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Evaluation of the HPV ISH Assay in Cervical Cancer

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Background : Human papillomavirus (HPV) infection can be detected by *in situ* hybridization (ISH), in which a punctate signal pattern indicates integrated HPV DNA and a diffuse pattern denotes the presence of episomal viral DNA. This study was conducted to evaluate the usefulness of an HPV ISH assay for invasive cervical cancer. **Methods :** The HPV ISH assay for high-risk HPV and immunohistochemical staining for $p16^{INK4a}$, p53, bcl-2, and Ki-67 were performed in a tissue microarray of 279 cervical cancers. **Results :** High-risk HPV ISH was positive in 194 (69.5%) of the samples. Punctate, diffuse, and mixed signal patterns were observed in 157 (56.3%), one (0.4%), and 36 cases (12.9%), respectively. Positive results in high-risk HPV ISH were associated with p16 and bcl-2 expression ($p = 0.01$ and $p < 0.01$, respectively). According to a Cox regression analysis, HPV infection and its surrogate immunohistochemical markers such as p16, bcl-2, and Ki-67 were not independent prognostic factors, but stage and grade were independent prognostic factors. **Conclusions :** Our results confirm that an HPV ISH assay is reasonably sensitive for HPV infection and that it might be useful to identify integrated HPV DNA in formalin-fixed and paraffin-embedded specimens. Further study encompassing HPV type, E2/E6 ratio, and therapeutic modality is necessary to understand the clinical meaning of HPV status in cervical cancer.

Key Words : HPV; *In situ* hybridization; Cervical cancer

Human papillomavirus (HPV) is the most important factor in cervical carcinogenesis, and infection by the virus seems to be an early event in malignant transformation.[1,2] Persistent infection with high-risk HPV has been identified as an essential, although insufficient, factor in the pathogenesis of cervical carcinoma.[3] High-risk HPV genomes replicate as episomal molecules in the normal viral life cycle. Although the HPV genome is consistently retained in an episomal state in early dysplastic low-grade lesions, in some advanced HPV-associated precancers and in the majority of high-risk HPV-associated carcinomas, the entire viral genome or fragments thereof are covalently integrated into the chromosomal DNA of the host cell. These observations suggest that integration of viral genes in severely dysplastic lesions strongly enhances neoplastic progression to invasive carcinomas.[4]

In situ hybridization (ISH) can detect HPV DNA in formalin-fixed, paraffin-embedded (FFPE) tissues and provides additional information about HPV integration status. Punctate staining in the nuclei is thought to indicate the presence of integrated HPV, while diffuse nuclear staining is thought to indicate the presence of episomal HPV genomes. However, the lower sensitivity of HPV ISH compared to other HPV molecular tests such as HPV polymerase chain reaction (PCR) has been a major limitation.[5] Recently, the INFORM HPV III (Ventana Medical Systems, Tucson, AZ, USA), a commercially available new generation of ISH probes, became available for HPV DNA testing in tissue specimens. The INFORM HPV III utilizes a stacked antibody approach (iView Blue Plus kit, Ventana Medical Systems) to enhance sensitivity in which the primary antibody is directed against a dinitrophenyl hapten, and signal amplification is generated through antibody stacks consisting of a secondary antibody and a biotinylated tertiary antibody.[6]

Specific host genes that are up-regulated in association with HPV infection, so called "surrogate markers," have been iden-

tified.[7] Among these, $p16^{INK4a}$ blocks the cyclin-dependent kinase 4/6 interaction with cyclin D1, thereby inhibiting progression through the G1/S transition checkpoint of the cell cycle. $p16^{INK4a}$ overexpression of results from a negative feedback linked to functional inactivation of pRb by HPV E7.[8]

The aim of the present study was to determine whether HPV ISH results can provide prognostic information and to evaluate the association between HPV ISH results and the expression of $p16^{INK4a}$, p53, bcl-2, and Ki-67 in cervical cancer specimens.

MATERIALS AND METHODS

Patients and tissue samples

We analyzed 279 cases of invasive cervical carcinoma treated between January 1997 and December 2004 at Seoul St. Mary's Hospital. The mean age of the patients with invasive cervical carcinoma was 49.4 years (range, 23 to 77 years). All slides were reviewed, and the clinicopathological data including histological type, grade, age at initial diagnosis, tumor stage, and survival were collected. The histological type of invasive cervical carcinoma was squamous cell carcinoma in 224 (80.3%), adenocarcinoma in 35 (12.5%) and adenosquamous carcinoma in 20 patients (7.2%). Tumor grade was determined by differentiation categories[9]: grade 1 (keratinizing tumors that are mainly differentiated with conspicuous keratin pearls) in 11 (3.9%), grade 2 (large cell non-keratinizing carcinomas with greater nuclear pleomorphism) in 243 (87.1%) and grade 3 (small cell non-keratinizing carcinoma with a high nuclear-cytoplasmic ratio) in 25 (9.0%). The tumor stage following surgical resection was stage I in 156 (55.9%), stage II in 117 (41.9%), stage III in four (1.4%) and stage IV in two patients (0.7%) (Table 1).

Table 1. Relationships between human papillomavirus (HPV) *in situ* hybridization (ISH) results and clinicopathological factors in cervical carcinomas

Characteristics		n	High-risk HPV ISH		p-value
			Negative (%)	Positive (%)	
Age (yr)	≤ 40	60	17 (28.3)	43 (71.7)	0.407
	> 40	219	68 (31.1)	151(68.9)	
Histologic type	SCC	224	64 (28.6)	160 (71.4)	0.354
	Adeno	35	14 (40)	21 (60)	
	ASC	20	7 (35)	13 (65)	
Grade	Well	11	2 (18.2)	9 (81.8)	0.660
	Moderate	243	75 (30.9)	168 (69.1)	
	Poorly	25	8 (32)	17 (68)	
Stage	I	156	46 (29.5)	110 (70.5)	0.146
	II	117	35 (29.9)	82 (70.1)	
	III	4	2 (50)	2 (50)	
	IV	2	2 (100)	0 (0)	
p16	Positive	249	70 (28.1)	179 (71.9)	0.014
	Negative	30	15 (50)	15 (50)	
bcl-2	Positive	102	19 (18.6)	83 (81.4)	0.001
	Negative	177	66 (37.3)	111 (62.7)	
p53	Positive	105	29 (27.6)	76 (72.4)	0.253
	Negative	174	56 (32.2)	118 (67.8)	
Ki-67	High	123	32 (26.0)	91 (74.0)	0.096
	Low	156	53 (34.0)	103 (66.0)	

n, number of patients; SCC, squamous cell carcinoma; Adeno, adenocarcinoma; ASC, adenosquamous carcinoma.

Tissue microarray (TMA)

To construct the TMA blocks, 3 mm-core biopsies were taken from viable, morphologically representative areas of paraffin-embedded tumor tissues and assembled on a recipient paraffin block, using a precision instrument (Micro Digital Co., Gunpo, Korea). After construction, 4 μm sections were cut, and histology was verified by hematoxylin-eosin staining.

High-risk HPV ISH

TMA sections were cut to a 4 μm thickness and mounted on positively charged glass slides for staining. INFORM HPV III Family 16 probe sets were performed according to the manufacturer's recommendations using the BenchMark™, Automated Slide Staining System (Ventana Medical Systems). The INFORM HPV III ISH Family 16 probe set contains a cocktail of HPV genomic probes in a formamide-based diluent. The probe cocktail has demonstrated affinity for the following genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. Nineteen cases of normal cervix obtained from hysterectomized uteri due to leiomyoma were used as a negative control. Two pathologists independently reviewed the HPV ISH slides using a multi-headed microscope, and a consensus was obtained after discussion. The HPV signal patterns in nuclei were classified as punctate, diffuse, or mixed. A punctate pattern, that is, distinct dot-like signals or scattered tiny particles in the nucleus, was considered to indicate integrated HPV, and a diffuse pattern, that is, large globular homogeneously dense staining in the nucleus, was considered to represent episomal HPV.

Immunohistochemistry (IHC) of p16, bcl-2, p53, and Ki-67

Four-micrometer sections of the paraffin-embedded tissue

arrays were deparaffinized, rehydrated in a graded series of alcohol and microwave-treated for 10 minutes in a citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. The antigen was retrieved with 0.01 M citrate buffer (pH 6.0) by heating the sample in a microwave vacuum histoprocessor (RHS-1, Milestone, Bergamo, Italy) at a controlled final temperature of 121℃ for 15 minutes. The tissue arrays were processed in an automatic IHC staining machine using standard protocols (Lab Vision Autostainer, Lab Vision Co., Fremont, CA, USA) with Dako ChemMate™ EnVision™ system (Dako, Carpinteria, CA, USA). The following antibodies were used: $p16^{INK4a}$ (1 : 50, E6H4, Dako), bcl-2 (1 : 50, Bcl-2-100, Invitrogen, Carlsbad, CA, USA), p53 (1 : 50, Dako), Ki-67 (1 : 50, MIB-1, Dako). The sections were visualized with 3-3′-diaminobenzidine and tissue arrays were counterstained with Mayer's hematoxylin. The p16 IHC was considered negative when less than 10% of the cancer cell nuclei showed positive staining, focally positive when 11-50% of the nuclei showed staining, and diffusely positive when > 51% of the nuclei was stained. The bcl-2 and p53 IHC were considered positive when more than 10% of the cytoplasm and nuclei showed positive staining, respectively. Ki-67 expression was scored semi-quantitatively based on the positive nuclear staining fraction of the tumor cells (score 0 = no staining, score 1+ = 1-10%, score 2+ = 11-25%, score 3+ = 26-50%, score 4+ = 51-100%). For purposes of statistical analysis, scores of 0 and 1 were considered as a low Ki-67 labeling index (LI) and scores of 2-4 were considered as high Ki-67 LI.

Statistical analyses

All statistical analyses were performed using SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA). The association between HPV infection and the expression of known HPV-associated proteins, p16, p53, bcl-2, and Ki-67 were analyzed using the chi-square test. Survival curves were plotted using the Kaplan-Meier method, and statistical significance was determined by the Breslow test. A multivariate analysis was performed with Cox's proportional hazard model. A p-value < 0.05 was considered significant.

RESULTS

High-risk HPV DNA detection by ISH

ISH reactions for high-risk HPV were positive in 194 (69.5%) and negative in 85 (30.5%) samples. Of the 194 HPV ISH positive cases, punctate, diffuse and mixed signal patterns were observed in 157 (56.3%), one (0.4%), and 36 cases (12.9%), respectively (Fig. 1).

IHC analysis for p16, bcl-2, p53, and Ki-67

Of the 279 cases, $p16^{INK4a}$, p53, and bcl-2 were detected in 249 (89.2%), 105 (37.6%), and 102 (36.6%) cases, respectively, and high Ki-67 LI was observed in 156 cases (55.9%) (Table 1, Fig. 2). High Ki-67 LI was correlated with bcl-2 positivity ($p = 0.00$, $r = 0.47$). $p16^{INK4a}$ positivity was correlated with bcl-2 positivity and high Ki-67 LI ($p = 0.001$, $r = 0.21$ and $p = 0.00$, $r = 0.35$, respectively).

Association between the HPV signal pattern and IHC results

A positive result for high-risk HPV ISH was associated with positivity for $p16^{INK4a}$ ($p = 0.01$) and bcl-2 ($p = 0.001$). A positive result for high-risk HPV ISH was not associated with p53 positivity or high Ki-67 LI ($p = 0.39$ and $p = 0.10$, respectively) (Table 1). Among high-risk HPV ISH-positive cases, cases displaying a diffuse signal (mixed + diffuse) were associated with $p16^{INK4a}$ positivity ($p = 0.04$) (Table 2).

Correlation between HPV status and clinicopathological parameters

No statistically significant correlation was observed between HPV status (high-risk HPV ISH-negative vs -positive) (Table 1) and clinicopathological factors including patient age, histological type, grade, or tumor stage, HPV signal pattern (punctate only vs diffuse + mixed), or clinicopathological factors.

The median length of follow-up after surgery was 59.2 months (mean, 60.8 months; range, 0.5 to 150.6 months). Within the observation period, 47 of 279 patients (16.8%) died of cancer-related causes. The 10-year survival rates for the high-risk HPV ISH-positive and -negative groups were 73% and 79%, respectively (Fig. 3D). Kaplan-Meier survival curves revealed a slightly worse survival rate in high-risk HPV ISH positive groups, but no statistically significant difference was found ($p = 0.73$).

Univariate analyses demonstrated shorter survival with higher stage, lymph node metastasis, histological type of non-squamous carcinoma, higher histological grade, p16 negativity, and low Ki-67 LI ($p = 0.00$, $p = 0.00$, $p = 0.03$, $p = 0.03$, $p = 0.03$,

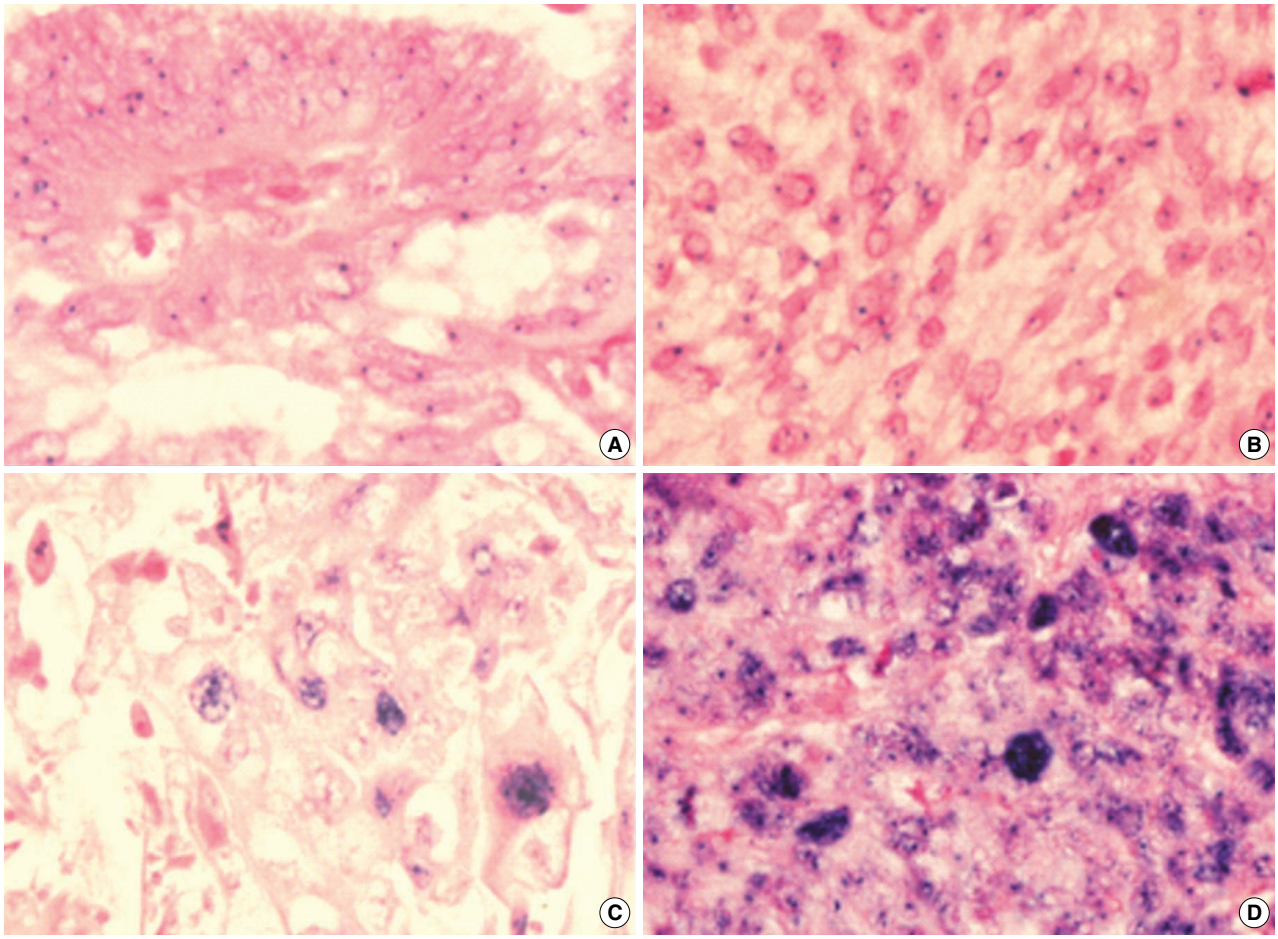

Fig. 1. High-risk human papillomavirus *in situ* hybridization signal patterns in cervical carcinoma. (A) Punctate staining pattern in adenocarcinoma. (B) Punctate staining pattern in squamous cell carcinoma. (C) Diffuse staining pattern. (D) Mixed staining pattern.

and p = 0.03, respectively) (Fig. 3). Neither bcl-2 positivity nor patient age (≤ 40 years vs > 40 years) was of prognostic significance (p = 0.17 and p = 057, respectively). In the multivariate analysis, tumor stage, lymph node metastasis, and histological grade were statistically significant independent prognostic factors for overall survival (Table 3).

DISCUSSION

In high-grade cervical intraepithelial neoplasia (CIN) and cervical carcinomas, high-risk HPV DNA is often found integrated into the host chromosome, whereas episomal HPV DNA is detected in nonprogressive intraepithelial lesions.[10,11] Integrated and episomal forms of the virus can be visualized in FFPE cervical tissue by ISH.[10,11] We analyzed 279 cases of invasive cervical carcinoma and found that 194 (69.5%) were positive for high-risk HPV. It has been reported that HPV can be detected using both ISH and PCR methods in old archival FFPE tissue, and that the detection rate is 66.1% and 59.4% of 180 cases, respectively. In that report, the detection rate was similar to that reported herein; however, the authors emphasized that considerable effort was spent determining the optimal conditions for each block. They adjusted the digestion conditions using a positive control probe to obtain the strongest signal in each case. In contrast, in the present study, we used a closed autostaining system, which was simple and quick.[12] Guo *et al.*[6] reported that the sensitivity of INFORM HPV in CIN lesions was 78.4% (69/88) and 75.9% (22/29) in cervical cancer. INFORM HPV is a chromogenic ISH test, and the threshold for HPV detection appears to be between 10 and 50 viral copies per cell when using FFPE samples.[13] HPV ISH can be applied to cervical smears and FFPE specimens, and HPV status can be interpreted in association with morphological tumor findings.

Kalof *et al.*[10] demonstrated the potential of ISH signal patterns to denote high-grade CIN; they found that both increased

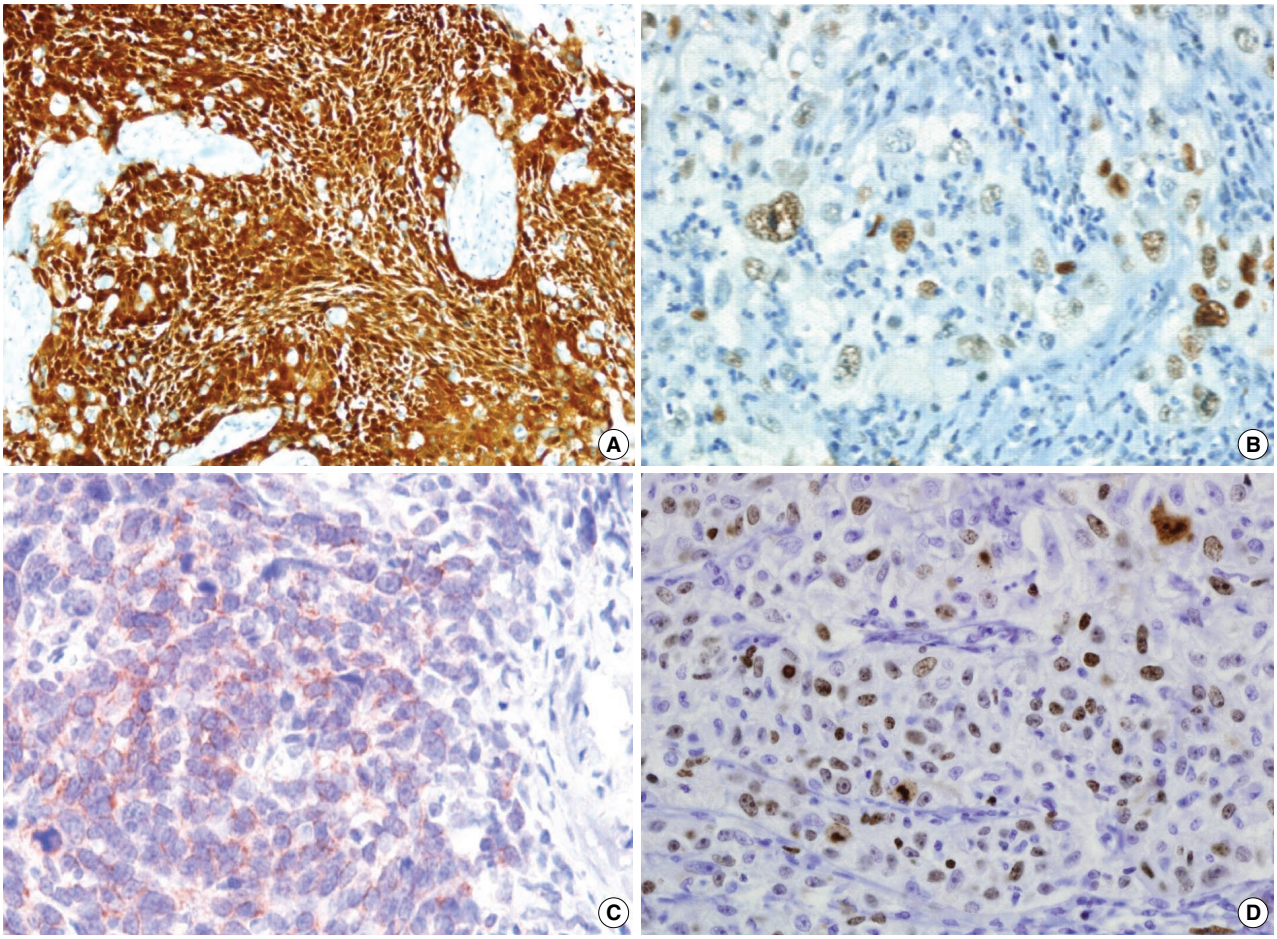

Fig. 2. $p16^{INK4a}$, bcl-2, p53, and Ki-67 immunoreactivity. (A) $p16^{INK4a}$ diffuse positive. (B) p53 positive. (C) bcl-2 positive. (D) High Ki-67 labeling index.

Table 2. Relationship between human papillomavirus (HPV) signal pattern and p16, bcl-2, and Ki-67 expression

		HPV signal pattern		
		Punctate only (%)	Mixed + Diffuse (%)	p-value
p16	Positive	142 (79.3)	37 (20.7)	0.037
	Negative	15 (100)	0 (0)	
bcl-2	Positive	64 (77.1)	19 (22.9)	0.162
	Negative	93 (83.8)	18 (16.2)	
p53	Positive	57 (74.0)	20 (26.0)	0.424
	Negative	102 (87.2)	15 (12.8)	
Ki-67	High	74 (81.3)	17 (18.7)	0.522
	Low	83 (80.6)	20 (19.4)	

Table 3. Cox multivariate analysis for invasive cervical carcinomas

	Hazard ratio	95% Confidence interval	p-value
Stage	2.122	1.356-3.320	0.001
Lymph node metastasis	3.174	1.664-6.053	0.000
Histologic grade	2.508	1.101-5.711	0.029
Histologic type	1.906	0.946-3.837	0.071
p16	0.663	0.286-1.537	0.338
Ki-67	0.607	0.307-1.198	0.150

$p16^{INK4a}$ immunoexpression and a punctate signal correlated well with high-grade CIN. Guo *et al.*[6] observed that the ISH assay using the INFORM HPV III probe was comparable to the PCR assay for detecting oncogenic HPV DNA in FFPE cervical tissue from patients with CINs. They concluded that the ISH assay using INFORM HPV III with improved sensitivity has the potential to be used in cervical tissue specimens with CINs. We also reported the usefulness of HPV ISH in CIN lesions, showing that HPV ISH is positive in 80.8% (21/26) of CINs and that the HPV ISH punctuated staining pattern is correlated with high grade CIN.[14] There are few studies on the clinical importance of HPV integration status in cervical cancer. In the present study, HPV ISH-positive cases displayed punctuate (18.6%), diffuse (0.5%), and mixed signal patterns in vary-

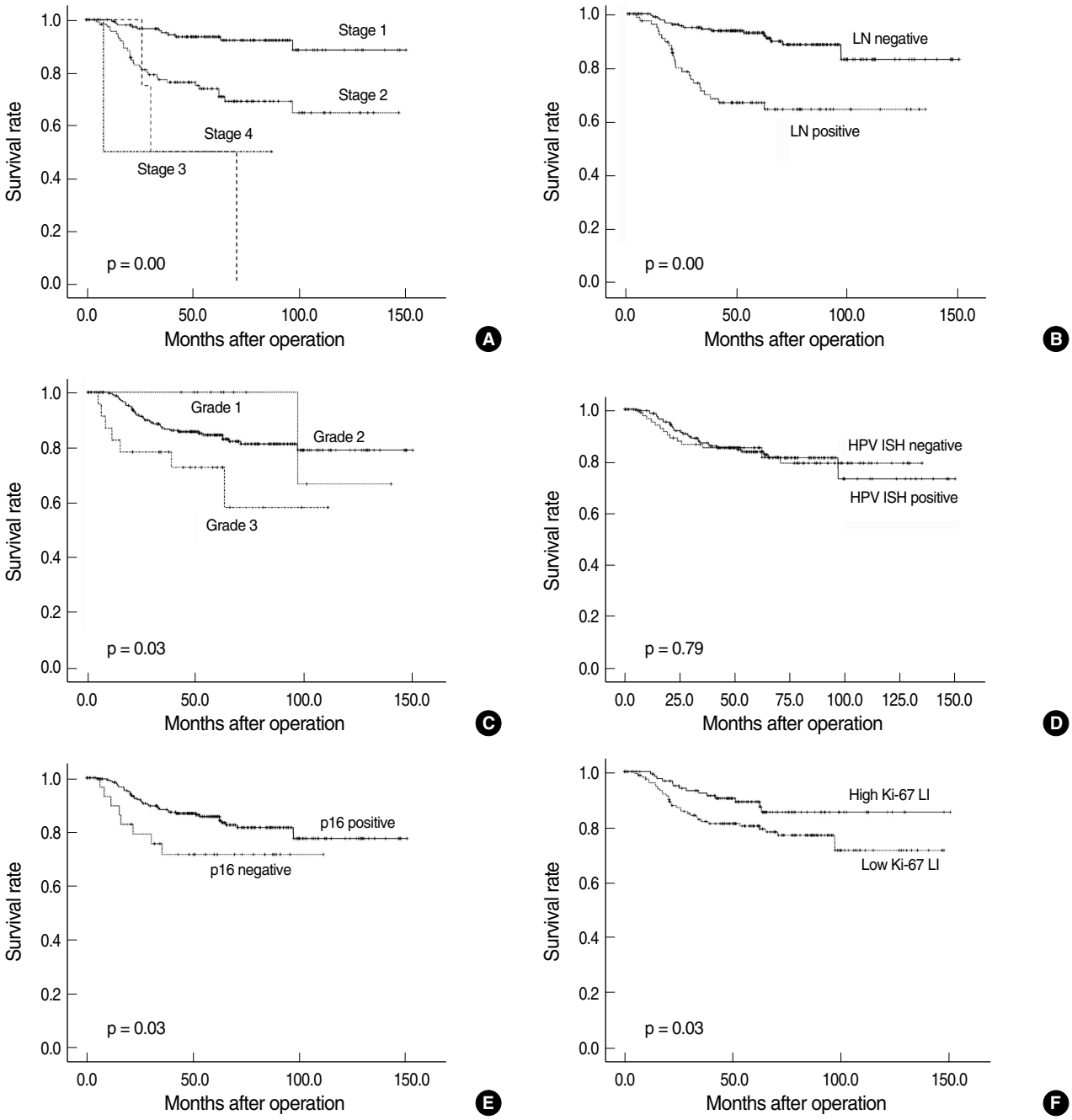

Fig. 3. Survival curves according to stage (A), lymph node (LN) status (B), grade (C), high-risk human papillomavirus (HPV) *in situ* hybridization (ISH) result (D), p16 immunohistochemistry results (E), and Ki-67 labeling index (LI) (F).

ing ratios (80.9%). No survival differences were observed between high-risk HPV ISH-positive and high-risk HPV ISH-negative patients, and among patients who were high-risk HPV ISH-positive, no survival differences were observed between those exhibiting a punctate HPV signal only and those with punctate and diffuse signals or a diffuse signal only. Using multiplex PCR, Qiu *et al.*[12] reported 7% of only integrated HPV 16 DNA, 38% of only episomal HPV DNA and 55% of both integrated and episomal HPV DNA in cervical cancer. Although the E2/E6 ratio correlates well with CIN or cervical carcinoma severity,[1,15-17] no significant differences were observed in the E2/E6 ratio between cases with a punctate signal only and cases with a mixed signal by HPV ISH.[6] In the present study, the multivariate analysis revealed that tumor stage, lymph node

metastasis and histological grade were prognostic factors in cervical carcinoma and that HPV integrated status or expression of surrogate markers such as p16 were not. Although HPV integration is important in the progression of CIN, more studies are needed to evaluate the potential role of HPV as a prognostic or predictive factor in invasive cervical cancers.

An HPV ISH-positive result was associated with p16 and bcl-2 positivity. Lorenzato *et al.*[18] demonstrated that the extent of $p16^{INK4a}$ positivity correlats with an increase in integrated HPV ISH signals with a resulting enhancement of cellular proliferation as assessed by Ki-67. In some studies, the Ki-67 proliferation index for CIN and cervical carcinoma associated with infection by high-risk HPV was greater than that in lesions unrelated to HPV.[19-22] In contrast, several other studies have reported no significant relationship between the Ki-67 proliferation index and HPV infection, which was a finding consistent with our results.[23,24] Bcl-2 is an inhibitor of apoptosis and prolongs cell life by suppressing apoptosis. In a previous study, increased bcl-2 protein expression was reported in cervical carcinoma cell lines containing mutated HPV E6-inactivated p53.[25] Another study also demonstrated a strong association between high-risk HPV type and bcl-2 expression in cervical cancer development.[26,27] Our finding is in agreement with that of Liang *et al.*[25] and Grace *et al.*,[26] who found a statistically significant correlation between bcl-2 expression and HPV infection.

Our results confirm that the INFORM HPV III Family 16 probe assay is reasonably sensitive for HPV infection and that it can be used to identify integrated HPV DNA in FFPE specimens. Our HPV results identified by HPV ISH were associated with the expression of HPV surrogate markers such as p16 and bcl-2, but not associated with prognostic factors such as stage, grade, or survival rate. Further study encompassing HPV type, E2/E6 ratio and therapeutic modality is necessary to understand the potential role of HPV infection in cervical cancer progression.

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