Short Communication

Absence of High-Risk Human Papillomavirus (HPV) Detection in Endocervical Adenocarcinoma with Gastric Morphology and Phenotype

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A subset of endocervical-type mucinous adenocarcinomas (ACs) of the uterine cervix exhibit a gastric phenotype and morphology, as reported in cases of minimal deviation AC in which the presence of human papillomavirus (HPV) has been rarely detected. To investigate the HPV-independent pathway of carcinogenesis in cases of gastric-type AC, we investigated the common high-risk HPV (hr-HPV) status in 52 nonsquamous cell carcinomas, using a PCR-based typing method and immunohistochemistry of p16^{INK4a} (a cyclin-dependent kinase inhibitor that is overexpressed in both cancerous and precancerous cervical tissue, making it an ideal biomarker for cervical cancer cases). Using novel morphological criteria, seven of 52 (13.5%) carcinomas were designated as gastric-type ACs, all of which were negative for both hr-HPV DNA and p16^{INK4a}. Nongastric-type ACs were frequently positive for both hr-HPV DNA (90%, 28/31) and p16^{INK4a} (94%, 29/31) with adenosquamous and neuroendocrine carcinomas demonstrating the presence of hr-HPV DNA in 86% (6/7) and 83% (5/6) of cases, respectively. In these two types of carcinoma, 86% (6/7) and 100% (6/6) were positive for p16^{INK4a}, respectively. Our data suggests that gastric-type AC appears to represent an oncogenic hr-HPV-independent neoplasm and therefore is a potential pitfall of HPV DNA testing and vaccination. (*Am J Pathol* 2010, 177:2169–2175; DOI: 10.2353/ajpath.2010.100323)

The incidence of uterine cervical cancer has been declining for the past three decades, largely due to widespread mass screening with Pap smears. This declining trend reflects the dramatic decrease of squamous cell carcinomas (SCCs), but recent studies have demonstrated an increase in both relative incidence and absolute number of adenocarcinoma (AC),^{1–5} with its incidence approaching 20% of all cervical cancers.^{1,4–7} This fact suggests that conventional sampling methods for Pap smears are not necessarily optimal for the early detection of ACs, and an effective tool is required for this particular purpose.⁸ The cause of the absolute increase of patients with ACs remains unknown.

High-risk human papillomavirus (hr-HPV) is currently considered to be implicated in the carcinogenesis of most ACs as well as SCCs of the cervix, with a detection rate reaching 85% in the former.^{9–12} Therefore, a combination of HPV DNA tests and cytology has been expected to contribute to improving sensitivity and specificity for the early detection of endocervical AC. On the other hand, however, some investigators have shed light on the existence of hr-HPV-negative endocervical ACs, which could be a serious diagnostic concern, and also indicates that endocervical AC is a heterogeneous group of neoplasms. Such putative hr-HPV-independent AC includes uncommon subtypes of AC, such as clear cell adenocarcinoma, mesonephric adenocarcinoma, and minimal deviation adenocarcinoma (MDA).^{12,13} Based on accumulated data in the literature,^{12–16} hr-HPV DNA is only rarely detected in cases of MDA, a well-differentiated variant of mucinous AC with a deceptively benign appearance but significantly aggressive clinical course.^{17,18}

A subset of mucinous AC shares gastric phenotype with MDA as evidenced by immunoreactivity for HIK1083

Supported by grants from Setsuro Fujii Memorial, The Osaka Foundation for Promotion of Fundamental Medical Research.

Accepted for publication July 8, 2010.

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and anti-MUC6, antibodies recognizing pyloric gland mucin of the stomach, and incidence of p16^{INK4a} immunoreactivity is significantly low in both MDA and the mucinous AC with gastric immunophenotype.^{19,20} Additionally, both neoplasms can be associated with lobular endocervical glandular hyperplasia (LEGH),¹⁹ which represents a pyloric gland metaplasia²¹ and has also been shown to be hr-HPV DNA-negative.^{16,22} These facts suggest that the mucinous AC with gastric immunophenotype, incorporating MDA as its well-differentiated form in the morphological spectrum, is a distinct entity arising via a hr-HPV-independent pathway. In a previous investigation we demonstrated that mucinous AC with gastric immunophenotype was morphologically distinct with a good interobserver reproducibility, characterized by neoplastic cells with conspicuous cell borders and clear and/or pale eosinophilic voluminous cytoplasm, and aggressive clinical behavior.^{20,23} MDA is not a common neoplasm, accounting for 1–3% of all endocervical ACs, but the gastric-type adenocarcinoma (GAC) appears in 10–30% of all endocervical ACs^{19,20} and thus may not be a negligible pitfall of HPV DNA tests and vaccination, and therapeutic strategy.

Following World Health Organization classification, GAC has been hitherto recognized as an endocervical-type mucinous AC because of a lack of definite criteria, and the exact hr-HPV DNA detection rate in this tumor is unknown. We conducted this study to investigate the HPV DNA status in cases of histopathologically defined GAC using novel diagnostic criteria and literature reviews on HPV detection rate in cases of AC published by Japanese investigators.

Materials and Methods

Case Selection

Ninety-one cases of invasive non-SCC, treated by surgery at Hyogo Cancer Center (HCC) from 2003–2006, were retrieved from the tumor registry. From these 91 cases, a total of 39 cases, including 34 cases with chemotherapy before hysterectomy, three cases without available tissue specimens, one case with AC *in situ* on pathology review, and one case in which only ovarian and omental metastases were removed, were excluded from the study to identify 52 patients (37 with FIGO stage IB, three with stage IIA, 10 with stage IIB, one with stage IVA, and one with stage IVB) who received primary surgical treatment. Two patients with stage IV disease, one with bladder wall involvement (IVA) and another with large bilateral ovarian metastases and disseminated nodules on the serosal surface of the diaphragm (stage IVB), were successfully treated with a complete resection of the tumors. The age of patients ranged from 26 to 78 years (mean, 48.5). Medical records were reviewed to obtain clinical information including the outcome of each patient. The current study was approved by the institutional review board of the HCC.

Pathological Evaluation

Representative sections on glass slides of 52 cases were reviewed by two of the authors (Y.M. and T.K.), board-certified pathologists specializing in gynecologic pathology and oncology, without any clinical information and original histopathological diagnosis. All tumors were classified following the current World Health Organization scheme (2003),¹⁸ and thereafter the pure AC group was further divided into GACs and non-GACs based on novel criteria which the authors described in a previous report.²⁰ GAC was defined as a mucinous adenocarcinoma displaying i) clear or pale eosinophilic cytoplasm, ii) voluminous cytoplasm, and iii) distinct cell borders. The “endocervical-type” mucinous AC, the major group of ACs in the current World Health Organization scheme (2003), was originally defined as mucinous AC which shows morphology similar to endocervical glands, but in reality any AC without evidence of specific direction of differentiation or morphology, including intestinal differentiation characterized by coexistence of goblet cells, have been recognized as ambiguously “endocervical-type” ACs. In the current study, therefore, we used the term “usual endocervical-type adenocarcinoma” (UEAC) for these tumors as suggested by Young et al.²⁴

DNA Extraction

In each case, a 10- μ m-thick section was cut from formalin-fixed and paraffin-embedded tissue blocks. These samples were incubated with proteinase K (1 mg/ml) for 18 hours at 56°C, heat inactivated, and added to a DNA purification column (Generation capture column kit; Gentra Systems Inc., Minneapolis, MN). The extraction of total DNA was performed according to the manufacturer's instructions. The concentration and the quality of the extracted DNA were determined by spectrophotometry. Five microliters of DNA solution was used as a template for polymerase chain reaction (PCR) amplification.

HPV DNA Detection and Typing

HPV sequence alignment and genotype-specific primer design was followed as previously reported by Nishiwaki et al.²⁵ For PCR with HPV genotype-specific primers, 16 HPV genotypes (genotypes 6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) were detected by multiplex PCR in a single tube. PCRs were performed with a multiplex PCR kit (Qiagen Inc., Valencia, CA), following the manufacturer's instructions, with minor modifications. Aminolevulinic- δ -synthase 1 (GenBank accession no. NM000688) was amplified as an internal positive control. An external positive control DNA fragment derived from *Brevundimonas diminuta* was cloned into plasmid pCRII-TOPO (Invitrogen, Carlsbad, CA) and added to the PCR mixtures along with external plasmid-specific primers.

Immunohistochemistry

Sections of 4 μ m thickness were cut from formalin-fixed and paraffin-embedded tissue blocks and mounted onto

Table 1. Age and Stage Distributions and Results of HIK1083 Immunohistochemistry in 52 Patients with Nonsquamous Cell Carcinoma of the Cervix

Histologic type	No.	Age (y)		FIGO stage		HIK1083 positivity	
		Mean	Range	IB	II–IV	No.	%
Adenocarcinoma	38	49	26–71	29	9	14	36.8
UEAC*	29	46	26–71	24	5	7	24.1
UEAC* + intestinal (mixed)	2	51	44–58	2	0	0	0.0
Gastric	7	57	40–69	3	4	7	100.0
Adenosquamous carcinoma	7	50	31–66	6	1	1	14.3
Adenoid basal carcinoma	1	72	—	0	1	0	0.0
Neuroendocrine tumors†	6	42	28–78	2	4	0	0.0

*UEAC: usual endocervical-type adenocarcinoma, including one case of villoglandular variant.

†Two cases of mixed neuroendocrine carcinoma and adenocarcinoma included.

silane-coated slides. Sections were deparaffinized in xylene and rehydrated through graded alcohols. Immunostaining was performed using the standard avidin-biotin complex (ABC) method. Gastric immunophenotype was determined using HIK1083 (Kanto Kagaku, Tokyo, Japan), a mouse monoclonal antibody recognizing pyloric gland mucin of the stomach. Sections were incubated with the antibody at a dilution of 1:100 without antigen retrieval at 4°C overnight. Immunoreactivity was regarded as positive when at least 1% of tumor areas showed positive staining. Immunohistochemistry for p16^{INK4a} was performed using a CINtec[®] Histology Kit, which used a mouse monoclonal antibody (clone E6H4; MTM Laboratories, Heidelberg, Germany). The result was interpreted as positive when cytoplasmic and/or nuclear staining was identified in 50% or more of tumor areas.

Literature Search for HPV Prevalence Rate within Cervical Adenocarcinoma in Japan

Using the database from the National Library of Medicine (PubMed; January 2000 to December 2009), searches for peer-reviewed journal articles written in English were conducted using a combination of search terms, such as “adenocarcinoma” or “adenosquamous” and “cervix” and “HPV”. The search was restricted to papers authored by Japanese investigators who used PCR techniques to detect HPV DNA from biopsies or surgical specimens of tumors. Studies that targeted uncommon histological

subtypes, specific HPV types such as types 16 and 18, and those analyzing fewer than ten cases were excluded. When data or data subsets from an identical study had been published in more than one article, only the publication with the largest sample size was included.

Statistical Analysis

Comparison of nominal and categorical data were performed by χ^2 analysis and Fisher's exact test. Nonnormally distributed parametric variables were compared by the Mann–Whitney *U*-test. Two-sided *P* values were reported.

Results

Histological Review

After review of 52 non-SCCs following the current World Health Organization scheme (2003), 38 tumors were reclassified as pure AC. Following the novel criteria, seven of 38 pure ACs (18%) were reclassified as GAC, with the remaining 31 pure ACs classified as non-GAC (Table 1). Of the 31 non-GACs, 28 were UEAC, two were combined UEAC and intestinal (mixed type) and one was a villoglandular type (Figure 1A). Seven non-SCCs were diagnosed as adenosquamous carcinoma (ASC), one as adenoid basal carcinoma, and six as neuroendocrine carcinoma (NEC), includ-

Figure 1. Usual endocervical-type adenocarcinoma (UEAC) and gastric-type adenocarcinoma (GAC). UEAC was composed of cells with mucin-deficient columnar cells, arranged in tubular or cribriform pattern (A), showing negative staining for HIK1083 immunohistochemistry (B), and diffuse nuclear and cytoplasmic staining for p16^{INK4a} (C). GAC was characterized by neoplastic glands composed of cells with abundant pale or pale eosinophilic cytoplasm with distinct cell borders (D). Neoplastic cells of GAC showing immunoreactivity for HIK1083 (E) and negative staining for p16^{INK4a} (F). A and D: Hematoxylin and eosin staining. Original magnification: $\times 200$ (A–F).

Table 2. Summary of HPV Status and p16^{INK4a} Immunoreactivity in 52 Nonsquamous Cell Carcinomas of the Cervix

Histologic type	No.	P16 ^{INK4a} positivity		HPV DNA positivity		HPV16*		HPV18*	
		No.	%	No.	%	No.	%	No.	%
Adenocarcinoma	38	29	76	28	74	13	46	15	54
UEAC [†]	29	27	93	26	90	13	50	13	50
UEAC [†] + intestinal (mixed)	2	2	100	2	100	0	0	2	100
Gastric	7	0	0	0	0	0	0	0	0
Adenosquamous carcinoma	7	6	86	6	86	2	33	4	67
Adenoid basal carcinoma	1	1	100	0	0	0	0	0	0
Neuroendocrine tumors [‡]	6	6	100	5	83	1	20	4	80

*Percentage indicates the proportion in HPV-positive cases.

[†]UEAC: usual endocervical-type adenocarcinoma, including one case of villoglandular variant.

[‡]Two cases of mixed neuroendocrine carcinoma and adenocarcinoma included.

ing small cell carcinoma and large cell neuroendocrine carcinoma, of which two were combined with AC.

Gastric Immunophenotype

HIK1083 was positive in all seven cases of GAC (Figure 1D), although the distribution was generally focal, whereas only seven of 31 non-GACs were positive (100% versus 23%; Table 1, Figure 1, B and E). One ASC was also focally positive, and six of seven ASCs, all seven NECs, and adenoid basal carcinomas were negative for HIK1083.

HPV DNA Detection, Typing, and p16^{INK4a} Immunoreactivity

HPV DNA (genotypes 6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) was detected in 39 of 52 non-SCCs (75%), and 28 of 38 pure ACs (74%), and was not detected in all GAC cases, in which immunoreactivity for p16^{INK4a} was also absent (Table 2, Figure 1F). The prevalence of HPV detection in non-GAC, ASC, and NEC cases was 90% (28/31), 86% (6/7), and 83% (5/6), respectively. In these three groups, p16^{INK4a} was strongly positive with a rate of 94% (29/31), 86% (6/7), and 100% (6/6), respectively (Figure 1C). With respect to HPV subtype, either type 16 or 18 was detected in all positive cases but no multiple infections were observed. In HIK1083-positive non-GAC and ASC cases, the prevalence was 86% (6/7) and 100% (1/1), respectively,

and these HPV-positive cases were also strongly positive for p16^{INK4a}.

Literature Review of HPV DNA Status in ACs and ASCs among Japanese Women

The data from five studies retrieved by the computer-assisted literature search,^{26–30} combined with those of the current study, were analyzed. In four studies, including our current study,^{26–28} AC and ASC were specifically investigated, while two other studies focused on only ASC.^{29,30} Consequently, data obtained from 124 pure ACs and 49 ASCs were collected (Table 3).^{26–28}

Prevalence of HPV DNA in pure ACs from the four studies ranged from 33%²⁸ to 74% (the current study), whereas in ASCs it ranged from 80%^{26,28} to 94%.²⁷ There was a significant difference in the prevalence between ACs and ASCs in the two studies (51% versus 94%, $P = 0.006$ ²⁷; 33% versus 80%, $P = 0.01$ ²⁸). In total, 67 of 124 pure ACs (54%) and 42 of 49 ASCs (86%) were positive for hr-HPV DNA and the difference between both was statistically significant ($P < 0.001$).

Discussion

Our data indicated that GAC, including MDA, is distinct from UEAC with regards to the implication of HPV in carcinogenesis. The majority of ACs of the uterine cervix have been considered to be associated with hr-HPV in-

Table 3. Data Summary of HPV DNA Status in Cases of Pure Adenocarcinoma and Adenosquamous Carcinoma in Japan

Author ref.	AC*+ASC [†]			AC*			ASC [†]			<i>P</i> value
	No.	HPV+		No.	HPV+		No.	HPV+		
		No.	%		No.	%		No.	%	
Nagai et al. ²⁷	54	35	64.8	37	19	51.4	17	16	94.1	0.006
Minaguchi et al. ²⁶	32	19	59.4	22	11	50.0	10	8	80.0	0.22
Sano et al. ²⁸	42	21	50.0	27	9	33.3	15	12	80.0	0.01
Kusanagi et al. (current study)	45	34	75.6	38	28	73.7	7	6	85.7	0.71
Total	173	109	63.0	124	67	54.0	49	42	85.7	<0.001

*AC: adenocarcinoma.

[†]ASC: adenosquamous carcinoma.

fection, although some investigators have suggested that uncommon subtypes such as MDA, serous and clear cell adenocarcinomas, are hr-HPV-independent. The current study has demonstrated that none of the seven GACs displayed either the common type hr-HPV DNA or p16^{INK4a} immunoreactivity, while in non-GAC cases, incidence of HPV DNA detection and p16^{INK4a} immunoreactivity were 90% (28/31) and 94% (29/31), respectively.

It is well known that hr-HPV plays a crucial role in the etiology of almost all cervical SCCs and the majority of ACs. As previously reported, the prevalence of hr-HPV DNA detection in endocervical AC varies significantly, but it is generally low compared with SCC.^{9–12} The low prevalence in cases of AC has been considered to stem from the technical difficulty in material sampling, sensitivity of HPV DNA detection methods, and erroneous inclusion of endometrial carcinoma arising in the lower uterine segment.¹⁰ Uncommon subtypes including clear cell, serous, and mesonephric adenocarcinomas are commonly reported to be HPV-independent^{12,31–34} and might have an impact on results, although these tumors are rare and thus their influence appears minimal.

MDA is an extremely well-differentiated form of mucinous AC, accounting for only 1–3% of all cervical ACs.³⁵ The unique aspects of this particular tumor are: i) there is only a rare association with HPV infection^{12,14,15,22,36}; ii) mutations in STK11, a tumor suppressor gene responsible for Peutz-Jeghers syndrome (PJS), which is identified in both PJS-associated and sporadic MDAs³⁴; and iii) a gastric-phenotype as evidenced by immunoreactivity for HIK1083 and/or anti-MUC6.^{37,38} On the other hand, Mikami et al demonstrated that a subset of mucinous ACs of “endocervical-type,” according to the current World Health Organization criteria (2003), can be positive for these two markers, and proposed the concept of GAC of the cervix to include MDA.¹⁹ They considered that GAC shows a spectrum of differentiation and MDA represented its well-differentiated form. Their observation indicates that mucinous ACs of the “endocervical-type” is in reality a heterogeneous group of neoplasm in terms of morphology and phenotype. Paradoxically the “endocervical-type” mucinous AC is more often mucin-deficient, and therefore the term UEAC, as proposed by Young et al, appears more appropriate rather than the ambiguous term “endocervical-type.”²⁴ Importantly, they insisted the separation of truly “mucinous” AC from UEAC because of the aggressive clinical behavior of the former.²⁴ The UEAC accounts for about 80% of ACs of the cervix, and truly “mucinous” ACs are considered to be less common tumors.²⁴ In this context, Kojima and colleagues recently demonstrated that the GAC is a morphologically distinct entity with decreased five-year disease-specific survival.²⁰ The GAC may represent a subset of “mucinous” ACs as defined by Young et al.²⁴

The morphologically defined GAC is characterized by less frequent p16^{INK4a} expression, compared with non-GAC, suggesting a HPV-independent nature of this particular subtype.^{19,20} Because absence of p16^{INK4a} expression can be a consequence of a variety of molecular modifications, including promoter hypermethylation,^{39–41} gene alteration,⁴² and regulation from upstream proteins,

direct evidence is yet to be found to prove this hypothesis. Two recent studies in the abstract forms have demonstrated significantly low prevalence of hr-HPV DNA in GACs diagnosed using the novel criteria outlined by Kojima et al,²⁰ with results of 13% (1/8)¹³ and 0% (0/3)⁴³, respectively. Therefore, the results obtained from the current study, combined with those from the two studies, appear to indicate no implication of hr-HPV in carcinogenesis of GAC.

The importance of recognition of GAC cannot be over-emphasized because of its rather common occurrence, worse prognosis, as well as potential pitfalls in HPV DNA tests. In our study from a single institution, the prevalence of GAC among all ACs was 18% (7/38), indicating it is not a rare tumor. A worse prognosis was clearly demonstrated by the authors,²⁰ although it remains undetermined whether decreased survival is due to the intrinsically aggressive nature of the tumor, resistance to adjuvant therapy, delay in diagnosis, or a combination of these factors.

The proportion of GACs might have an impact on the hr-HPV DNA detection rate in all ACs. Miura and colleagues reviewed papers on the detection of hr-HPV in cervical cancer among Japanese women and demonstrated that hr-HPV positivity in cases of AC and ASC were significantly low compared with that in SCC (70% versus 91%).⁴⁴ In contrast, worldwide there appears to be no significant difference in detection rates of hr-HPV DNA between AC, ASC, and SCC groups.⁴⁵ The prevalence in cases of AC and ASC from the current review of four Japanese papers was 63% (109/179) and was very close to the results shown by Miura et al. The prevalence in pure ACs was quite low (54%) among Japanese women, whereas in ASCs, it was closer to that of other countries (85.7%, 42/49, $P < 0.001$) as published in recent papers (ranging 85–95%).^{29,30} Therefore, the less frequent hr-HPV DNA detection in cases of non-SCC among Japanese women was considered to be a function of hr-HPV status in pure ACs. In the current study almost all UEACs, ASCs, and NECs were positive for hr-HPV types 16 or 18 as previously reported,^{10,12} and all GACs were negative for HPV and did not exhibit p16^{INK4a} immunoreactivity. This fact prompts us to consider that the higher incidence of GAC in Japan could explain the lower incidence of hr-HPV detection compared with other countries. To prove this hypothesis, further study is required to demonstrate the incidence of GACs among Western as well as “hot zone” countries.

Whether HPV status has an impact on outcome among patients with AC is still controversial, because hr-HPV-unrelated AC has been overlooked and not thoroughly investigated. Uchiyama et al dealt with this issue and showed that hr-HPV-negative AC had a poorer prognosis compared with hr-HPV-positive AC through univariate analysis.⁴⁶ Parker et al showed that p53 protein overexpression is much more common among hr-HPV-negative ACs,⁴⁷ and Suzuki et al reported radioresistance of ACs overexpressing the p53 protein.⁴⁸ These observations suggest an aggressive nature of hr-HPV-unrelated ACs. Frequent p53 protein overexpression has also been reported in cases of MDA.⁴⁹ Therefore, in cases of GAC,

p53 as well as a variety of gene alterations might play a role in carcinogenesis distinct from HPV-related AC. A more detailed understanding of its molecular mechanisms is expected to improve the outcome from treatment.

Finally, the definition of GAC may require further refinement, and the novel morphological criteria should be verified. The authors have demonstrated a good agreement in recognizing GAC, with a mean kappa value of 0.66 (range 0.33–0.93), which is comparable to that for UEAC among 28 pathologists.²³ Another criticism would be on the existence of a minor subset of HIK1083-positive UEAC. Although in the previous study we demonstrated an impact of both gastric immunophenotype and morphology on patient outcome, it might be conceivable that morphological expression is much more crucial in recognizing GAC because i) HIK1083 can be only focally positive, and ii) p16^{INK4a}-positive ACs *in situ* and UEAC can also be positive for HIK1083 and/or MUC6, possibly as a consequence of focal heterodifferentiation in the tumor,¹⁹ indicating the possibility that a minor subset of hr-HPV-related UEAC with gastric immunophenotype does indeed exist. Although there is a gray zone in recognizing GAC, accumulated data, including ours that 86% (6/7) of HIK1083-positive non-GACs harbor hr-HPV, suggests that clear-cut gastric morphology mostly indicates absence of hr-HPV and may predict unfavorable outcomes. Therefore, GAC should be well defined by morphology for routine practice and research purposes at this point, and further refinement might be possible using more sophisticated techniques such as cDNA microarray analysis.

In summary, we have demonstrated that GAC is a hr-HPV-independent neoplasm and is possibly the reason for the low prevalence of hr-HPV in cases of cervical AC in Japan. HPV DNA tests and vaccination are expected to control and prevent AC as well as SCC, both of which have been strongly associated with hr-HPV, but their limitations and pitfalls should be recognized. Special consideration and strategies are needed for the treatment of GACs and some uncommon types of AC.

Acknowledgments

We thank Dr. Chiho Obayashi (HCC) for providing specimens, Dr. Morie Nishiwaki (Genetic Lab Co. Ltd.) for advice on HPV detection, and Isako Ushio, Yoshie Kinoshita, and Eiji Katayama for technical support.

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