with HPV DNA testing. However, considering the global socioeconomic background, we can postulate that p16 and p16/ Ki-67 IHC can be used as a next step after positive cytology for ASC-US or LSIL specimens in low-income countries, instead of HPV DNA testing. Alternatively, if HPV DNA testing is covered

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by insurance, p16 or p16/Ki-67 DS and HPV DNA co-testing can be performed. In middle- and high-income countries, hTERC amplification can be performed as an adjunctive test to HPV DNA testing in women with ASC-US and LSIL.

Keywords: cervical cancer; HPV; p16; Ki-67; telomerase; fibronectin; progression

1. Introduction

Human papillomaviruses (HPVs) are common sexually transmitted infectious agents described as non-enveloped, double-stranded, circular DNA viruses belonging to the Papovaviridae family [1]. Approximately 90% of HPV infections are transient and become undetectable in 1–2 years. However, persistent infections with oncogenic HPV types have been associated with the progression of the disease [2,3]. According to epidemiological data, 12 mucosal alpha HPVs are categorized as high-risk HPV (HR-HPV) types and are responsible for several anogenital and head and neck cancers [4]. HPV16 and 18 are the most carcinogenic types: HPV16 has been associated with 50–60% of cervical cancers (CCs), HPV18 with 10–15% of CCs and the remaining HR-HPV types have been implicated in 25–40% of CCs [5,6].

CC is the fourth most frequently diagnosed cancer worldwide [7] and according to the WHO it is the fourth leading cause of death in women with cancer, with an estimated annual incidence of 604,000 cases and 342,000 deaths reported worldwide in 2020 [8]. The progression of a persistent HPV infection to cancer usually takes 15–20 years and it is preventable by the optimal application of secondary prevention programs [9]. CC screening is recommended to be initiated at the age of 21 years via cytology every three years or, for women aged 30–65 years, cytology in combination with HR-HPV testing every five years. Screening can be discontinued in women with a hysterectomy or women older than 65 years who have a history of regular screening with negative results [10–12].

Cytology-based screening, also known as the Papanicolau smear (Pap smear) test, was first introduced in 1940 by Georgios Papanicolau as a CC screening tool. Conventionally, microscopic evaluation is performed on cervical cells obtained from cervical scraping after fixing them on a glass slide. Another cytology-based screening method is liquid-based cytology (LBC), by which cervical cells are suspended in a liquid medium and then filtered and transferred onto a monolayer for microscopic evaluation [11,13,14]. Both methods have shown similar sensitivity, specificity, positive predictive value, negative predictive value and accuracy for the detection of cervical intraepithelial neoplasia (CIN) 2 or higher [15].

Cytological findings have been classified according to the Bethesda system [16], which was updated in 2014 [17] and includes the following categories: atypical squamous cells of undetermined significance (ASC-US); atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H); low-grade squamous intraepithelial lesion (LSIL—corresponding to mild dysplasia/ CIN 1); high-grade squamous intraepithelial lesion (HSIL—corresponding to moderate or severe dysplasia, CIS; CIN 2 or CIN 3) and squamous cell carcinoma (SCC) [17]. ASC-US and LSIL are generally considered transient lesions of the cervical epithelium, although an important proportion of women with ASC-US and LSIL have underlying CIN2 or 3 and an increased risk for developing CC [18]. Rigurous triage of women with ASCUS or LSIL is warranted for early diagnosis and treatment of CIN2 or 3 lesions, as well as for minimizing unnecessary biopsies, especially in young women who wish to conceive. The Pap smear test is currently used as a first step in the CC screening method and more recently, HR-HPV co-testing has been integrated into cervical cancer screening guidelines [19]. Despite existing protocols, CC maintains high incidence and mortality rates, which is why several adjunctive biomarkers and their role in accurately predicting progression to CC have been studied. For this purpose, we have summarized current scientific data on the role of p16/Ki-67 immunohistostaining (IHC), telomerase and fibronectin biomarkers.

The main role of p16/Ki-67 IHC in the triage of HPV-positive women is to distinguish between those with underlying high- and low-grade cervical lesions, which aids in determining the necessity for immediate colposcopy referrals [20]. It is cost-effective, highly reproducible and has a relatively low technical complexity [13], which makes it easily accessible and widely used.

Telomerase up-regulation is known to arrest cellular apoptosis, thus having a central role in malignant proliferation [21,22]. Moreover, the E6/E7 oncogene encoding the HPV proto-oncoprotein can up-regulate telomerase activity by human telomerase RNA component (hTERC) gene amplification. Studies have shown an important correlation between HR-HPV infection and hTERC up-regulation in CC progression [23–26]. Telomerase activity as a prognostic biomarker in CC has been demonstrated through numerous studies and it is generally recommended as an ancillary biomarker in CC screening, after cytology and HPV DNA detection.

Fibronectin (FN1) is a glycoprotein component of the extracellular matrix that plays an important part in cell growth, cell adhesion and differentiation [27]. A few studies have discussed its potential role in different malignancies such as hepatocellular, renal, gastrointestinal, head and neck cancers [28,29]. We further discuss the literature published so far.

2. Materials and Methods

2.1. Study Selection

We conducted a systematic review of the literature following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. We searched the PubMed database for studies published between 2011 and 2022 using the term *cervical cancer* in combination with the following terms: *telomerase*, *fibronectin*, *p16*, *ki-67*, *HPV.* The last search was run on 25th March 2022. There was no limit to study design.

2.2. Data Extraction

Two investigators independently selected relevant articles according to predefined inclusion and exclusion criteria, as described above. Disagreements were resolved by discussion, with a prior arrangement that any unsettled discrepancy would be determined by a third author.

2.3. Inclusion Criteria

Eligibility was restricted to studies in which p16, ki-67, telomerase and fibronectin positivity were correlated with histopathologic modifications in cervical specimens classified according to the Bethesda system. The relationship between the grade of cervical dysplasia and mentioned markers was analyzed. Only articles in English were selected. Only studies in which telomerase activity and HPV detection were performed by genomic amplification techniques and not by staining procedures were included. Other potentially relevant articles were identified by manually checking the references of the articles included.

2.4. Exclusion Criteria

We excluded the following studies: those where the number of patients was either not specified or expressed as different age frequencies; those where the main inclusion criterion was only HPV-positive patients; those where different comparisons were drawn, either between the sensitivity and specificity of various detection methods, or between self-sampled specimens and samples collected by healthcare professionals. Additionally, studies in which p16 and ki-67 staining were assessed only according to the level of expression and not as either positive or negative specimens were excluded, given the great heterogeneity of histopathological assessment techniques and grading systems [30,31].

2.5. Data Synthesis and Statistical Analysis

Pertinent data were selected in the form of: number of biopsy specimens analyzed, number of specimens for each category of the Bethesda classification system, number of

HPV-positive specimens, HPV types detected, number of p16-, Ki-67-, telomerase- and fibronectin-positive specimens.

2.6. Limitations

The limitations of this review lie in study heterogeneity, which is reflected in the different scoring systems used for cervical modifications, for HPV detection, and for p16/ki-67 staining positivity. In order to limit bias in reporting, we objectively summarized relevant data from the literature in Tables 1–4. We only included studies where a definitive histopathologic diagnosis was provided and cervical dysplasia was classified according to either of the Bethesda systems [16,17]. Non-neoplastic lesions (NNL) included any type of modification, including inflammation (cervicitis), infection and atypical metaplasia, which was mentioned in just one study [32]. Cervical cancer (CC) was ascribed to both squamous cell carcinoma, either in situ or invasive, and adenocarcinoma, considering that most studies included both types of cervical cancer under this common nomenclature.

3. Results

A total of 853 records were initially identified in the literature search, of which 34 were duplicates and 728 did not meet the inclusion criteria, thus being further excluded (Figure 1). A total of 20,877 biopsy specimens were investigated, of which there were 7174 for p16 IHC, 745 for Ki-67 IHC, 5329 for p16/ ki-67 dual staining (DS) and 9084 for telomerase up-regulation.

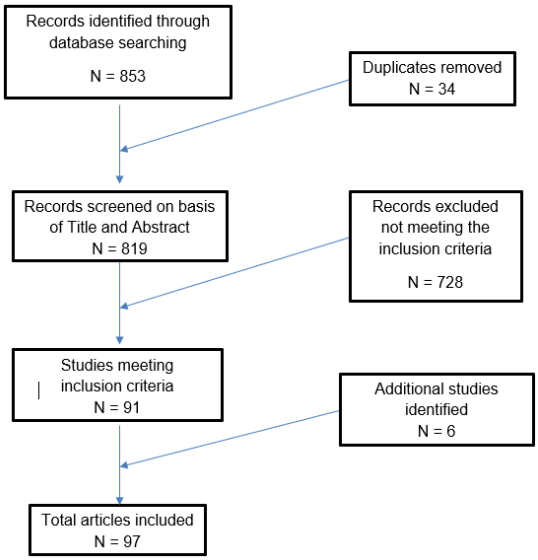

Figure 1. Literature search and article selection.

p16 staining

Nineteen studies had relevant data regarding p16 IHC, totaling 7174 biopsy specimens: 1375 NNL, 1857 CIN1, 2 CIN 1/2, 1923 CIN2, 43 CIN2/3, 1664 CIN3, 310 CC. p16 was positive in 3813/7069 biopsy specimens: 2.54% NNL, 15.02% CIN1, 0.05% CIN1/2, 35.79% CIN2, 0.91% CIN2/3, 38.18% CIN3, 6.74% CC. HPV genotyping was positive in 4486/6335 biopsy specimens: 5.84% NNL, 19.30% CIN1, 0.02% CIN1/2, 35.95% CIN2, 0.53% CIN2/3, 32.56% CIN3, 4.34% CC (Table 1).

The majority of the studies demonstrated a directly proportional increase in the likelihood of p16 positive staining and the severity of cervical dysplasia [6,33–43]. Similarly, the number of HPV-positive specimens increased with the degree of intraepithelial lesion [6,33–37,43]. Moreover, higher sensitivity and specificity rates were demonstrated for the combination of HR-HPV detection and p16 IHC in the early diagnosis of cervical lesions, as compared with either test alone: p16 sensitivity (Se) = 95.83% and specificity

(SP) = 65.34%, HR-HPV Se = 91.67% and Sp = 53.4%, combination p16 and HR-HPV testing Se = 89.58% and Sp = 72.73% [33].

Additionally, Alhamlan et al. [44] found that p16 IHC was a significant negative predictor of survival. In a retrospective, cross-sectional study conducted on 315 cervical biopsy specimens collected from women aged 23–95 years old who were also PCR-tested for HPV L1 protein, p16 overexpression correlated with poorer survival rates (multivariate Cox regression, hazard ratio, 3.2; 95% CI, 1.1–8.8). Conversely, a multivariate Cox regression analysis showed that HPV-positive cervical cancer (CC) had better survival rates, whereas HPV-negative CC was linked to significantly worse disease-free survival [36]. Similar findings were reported in the literature [45–47].

Table 1. p16 IHC in cervical tissue biopsy specimens.

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16 Positive IHC and Correlation with Biopsy Results
Alhamlan et al., 2021 [44]	315, of which: 82 NNL 54 CIN1 16 CIN2 45 CIN3 118 CC	96/315, of which: 6/82 NNL 6/54 CIN1 3/16 CIN2 17/45 CIN3 64/118 CC	111/212, of which: 2/54 NNL 9/37 CIN1 7/10 CIN2 20/25 CIN3 73/84 CC
Castle et al., 2019 [6]	4010, of which: 283 NNL 934 CIN1 1512 CIN2 1208 CIN3 73 CC	3172/4010 59/283 NNL 507/934 CIN1 1386/1512 CIN2 1154/1208 CIN3 66/73 CC, of which: 1283/3172 HPV16: 9/283 NNL 67/934 CIN1 506/1512 CIN2 658/1208 CIN3 43/73 CC 242/3172 HPV 18/45: 7/283 NNL 49/934 CIN1 111/1512 CIN2 65/1208 CIN3 10/73 CC 1357/3172 OHR-HPV: 28/283 NNL 270/934 CIN1 659/1512 CIN2 390/1208 CIN3 10/73 CC 213/3172 IR-HPV: 11/283 NNL 82/934 CIN1 85/1512 CIN2 34/1208 CIN3 2/73 CC 76/3172 LR-HPV: 4/283 NNL 39/934 CIN1 25/1512 CIN2 7/1208 CIN3 1/73 CC	2520/4010, of which: 21/283 NNL 248/934 CIN1 1087/1512 CIN2 1095/1208 CIN3 69/73 CC

Table 1. *Cont.*

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16 Positive IHC and Correlation with Biopsy Results
Haltas et al., 2012 [48]	64, of which: 8 NNL 26 CIN1 19 CIN2 8 CIN3 3 CC	N/A	37/64, of which: 0/8 NNL 12/26 CIN1 15/19 CIN2 7/8 CIN3 3/3 CC
Huang et al., 2011 [33]	272, of which: 82 NNL 94 CIN1 41 CIN2 28 CIN3 27 CC	170/272, of which: (HR-HPV) 19/82 NNL 63/94 CIN1 35/41 CIN2 27/28 CIN3 26/27 CC	153/272, of which: 14/82 NNL 47/94 CIN1 37/41 CIN2 28/28 CIN3 27/27 CC
Indarti et al., 2013 [34]	30, of which: 11 CIN1 9 CIN2 10 CIN3	14/30, of which: 0/11 CIN1 5/9 CIN2 9/10 CIN3	17/30 0/11 CIN1 7/9 CIN2 10/10 CIN3
Liao et al., 2013 [35]	463, of which: 187 NNL 171 CIN1 53 CIN2 49 CIN3 3 CC	248/463 29/187 NNL 124/171 CIN1 45/53 CIN2 47/43 CIN3 3/3 CC	160/463 5/187 NNL 73/171 CIN1 40/53 CIN2 39/49 CIN3 3/3 CC
Ma et al., 2011 [36]	131, of which: 79 NNL 26 CIN1 23 CIN2/3 3 CC	88/131 HR-HPV, of which: 43/79 NNL 21/26 CIN1 21/23 CIN2/3 3/3 CC	49/131 10/79 NNL 16/26 CIN1 20/23 CIN2/3 3/3 CC
Pabuccu et al., 2017 [49]	27, of which: 14 NNL 5 CIN1 8 CIN2/3	N/A	13/27 1/14 NNL 5/5 CIN1 7/8 CIN2/3
Pacchiarotti et al., 2014 [50]	577, of which: 312 NNL 159 CIN1 39 CIN2 58 CIN3 9 CC	N/A	193/577, of which: 6/312 NNL 91/159 CIN1 36/39 CIN2 53/58 CIN3 7/9 CC
Sarma et al., 2017 [51]	110, of which: 25 NNL 25 CIN1 21 CIN2 12 CIN3 27 CC	N/A	60/110, of which: 2/25 NNL 8/25 CIN1 11/21 CIN2 12/12 CIN3 27/27 CC
Tsoumpou et al., 2011 [52]	126, of which: 12 NNL 66 CIN1 36 CIN2 12 CIN3	64/126, of which: 28/78 NNL/CIN1 36/48 CIN2/3	28/126, of which: 8/78 NNL/CIN1 20/48 CIN2/3

Table 1. *Cont.*

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16 Positive IHC and Correlation with Biopsy Results
Valasoulis et al., 2013 [37]	200, of which: 23 NNL 79 CIN1 50 CIN2 48 CIN3	133/200 HPV: 6/23 NNL 41/79 CIN1 41/50 CIN2 45/48 CIN3 118/200 HR-HPV: 5/23 NNL 30/79 CIN1 38/50 CIN2 45/48 CIN3 60/200 HPV16/18: 0/23 NNL 14/79 CIN1 17/50 CIN2 29/48 CIN3	53/200, of which: 2/23 NNL 12/79 CIN1 17/50 CIN2 22/48 CIN3
van Baars et al., 2015 [39]	104, of which: 25 NNL 11 CIN1 23 CIN2 45 CIN3	90/104, of which: 13/25 NNL 11/11 CIN1 23/23 CIN2 43/45 CIN3	76/104, of which: 0/25 NNL 8/11 CIN1 23/23 CIN2 45/45 CIN3

p16/Ki-67 DS

Seventeen studies had relevant data regarding p16/Ki-67 DS, totaling 5329 biopsy specimens: 2704 NNL, 936 CIN1, 2 CIN1/2, 655 CIN2, 12 CIN2/3, 810 CIN3, 210 CC. p16/Ki-67 DS was positive in 2327/5300 biopsy specimens: 20.24% NNL, 15.68% CIN1, 22.04% CIN2, 0.34% CIN2/3, 30.46% CIN3, 8.51% CC. HPV genotyping was positive in 2376/4883 biopsy specimens: 28.78% NNL, 17.97% CIN1, 0.04% CIN1/2, 18.01% CIN2, 0.37% CIN2/3, 24.41% CIN3, 7.53% CC.

p16/Ki-67 IHC has been used most frequently throughout the studies. Similar to p16 and Ki-67 IHC alone, an increase in the number of DS-positive biopsy specimens was correlated with a more severe histological diagnosis [32,41–43,53–63] and with HPV DNA positivity [41–43,58,59,63] (Table 2).

Table 2. p16/Ki-67 DS in cervical tissue biopsy specimens.

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16/Ki67 Positive IHC and Correlation with Biopsy Results
Celewicz et al., 2018 [53]	43, of which: 17 NNL 5 CIN1 10 CIN2 8 CIN3 3 CC	NA	30/43, of which: 9/17 NNL 2/5 CIN1 9/10 CIN2 7/8 CIN3 3/3 CC
Diouf et al., 2020 [54]	69, of which: 30 NNL 14 CIN1 3 CIN2 3 CIN3 19 CC	30/38, of which: 1/7 NNL 4/6 CIN1 6/6 CIN2/3 19/19 CC	32/46, of which: 1/7 NNL 6/14 CIN1 6/6 CIN2/3 19/19 CC

Table 2. *Cont.*

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16/Ki67 Positive IHC and Correlation with Biopsy Results
Donà et al., 2012 [64]	113, of which: 14 NNL 35 CIN1 24 CIN2 37 CIN3 3 CC	95/107 5/13 NNL 31/33 CIN1 23/24 CIN2 36/37 CIN3/CC 84/107 HR-HPV 3/13 NNL 25/33 CIN1 20/24 CIN2 36/37 CIN3/CC 11/107 O-HPV 2/13 NNL 6/33 CIN1 3/24 CIN2 0/37 CIN3/CC	62/107, of which: 0/13 NNL 13/33 CIN1 17/24 CIN2 32/37 CIN3/CC
El-Zein et al., 2020 [55]	492, of which: 134 NNL 130 CIN1 99 CIN2 121 CIN3 8 CC	321/492, of which: 47/134 NNL 69/130 CIN1 86/99 CIN2 111/121 CIN3 8/8 CC 119/492 HPV16: 7/134 NNL 17/130 CIN 37/99 CIN2 55/121 CIN3 3/8 CC 26/492 HPV18: 6/134 NNL 4/130 CIN1 5/99 CIN2 5/121 CIN3 6/8 CC 139/492 HPV16/18: 12/134 NNL 20/130 CIN1 41/99 CIN2 58/121 CIN3 8/8 CC 235/492 OHR-HPV: 41/134 NNL 63/130 CIN1 58/99 CIN2 70/121 CIN3 3/8 CC 321/492 ANY HR-HPV: 47/134 NNL 69/130 CIN1 86/99 CIN2 111/121 CIN3 8/8 CC	279/492, of which: 41/134 NNL 54/130 CIN1 72/99 CIN2 105/121 CIN3 7/8 CC
Frega et al., 2019 [56]	78, of which: 53 CIN2 25 CIN3	73/78, of which: 50/53 CIN2 23/25 CIN3	74/78, of which: 50/53 CIN2 24/25 CIN3

Table 2. *Cont.*

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16/Ki67 Positive IHC and Correlation with Biopsy Results
Liu et al., 2020 [65]	305, of which: 90 NNL 48 CIN1 35 CIN2 117 CIN3 15 ICC	N/A	165/305, of which: 3/90 NNL 8/48 CIN1 26/35 CIN2 113/117 CIN3 15/15 CC
Ngugi et al., 2015 [57]	22, of which: 12 NNL 2 CIN1 2 CIN2 6 CIN3	21/22 HR-HPV, of which: 11/12 NNL 2/2 CIN1 2/2 CIN2 6/6 CIN3	8/22, of which: 1/12 NNL 0/2 CIN1 1/2 CIN2 6/6 CIN3
Waldstrøm et al., 2013 [59]	226, of which: 42 NNL 97 CIN1 41 CIN2 45 CIN3 1 CC	174/226, of which: 28/42 NNL 66/97 CIN1 36/41 CIN2 43/45 CIN3 1/1 CC	154/226, of which: 23/42 NNL 54/97 CIN1 33/41 CIN2 43/45 CIN3 1/1 CC
Wentzensen et al., 2012 [32]	623, of which: 137 NNL 228 CIN1 169 CIN2 83 CIN3 6 CC	171/623 HPV16, of which: 24/137 NNL 31/228 CIN1 60/169 CIN2 53/83 CIN3 3/6 CC	371/623, of which: 42/137 NNL 106/228 CIN1 140/169 CIN2 77/83 CIN3 6/6 CC
Yu et al., 2016 [60]	1290, of which: 996 NNL 63 CIN1 42 CIN2 119 CIN3 70 CC	463/1290, of which: 204/996 NNL 41/63 CIN1 40/42 CIN2 111/119 CIN3 67/70 CC	427/1290, of which: 183/996 NNL 34/63 CIN1 34/42 CIN2 111/119 CIN3 65/70 CC
Yu et al., 2016 [61]	701, of which: 640 NNL 46 CIN1 11 CIN2 4 CIN3	173/701, of which: 126/640 NNL 32/46 CIN1 11/11 CIN2 4/4 CIN3	149/701, of which: 111/640 NNL 26/46 CIN1 8/11 CIN2 4/4 CIN3
Zhang et al., 2019 [62]	537, of which: 298 NNL 29 CIN 49 CIN2 111 CIN3 50 CC	294/537, of which: 76/298 NNL 18/29 CIN 45/49 CIN2 106/111 CIN3 49/50 CC 168/537 HPV16/18 23/298 NNL 8/29 CIN 16/49 CIN2 80/111 CIN3 41/50 CC 168/537 O-HPV 59/298 NNL 10/29 CIN 34/49 CIN2 50/111 CIN3 15/50 CC	234/537, of which: 39/298 NNL 10/29 CIN 38/49 CIN2 99/111 CIN3 48/50 CC

Table 2. *Cont.*

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16/Ki67 Positive IHC and Correlation with Biopsy Results
Zhu et al., 2019 [63]	300, of which: 138 NNL 108 CIN1 29 CIN2 22 CIN3 3 CC	256/300, of which: 103/138 NILM 100/108 CIN1 28/29 CIN2 22/22 CIN3 3/3 CC	96/300, of which: 3/138 NILM 40/108 CIN1 28/29 CIN2 22/22 CIN3 3/3 CC

Ki-67 staining

Six studies had data regarding Ki-67 IHC alone, totaling 745 biopsy specimens, of which: 243 NNL, 196 CIN1, 2 CIN1/2, 104 CIN2, 12 CIN2/3, 141 CIN3, 47 CC. Ki-67 was positive in 384/654 biopsy specimens: 17.18% NNL, 18.48% CIN1, 0.26% CIN1/2, 22.39% CIN2, 2.60% CIN2/3, 27.60% CIN3, 13.54% CC. HPV genotyping was positive in 411/684 specimens: 21.16% NNL, 22.62% CIN1, 0.24% CIN1/2, 18.24% CIN2, 0.73% CIN2/3, 28.95% CIN3, 8.02% CC.

Ki-67 was generally expressed in combination with p16 IHC, as DS positivity. Where data were available, a direct proportionality relation between Ki-67 expression alone and the severity of intraepithelial lesion was demonstrated [40–43,66], as well as between Ki-67 expression and HPV DNA positivity [40–43] (Table 3).

Telomerase

Seventeen studies had data regarding telomerase up-regulation detected via fluorescence in situ hybridization (FISH) of hTERC amplification, totaling 9084 biopsy specimens: 1998 NNL, 2423 CIN1, 65 CIN1/2, 1617 CIN2, 120 CIN2/3, 1832 CIN3, 1029 CC. Telomerase was detected in 4337/9084 biopsy specimens: 4.28% NNL, 12.12% CIN1, 0.94% CIN1/2, 24.53% CIN2, 1.86% CIN2/3, 34.67% CIN3, 22.09% CC. HPV genotyping was positive in 2872/ 3937 biopsy specimens: 12.74% NNL, 23.39% CIN1, 1.11% CIN1/2, 17.79% CIN2, 2.09% CIN2/3, 25.52% CIN3, 11.90% CC (Table 4).

Throughout the studies, telomerase activity increased with the severity of cervical dysplasia [67–80]. Furthermore, significant differences in telomerase activity levels between L-SIL versus H-SIL, L-SIL versus CC and H-SIL versus CC, with higher activity levels in the more advanced groups, were demonstrated [22,81,82]. Similarly, He et al. [69] showed significant differences in the frequency of genomic amplification of hTERC between NNL and CIN2/CIN3/SCC, between CIN1 and CIN2/CIN3/SCC, as well as between CIN2 and SCC lesions. Additionally, Chen et al. [68], further demonstrated the superiority in terms of sensitivity and specificity of hTERC and HPV DNA co-testing when compared with hTERC amplification testing alone, for cervical cancer screening: hTERC Se = 90.0% and SP = 89.6%, HPV DNA Se = 100% and Sp = 44.0%, combination hTERC and HPV DNA Se = 90.0% and Sp = 92.2% [33].

Table 3. p16, Ki-67 and DS IHC in cervical tissue biopsy specimens.

Reference Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16 Positive IHC and Correlation with Biopsy Results	KI-67 Positive IHC and Correlation with Biopsy Results	DS Positive IHC and Correlation with Biopsy Results
Chang et al., 2014 [40]	143, of which: 77 NNL 33 CIN 6 CIN2 22 CIN3 5 CC	70/143, of which: 23/77 NNL 21/33 CIN1 4/6 CIN2 18/22 CIN3 4/5 CC	31/141, of which: 5/75 NNL 3/33 CIN1 4/6 CIN2 15/21 CIN3 4/5 CC	29/124 of which: 2 /69 NNL 2/27 CIN1 4/5 CIN2 17/19 CIN3 4/4 CC	NA
Gatta et al., 2011 [67]	72, of which: 10 NNL (controls) 32 CIN1 10 CIN2 10 CIN3 10 CC	9/72, of which: 0/10 NNL 8/32 CIN1 1/10 CIN2 0/10 CIN3 0/10 CC	41/72, of which: 0/10 NNL 11/32 CIN1 10/10 CIN2 10/10 CIN3 10/10 CC	N/A	NA
Jackson et al., 2012 [43]	97, of which: 39 NNL 46 CIN1 12 CIN2/3	17/36, of which: 4/9 NNL 10/24 CIN1 3/3 CIN2/3	14/97, of which: 1/39 NNL1 5/46 CIN1 8/12 CIN2/3	25//97, of which: 4/39 NNL 11/46 CIN1 10/12 CIN2/3	13/97, of which: 1/39 NNL 4/46 CIN1 8/12 CIN2/3
Koo et al., 2013 [41]	70, of which: 27 NNL 6 CIN1 20 CIN2 17 CIN3	36/70 HR-HPV: 9/27 NNL 2/6 CIN1 14/20 CIN2 11/17 CIN3 of which: 18/36 HPV 16/18: 3/9 NNL 0/2 CIN1 7/14 CIN2 8/11 CIN3	50/70, of which: 15/27 NNL 2/6 CIN1 16/20 CIN2 17/17 CIN3	48/70, of which: 16/27 NNL 2/6 CIN1 14/20 CIN2 16/17 CIN3	43/70, of which: 4/27 NNL 4/6 CIN1 18/20 CIN2 17/17 CIN3
Li et al., 2019 [42]	350, of which: 84 NNL 77 CIN1 68 CIN2 89 CIN3 32 CC	271/350, of which: 49/84 NNL 50/77 CIN1 56/68 CIN2 87/89 CIN3 29/32 CC 271/350, of which: 141/350 HPV16 16/350 HPV 18 16/350 HPV 31 21/350 HPV 33	197/350, of which: 9/84 NNL 22/77 CIN1 55/68 CIN2 80/89 CIN3 31/32 CC	276/350, of which: 41/84 NNL 56/77 CIN1 60/68 CIN2 87/89 CIN3 32/32 CC	185/350 8/84 NNL 17/77 CIN1 50/68 CIN2 79/89 CIN3 31/32 CC

Table 3. *Cont.*

Reference Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	p16 Positive IHC and Correlation with Biopsy Results	KI-67 Positive IHC	DS Positive IHC and Correlation with Biopsy Results
		13/350 HPV 35 13/350 HPV 39 3/350 HPV 45 16/350 HPV 51 56/350 HPV 52 10/350 HPV 56 61/350 HPV 58 8/350 HPV 59 11/350 HPV 68			
Toll et al., 2014 [66]	13, of which: 6 NNL 2 CIN1 2 CIN1/2 3 CIN3	8/13, of which: 2/6 NNL 2/2 CIN1 1/2 CIN1/2 3/3 CIN3	10/13, of which: 4/6 NNL 1/2 CIN1 2/2 CIN1/2 3/3 CIN3	6/13, of which: 3/6 NNL 0/2 CIN1 1/2 CIN1/2 2/3 CIN3	5/13, of which: 2/6 NNL 0/2 CIN1 1/2 CIN1/2 2/3 CIN3

Table 4. hTERC up-regulation in cervical tissue biopsy specimens.

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	hTERC up-Regulation and Correlation with Biopsy Results
Chen et al., 2012 [68]	243, of which: NNL = 164 CIN1 = 29 CIN2 = 21 CIN3 = 22 CC = 7	158/243, of which: NNL = 84/164 CIN1 = 24/29 CIN2 = 21/21 CIN3 = 22/22 CC = 7/7	55/243, of which: NNL = 15/164 CIN1 = 5/29 CIN2 = 6/21 CIN3 = 22/22 CC = 7/7
He et al., 2012 [69]	175, of which: NNL = 24 CIN1 = 34 CIN2 = 36 CIN3 = 33 CC = 48	N/A	86/175, of which: NNL = 0/24 CIN1 = 5/34 CIN2 = 18/36 CIN3 = 23/33 CC = 40/48
He et al., 2020 [70]	135, of which: CIN 1/2 = 65 CIN3 = 39 CC = 31	97/135 CIN1/2 = 32/65 CIN3 = 35/39 CC = 30/31	109/135 CIN 1/2 = 41/65 CIN3 = 37/39 CC = 31/31
Ji et al., 2019 [71]	213, of which: NNL = 159 CIN1 = 31 CIN2 = 14 CIN3 = 7 CC = 2	103/213 75 HR, 28 LR, of which: NNL = 41 HR, 25 LR/159 CIN1 = 16 HR, 2 LR/31 CIN2 = 10 HR, 1 LR/14 CIN3 = 6 HR/7 CC = 2 HR/2	64/213, of which: NNL = 29/159 CIN1 = 18/31 CIN2 = 9/14 CIN3 = 6/7 CC = 2/2
Jiang et al., 2010 [72]	6726, of which: NNL = 1257 CIN1 = 2054 CIN2 = 1387 CIN3 = 1410 CC = 618	1752/2313, of which: NNL = 156/385 CIN1 = 560/794 CIN2 = 406/461 CIN3 = 490/522 CC = 140/151	3250/6726, of which: NNL = 124/1257 CIN1 = 428/2054 CIN2 = 952/1387 CIN3 = 1162/1410 CC = 584/618
Jin et al., 2011 [73]	130, of which: NNL = 52 CIN1 = 33 CIN2 = 9 CIN3 = 26 CC = 10	N/A	46/130, of which: NNL = 2/52 CIN1 = 6/33 CIN2 = 6/9 CIN3 = 22/26 CC = 10/10
Koeneman et al., 2019 [83]	19, of which: CIN2 = 3 CIN3 = 16	19/19	15/19, of which: CIN 2 = 3/3 CIN 3 = 12/16
Kudela et al., 2018 [74]	111, of which: NNL = 27 CIN1 = 15 CIN2 = 24 CIN3 = 25 CC = 20	90/111, of which: NNL = 14/27 CIN1 = 7/15 CIN2 = 24/24 CIN3/CIS = 25/25 CC = 20/20	58/111, of which: NNL = 1/27 CIN1 = 4/15 CIN2 = 11/24 CIN3 = 21/25 CC = 20/20
Kuglik et al., 2015 [75]	74, of which: NNL = 12 CIN1 = 6 CIN2 = 6 CIN3 = 12 CC = 38	64/74, of which: NNL = 10/12 CIN1 = 3/6 CIN2 = 3/6 CIN3 = 10/12 CC = 34/38	23/74, of which: NNL = 3/12 CIN1 = 1/6 CIN2 = 3/6 CIN3 = 7/12 CC = 33/38

Table 4. *Cont.*

Reference, Year	Number of Biopsy Specimens	HPV Detection and Correlation with Biopsy Results	hTERC up-Regulation and Correlation with Biopsy Results
Li et al., 2014 [77]	171, of which: NNL = 64 CIN1 = 26 CIN2 = 29 CIN3 = 36 CC = 16	N/A	67/171, of which: NNL = 6/64 CIN1 = 6/26 CIN2 = 15/29 CIN3 = 26/36 CC = 14/16
Liu et al., 2012 [23]	114, of which: NNL = 27 CIN1 = 26 CIN2 = 16 CIN3 = 24 CC = 21	77/114	51/114 NNL = 0/26 CIN1 = 4/19 CIN2 = 6/12 CIN 3 = 22/27 CC = 19/19
Liu et al., 2019 [24]	150, of which: NNL = 32 CIN1 = 38 CIN2/3 = 66 CC = 14	108/150, of which: NNL = 10/32 CIN1 = 25/38 CIN2/3 = 60/66 CC = 13/14	64/150 NNL = 4/32 CIN1 = 13/38 CIN2/3 = 35/66 CC = 12/14
Xiang et al., 2012 [21]	92, of which: NNL = 20 CIN3 = 14 CC = 58	N/A	62/92 NNL = 0/20 CIN3 = 12/14 CC = 50/58
Yin et al., 2012 [78]	166, of which: NNL = 40 CIN1 = 27 CIN2/3 = 54 CC = 45	N/A	101/166 NNL = 0/40 CIN1 = 12/27 CIN2/3 = 46/54 CC = 43/45
Zappacosta et al., 2015 [25]	54, of which: NNL = 8 CIN1 = 26 CIN2 = 9 CIN3 = 11	52/54	20/54, of which: NNL = 0/8 CIN1 = 6/26 CIN2 = 6/9 CIN3 = 8/11
Zheng et al., 2013 [79]	373, of which: NNL = 89 CIN1 = 36 CIN2 = 43 CIN3 = 129 CC = 76	267/373, of which: NNL = 26/89 CIN1 = 19/36 CIN2 = 32/43 CIN3 = 119/129 CC = 71/76	192/373, of which: NNL = 0/89 CIN1 = 5/36 CIN2 = 18/43 CIN3 = 101/129 CC = 68/76
Zhu et al., 2018 [80]	138, of which: NNL = 23 CIN1 = 42 CIN2 = 20 CIN3 = 28 CC = 25	85/138, of which: NNL = 4/23 CIN1 = 16/42 CIN2 = 14/20 CIN3 = 26/28 CC = 25/25	74/138, of which: NNL = 2/23 CIN1 = 13/42 CIN2 = 11/20 CIN3 = 23/28 CC = 25/25

Fibronectin

Zhou et al. [84] performed a comparative study assessing the levels of FN1 expression in 94 paired patients with CC by quantitative real-time polymerase chain reaction (qRT-PCR). They found significantly higher FN1 levels in cervical cancer tissues than in adjacent normal tissues. Furthermore, higher FN1 expression was correlated with poor prognosis.

4. Discussion

Currently, cervical cytology (Pap smear) is the standard screening test for CC and precancerous lesions [11]. For ASC-US and ASC-H lesions, a combination of Pap smear and HR-HPV analysis is generally recommended as a triage step before colposcopy [66]. However, these tests have low applicability: Pap smear can only identify abnormal cell morphologies and most HPV infections are self-limited, thus neither test has predictive value [22].

Despite being considered transitory, low-grade lesions, a critically large number of ASC-US and LSIL specimens had underlying CIN2 and CIN3 morphologic changes, which carry a high risk for malignant transformation [18]. Consequently, adjunctive biomarkers have been investigated in order to increase the accuracy of CC screening and to guide selection of the most appropriate treatment option.

P16/Ki-67 staining

p16inka (p16) is a cyclin-dependent kinase inhibitor involved in the normal cycle of somatic cells and acts as a tumor suppressor [44]. p16 overexpression is associated with keeping the retinoblastoma protein (Rbp) in an unphosphorylated state which deaccelerates cell cycle progression from G1 to S phase [85,86]. Viral oncogenes E6 and E7 are known to be drivers of proliferation, promoting and maintaining the malignant growth of cervical cells in the process of high-risk HPV-linked carcinogenesis [13,87]. p16 protein is considered a surrogate biomarker for the transforming activity of high-risk HPV and it can be detected via IHC staining of cytology or histology specimens [88,89]. p16-positivity is defined as strong and diffuse staining, meaning nuclear and/or nuclear plus cytoplasmic expression affecting the basal and para-basal cell layers and extending to the surface of the squamous epithelium on histological sections [90].

Ki-67 is a non-histone cell cycle progression antigen expressed only during the active phases of the cell cycle (G1, S, G2 and mitosis) and it is described as a biomarker for determining the growth fraction of a tumor [91]. According to IHC studies, Ki-67 is normally expressed in the basal and para-basal layers of the epithelium, whereas high-grade CIN lesions containing abnormally proliferating cells appear as increased Ki-67 staining in all layers of the squamous epithelium [19]. Isolated expression of p16 or Ki-67 within a cell may be considered physiologic, whereas simultaneous positive staining of the two biomarkers is linked with cell cycle dysregulation associated with a transformative high-risk HPV infection [13]. Co-expression of p16 and the cell cycle progression biomarker Ki-67 in one cell allows for the unequivocal identification of HPV-transformed epithelial cells and can be detected via dual immunostaining (DS) of p16/Ki-67 [92].

Additionally, p16/Ki-67 DS positivity was strongly associated with HR-HPV persistence and the presence of CIN2+ lesions [57]. One study found that p16/Ki-67 DS had sensitivity and specificity rates of 93.2% and 46.1%, respectively, for CIN3+ detection and these increased to 97.2% and 60.0% in women older than 30 years; for women with HR-HPV-positive ASC-US and LSIL, sensitivity and specificity rates were as high as 90.6% and 48.6%, respectively, which might make p16/Ki-67 DS a potent biomarker for LSIL triage [22]. Additionally, Ma et al. [36] showed that p16 immunostaining had significantly higher specificity and accuracy in predicting high-grade CIN and CC in ASC-US and LSIL specimens, as compared with HR-HPV DNA testing.

In a prospective, cross-sectional study on 599 patients, Liu et al. [65] compared the clinical performance of Pap smear, HPV DNA testing and p16/Ki-67 DS for the detection of CIN2+/VAIN2+. They found that for women who tested positive for HR-HPV and had a Pap smear $\geq$ ASC-US, DS reduced the number of unnecessary colposcopy referrals from 274 to 181. Additionally, DS identified four high-grade lesions that had initially negative colposcopy-guided biopsy results.

A recent meta-analysis evaluating the accuracy of p16 and p16/Ki-67 DS versus HR-HPV testing for the detection of CIN2+/CIN3+ in women with ASC-US and LSIL found

that p16 staining and p16/Ki-67 DS were more specific than HR-HPV DNA testing, whereas p16 staining was less sensitive and DS has similar sensitivity [93].

Throughout the studies, however, sensitivity (Se) rates remained above 90%, whereas specificity (Sp) rates were below 50% [22], which indicates a high risk of unnecessary biopsy referrals. p16 IHC had significantly higher specificity and accuracy rates in predicting high-grade CIN and CC in ASC-US and LSIL specimens, as compared with HR-HPV DNA testing [36]. Additionally, p16 and HR-HPV co-testing had Se = 89.58% and Sp = 72.73% [33]. However, no studies analyzing the combined Se and Sp rates of cytology, HPV DNA testing and DS have been performed. On the other hand, p16 IHC was shown to be a significant negative predictor of survival [44], whereas HPV-positive CC had better survival rates [45–47]. Finally, according to The Lower Anogenital Squamous Terminology (LAST), p16 IHC is recommended for distinguishing between H-SIL and benign lesions mimicking precancerous lesions (immature squamous metaplasia, atrophy, repair changes and tangentially sectioned specimens) and also for the assessment of morphologically equivocal cases interpreted as L-SIL versus H-SIL [94].

Hence, given the current literature, it can be postulated that DS can be used ancillary to, or instead of HPV DNA detection, for women with ASC-US and LSIL. Additionally, p16 IHC can be used as a negative survival predictor for women with CC [44].

Telomerase

Telomerase is a ribonucleoprotein enzyme complex that adds 50-TTAGGG-30 repeats to the chromosomal ends known as telomeres, which play an important part in maintaining chromosomal stability during DNA replication [21,23,24]. Human telomerase consists of three subunits: one RNA component (hTERC), which functions as a template for DNA replication; one of unknown function (TP1) and the human telomerase reverse transcriptase (hTERT) [95,96]. hTERC gene expression is consistent with telomerase activity and it is generally expressed in many normal tissues [24]. However, telomerase up-regulation can reflect a malignant process as it stops cellular apoptosis, consequently leading to tumorigenesis [21,22]. The majority of studies have demonstrated the importance of increased telomerase activity as a prognostic marker in CC, its level being positively correlated with viral load, clinical stage, tumor size and lymph node metastases [96]. The activity of telomerase might be a potential method for the differential diagnosis between low-grade and high-grade precancerous cervical neoplasia, reaching Se and Sp rates of over 90% [21,23,97,98]. HR-HPV positivity and increased hTERC activity have been linked to more aggressive CC and might have an important role in future screening algorithms [23–25].

Furthermore, it has been suggested that hTERC amplification be used as a triage test, ancillary to HPV DNA in ASC-US and LSIL cytological samples, as a predictor of progression to more severe cervical neoplasia [21]. Studies have shown that increased telomerase activity detected by FISH analysis increased with the degree of cervical dysplasia [21]. In addition, hTERC FISH analysis significantly improved the specificity and positive predictive value of HPV DNA testing in differentiating CIN2+ from CIN2 cytological samples [25,79]. Currently, the determination of telomerase activity is not used in routine screening tests, but most authors have proposed that this method become part of future screening tests for cervical dysplasia [24,77,80,96].

Moreover, the combination of cytology, HPV DNA testing and hTERC amplification reached Se and Sp levels as high as 100% and 98.11%, respectively [68,71]. This makes hTERC an important adjunctive biomarker for CC screening and it can be recommended as an ancillary test to cytology and HPV DNA detection in women with ASC-US and LSIL lesions.

Fibronectin

Fibronectin is an extracellular matrix glycoprotein that plays a major role in cell differentiation, growth and migration. Furthermore, it is involved in the processes of wound healing and embryonic development, as well as oncogenic transformation. The highest levels of fibronectin expression were detected in colorectal, renal and esophageal cancers and were associated with poor prognosis [84]. Few studies have shown a significantly

higher expression of fibronectin in cervical cancer tissues compared with adjacent normal tissues, but further evidence is lacking [84,99]. Consequently, the role of fibronectin as a prognostic marker in patients with CC requires additional investigation and might have potential diagnostic and therapeutic implications.

5. Challenges and Future Scope

CC screening and HPV vaccination campaigns are the pillars of CC prevention. However, given the financial, political and educational differences worldwide, strategies for CC prevention cannot be implemented homogenously. Access to medical care, information campaigns and health financing influence the addressability of CC screening and the treatment options. Hence, there is continuous research for more reliable and accessible biomarkers that can be used irrespective of the socioeconomic background of each country.

6. Conclusions

Currently, cervical cytology and HR-HPV analysis are the well-known and widely accepted screening tests for CC and precancerous lesions. However, they cannot be used to predict lesion progression to high-risk intraepithelial neoplasia. ASC-US and LSIL specimens can have underlying CIN2 and CIN3 morphologic changes, which carry a high risk for CC progression, which emphasizes the need for adjunctive biomarkers with predictive value.

p16 IHC had significantly higher specificity and accuracy rates than HPV DNA testing in predicting high-grade cervical dysplasia and CC in ASC-US and LSIL specimens. Thus, p16 IHC can be used as an alternative to HPV DNA testing in low-income countries for women with ASC-US and LSIL cytology. However, p16 and HPV DNA co-testing have better sensitivity and specificity rates (Se = 89.58% and Sp = 72.73%), which lowers the number of unnecessary colposcopy referrals, but each case should be investigated according to financial availability. Additionally, p16 can be used as a negative survival predictor for women with CC.

The combination of cytology, HPV DNA testing and hTERC FISH amplification reached sensitivity and specificity levels as high as 100% and 98.11%, respectively, which make hTERC an important, although expensive, adjunctive biomarker for CC screening. It can be recommended as an ancillary test to cytology and HPV DNA detection in women with ASC-US and LSIL lesions, in medium- and high-income countries.

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