Microbiology and Immunology

Microbiol Immunol 2012; **56**: 128–133
doi:10.1111/j.1348-0421.2011.00406.x

NOTE

Novel multiplexed genotyping of human papillomavirus using a VeraCode-allele-specific primer extension method

Yuri Kitamura-Muramatsu[1], Rika Kusumoto-Matsuo[2], Kazunari Kondo[3], Seiichiro Mori[2], Susumu Saito[1], Yusuke Tsukahara[1] and Iwao Kukimoto[2]

[1]Riken Genesis Co., Ltd, Riken Yokohama Institute, East Research Building 3F, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, [2]Pathogen Genomics Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-murayama, Tokyo 208-0011 and [3]NTT Medical Center Tokyo, 5-9-22 Higashi-gotanda, Shinagawa-ku, Tokyo 141-8625, Japan

ABSTRACT

A VeraCode-allele-specific primer extension (ASPE) method was applied to the detection and genotyping of human papillomavirus (HPV)-DNA. Oligonucleotide primers containing HPV-type-specific L1 sequences were annealed to HPV-DNA amplified by PGMY-PCR, followed by ASPE to label the DNA with biotinylated nucleotides. The labeled DNA was captured by VeraCode beads through hybridization, stained with a streptavidin-conjugated fluorophore, and detected by an Illumina BeadXpress® reader. By using this system, 16 clinically important HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) were correctly genotyped in a multiplex format. The VeraCode-ASPE genotyping of clinical DNA samples yielded identical results with those obtained by validated PGMY-reverse blot hybridization assay, providing a new platform for high-throughput genotyping required for HPV epidemiological surveys.

Key words allele-specific primer extension, genotyping, human papillomavirus, multiplex assay.

Human papillomaviruses (HPV) are recognized as the causative agents of cervical cancer, its precursor lesions, and other anogenital cancers (1). Among more than 100 HPV types so far identified, nearly 40 types infecting the anogenital mucosa are classified as either low- or high-risk types on the basis of their oncogenic potentials (2). A previous large-scale case–control study revealed 15 high-risk types, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, which are closely linked to the development of cervical cancer, with HPV16 the predominant high-risk type worldwide (3). In contrast, low-risk HPV types, including HPV6 and 11, are associated almost exclusively with benign lesions. Due to the lack of a cell culture system to isolate HPV from clinical samples, detection of HPV-DNA is the only reliable means for diagnosis of HPV infection. HPV genotyping is of particular importance for understanding the natural history of HPV infection and management of cervical cancers. In addition, with the worldwide introduction of HPV vaccines that target the two prominent high-risk types, HPV16 and 18, there is a growing demand for reliable and practical HPV genotyping to monitor HPV prevalence and vaccine efficacy at both individual and population levels.

Various molecular techniques have been developed for detection of HPV-DNA, most of which rely on amplification of HPV-DNA by PCR. The PCR of HPV-DNA generally utilizes degenerate/consensus primer systems, such as MY09/11 (4), PGMY09/11 (5), GP5+/6+ (6), or SPF (7), all of which are designed to amplify the L1 region of the HPV genome. For HPV genotyping, PCR is followed

Correspondence
Iwao Kukimoto, Pathogen Genomics Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-murayama, Tokyo 208-0011, Japan.
Tel: +81 42 561 0771; fax: +81 42 567 5632; email: ikuki@nih.go.jp

Received 8 September 2011; accepted 3 November 2011.

List of Abbreviations: ASPE, allele-specific primer extension; HPV, human papillomavirus; MFI, median fluorescence intensity; RBH, reverse blot hybridization; RLB, reverse line blot; SNP, single nucleotide polymorphism; WHO, World Health Organization.

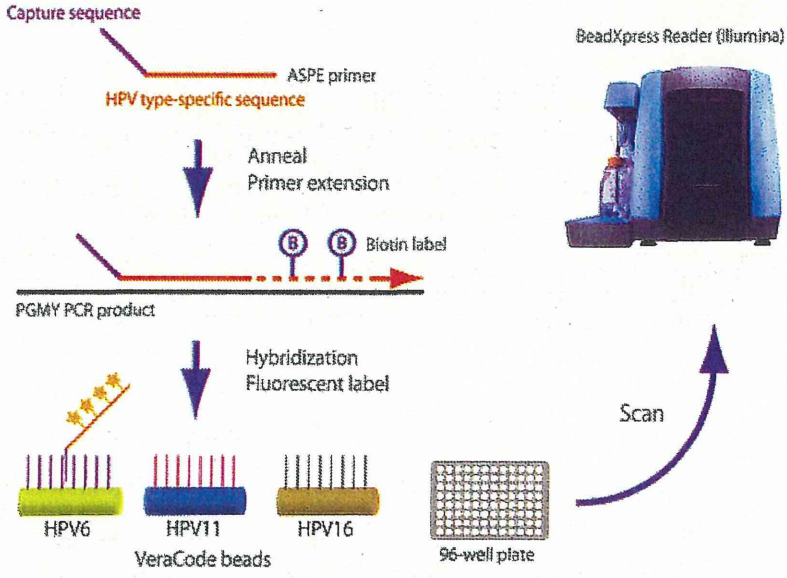

Fig. 1. Outline of VeraCode-ASPE HPV genotyping. HPV-type-specific ASPE primers are annealed to PGMY-PCR products, followed by primer extension by DNA polymerase to label DNA with biotin-14-dCTP. The biotin-labeled DNA is hybridized with VeraCode beads via a capture sequence at the 5′ end of the ASPE primer, and labeled with a streptavidin-fluorophore conjugate to allow detection of fluorescent signals by a BeadXpress® reader in a 96-well plate format. Each VeraCode bead contains digitally assigned barcodes so that the BeadXpress® reader can identify HPV-type-specific signals from each VeraCode-bead, enabling HPV genotyping with digital read-out. The BeadXpress® reader photograph is used with the permission of Illumina Inc. (San Diego, CA, USA).

by sequence analysis, restriction fragment length polymorphism analysis, or hybridization with type-specific oligonucleotide probes by a membrane-based RLB assay. Of the various HPV genotyping assays, the RLB assay has the advantage of being able to detect multiple HPV-type infections with greater sensitivity. Several RLB assays combined with different PCR schemes have been established and used for HPV research and diagnostic purposes (8–10). However, the RLB assays are relatively laborious, are limited to a maximum of about 40 samples per assay, and depend on visual read-out of the hybridization signal. To overcome these drawbacks, HPV genotyping using Luminex® suspension array technology has been developed (11–14). The Luminex®-based genotyping coupled with GP5+/6+ PCR allowed sensitive and specific genotyping of 27 mucosal HPV types in a 96-well plate format with a digital read-out (13). Moreover, a modified version of GP5+/6+ PCR was successfully introduced into the Luminex®-based assay, and showed improved sensitivity (15).

A VeraCode-ASPE method was first developed for the detection of SNP in the human genome (16) and has been applied to multiplex SNP genotyping on the Illumina BeadXpress® platform (17, 18). The ASPE primer is composed of two distinct regions: the 5′ region that contains the capture sequence, which is used in a subsequent hybridization reaction, and the 3′ region that contains the genomic target region with a SNP nucleotide at the extreme 3′ end. For SNP genotyping, the ASPE primer that matches the SNP nucleotide to the genome is extended by the primer extension reaction and is thus labeled with biotinylated nucleotides. After the primer extension, the products are mixed with VeraCode beads, so that the capture sequence on the primer hybridizes to its complementary sequence attached to the VeraCode beads. Labeling is then carried out with a streptavidin-fluorophore conjugate, followed by scanning and detection of the fluorescent signal using an Illumina BeadXpress® reader (Illumina Inc., San Diego, CA, USA).

In this work, the VeraCode-ASPE method on the Illumina BeadXpress® platform was evaluated for its suitability as a method to detect and genotype HPV-DNA (Fig. 1). The HPV-DNA amplified by PGMY-PCR was selected as a target for the VeraCode-ASPE genotyping, as PGMY-PCR has been validated as a sensitive and specific means for HPV-DNA amplification (19, 20). HPV-type-specific ASPE primers were designed to target the PCR amplicons of 16 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) in the 3′ region (Table 1), and with type-specific capture sequences in the 5′ region. The T_m values of the HPV-type-specific sequences, the lengths of which ranged from 19 to 28 bases, were adjusted to be between 54°C and 66°C using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) thus allowing similar annealing profiles.

HPV-DNA, which was provided by the HPV laboratory network in the WHO as a quality-assured authentic panel for validation of HPV genotyping, was used to assess the sensitivity and specificity of the VeraCode-ASPE HPV genotyping. Fifty copies of HPV16- and 18-DNA and 500 copies of the other 14 HPV-type DNAs from the panel were subjected to PGMY-PCR with AmpliTaq Gold® polymerase (Applied Biosystems, Foster City, CA,

Table 1. HPV-type-specific sequences in ASPE primers for VeraCode-ASPE genotyping

Type	DNA sequence (5′–3′)	Tm (°C)
HPV6	CATCCGTAACTACATCTTCCA	55.3
HPV11	GCATCTGTGTCTAAATCTGCTAC	55.9
HPV16	AGTACCTACGACATGGGGAG	56.6
HPV18	CCAGGTACAGGAGACTGTGTA	55.3
HPV31	TGCAATTGCAAACAGTGATAC	57.3
HPV33	CTTTATGCACACAAGTAACTAGTGA	55.7
HPV35	TGTTCTGCTGTGTCTTCTAGTGA	57.8
HPV39	CCAACTTTACATTATCTACCTCTATAGA	55.3
HPV45	CACAAAATCCTGTGCCAAGT	58.6
HPV51	ACTGCTGCGGTTTCCCCAA	65.6
HPV52	GGAATACCTTCGTCATGGC	57.9
HPV56	TGATGCACGAAAAATTAATCAG	57.9
HPV58	TGACATTATGCACTGAAGTAACTAAG	57.0
HPV59	AAAGAATATGCCAGACATGTG	55.3
HPV66	AGTTAATGTGCTTTTAGCTGC	54.3
HPV68	GCTGTACCAAATATTTATGATCCTAA	57.1

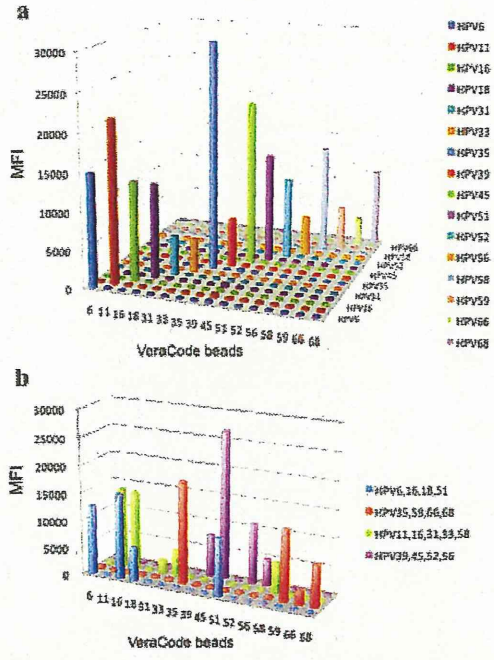

Fig. 2. VeraCode-ASPE genotyping of the WHO HPV-DNA proficiency panel. (a) PGMY-PCR products derived from the panel DNA containing each single-type HPV-DNA were subjected to the VeraCode-ASPE reaction and analyzed by the BeadXpress® reader. The signals from each VeraCode bead are shown as the MFI. The VeraCode bead number indicates the HPV type number that is assigned to each bead. (b) PGMY-PCR products derived from the panel DNA containing multiple-type HPV-DNA were subjected to the VeraCode-ASPE reaction and analyzed by the BeadXpress® reader.

USA) as described (21). One-third of the PCR products was treated with 2 U shrimp alkaline phosphatase and 5 U exonuclease I at 37°C for 45 min, followed by the ASPE reaction in a mixture containing 1× PCR buffer II (Roche, Indianapolis, IN, USA), 2.5 mM $MgCl_2$, 5 μM of each dATP, dGTP and dTTP, 7.5 μM biotin-14-dCTP, 0.05 μM of each ASPE primer, 0.5 U AmpliTaq Gold® polymerase, with denaturation at 95°C for 10 min followed by 50 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec. The reaction products were then incubated with the VeraCode bead mixture for 1 hr at 45°C in a VeraCode-bead plate, followed by staining with streptavidin-Alexa-647 in a buffer consisting of 3× standard saline citrate (SSC) and 0.1% Tween 20 for 15 min at room temperature. The VeraCode-bead plate was subjected to scanning by the BeadXpress® reader, and the read-out was expressed as the MFI obtained from each HPV type-assigned bead. As shown in Figure 2a, the 16 types of HPV-DNA were specifically detected with signals from their corresponding VeraCode beads. Signal values from non-target HPV-DNAs were as low as those from DNA-negative samples, and were classified as background noises. Furthermore, when the panel DNA containing a mixture of HPV-DNA was analyzed, corresponding signals from included HPV types were correctly detected (Fig. 2b), which indicates that VeraCode-ASPE typing is applicable to the simultaneous detection of multiple HPV-type DNAs.

To test the suitability of this assay for diagnostic purposes, DNA samples prepared from clinical specimens were analyzed by VeraCode-ASPE HPV genotyping. DNA was purified using the QIAamp® DNA blood kit (QIAGEN, Hilden, Germany) from cervical exfoliated cells that had been collected from outpatients with their informed consent for HPV genotyping. The study design was approved by the institutional review board of the NTT Medical Center, Tokyo. DNA samples were previously genotyped by PGMY-reverse blot hybridization (PGMY-RBH) assay, which had been validated as to be sensitive and specific for genotyping of the 16 HPV types in the studies of the WHO HPV-DNA proficiency panel (20). The same PGMY-PCR products derived from these DNA samples were subjected to VeraCode-ASPE HPV genotyping as carried out for the WHO HPV-DNA panel. A positive result was defined as a signal value more than three-fold the average background value for each HPV-type-specific VeraCode bead. Of 50 clinical samples analyzed by the VeraCode-ASPE assay, 20 samples gave HPV-positive results, whereas the remaining 30 samples were judged to be negative. Table 2 shows raw MFI data and typing results of the VeraCode-ASPE assay with 20 positive samples and one negative sample. Overall, the typing results were identical to those obtained by the PGMY-RBH assay, which strongly suggests that the VeraCode-ASPE assay can

Table 2. VeraCode-ASPE genotyping of DNA from clinical specimens

DNA no.	HPV6 HPV45 Typing result	HPV11 HPV51	HPV16 HPV52	HPV18 HPV56	HPV31 HPV58	HPV33 HPV59	HPV35 HPV66	HPV39 HPV68
#1644	46	50	47	102	53	47	53	78
	58	**28537**	50	51	48	71	424	147
	HPV51							
#1646	233	185	209	298	194	207	204	224
	213	320	237	**14999**	243	215	672	262
	HPV56							
#1647	203	237	**14047**	281	219	215	191	240
	217	297	200	224	217	**19118**	647	307
	HPV16, 59							
#1650	221	243	214	296	246	255	221	231
	253	323	**12271**	260	251	229	669	333
	HPV52							
#1652	47	54	51	140	57	46	56	97
	59	140	**24978**	55	56	55	608	173
	HPV52							
#1654	201	212	186	316	206	202	216	258
	821	289	219	191	**14963**	197	743	312
	HPV45, 58							
#1657	389	383	318	434	353	363	334	**1570**
	363	445	**46218**	395	364	346	825	431
	HPV39, 52							
#1661	120	130	128	238	166	136	149	**4599**
	156	221	151	134	123	137	622	255
	HPV39							
#1662	242	221	**45495**	244	186	223	238	270
	229	279	**26066**	209	230	231	518	286
	HPV16, 52							
#1666	204	252	**2654**	301	236	191	225	265
	223	330	226	243	**16990**	225	556	**5996**
	HPV16, 58, 68							
#1668	81	83	**38858**	135	92	84	86	121
	95	148	**10338**	84	94	83	435	178
	HPV16, 52							
#1669	118	156	122	189	**7566**	129	148	151
	143	207	140	131	**30234**	151	449	224
	HPV31, 58							
#1672	209	209	214	281	220	203	170	241
	219	266	216	201	**22802**	218	578	290
	HPV58							
#1676	91	92	96	169	96	87	92	121
	100	**12816**	89	99	96	97	491	190
	HPV51							
#1679	279	274	240	**36487**	238	268	278	270
	257	325	293	232	276	265	519	354
	HPV18							
#1683	241	278	**39325**	312	276	232	246	266
	249	321	251	233	258	226	552	337
	HPV16							
#1684	91	87	104	130	88	87	94	103
	96	145	89	101	86	91	**26638**	160
	HPV66							
#1685	151	129	**6983**	237	150	122	137	174
	150	221	146	145	145	139	559	233
	HPV16							

Continued.

Table 2. *Continued*

DNA no.	HPV6 HPV45 Typing result	HPV11 HPV51	HPV16 HPV52	HPV18 HPV56	HPV31 HPV58	HPV33 HPV59	HPV35 HPV66	HPV39 HPV68
#1686	196	198	198	289	169	204	235	203
	208	276	202	187	186	**20644**	574	275
	HPV59							
#1691	230	205	230	346	227	234	216	222
	250	275	225	**13139**	226	241	552	317
	HPV56							
#1645	150	138	142	252	151	136	148	183
	147	219	143	148	134	131	659	229
	negative							
DNA(–)	188	205	202	289	195	198	213	211
	217	264	206	220	204	222	457	259
Cut-off	565	615	607	868	584	595	638	634
	650	791	618	659	611	667	1372	778

Signal values above cut-off values are indicated in bold letters.

substitute for the reverse blot hybridization on the same platform of PGMY-PCR.

The principle of the allele-specific primer extension was previously used in tag-array-based HPV genotyping (22, 23); however, the array format of this assay hampers its application to high-throughput HPV genotyping. In contrast, the 96-well plate format of the VeraCode-ASPE method enables HPV genotyping for large amounts of clinical samples. Furthermore, there are a total of 144 different sets of VeraCode beads, and thus it is possible to include more HPV types in the VeraCode-ASPE genotyping format. In conclusion, the VeraCode-ASPE genotyping is a powerful new tool for the high-throughput HPV genotyping that will be required for large-scale surveillance of HPV-type distribution at the population level in the near future.

ACKNOWLEDGMENTS

This work received financial support from the Ministry of Health, Labor and Welfare in Japan, and the WHO HPV laboratory network. We thank Dr Roland Sahli at Centre Hospitalier Universitaire Vaudois in Lausanne for technical support for the introduction of the PGMY-RBH assay.

DISCLOSURE

The authors did not receive any financial support from the companies whose products were used in this work. The authors have no conflict of interest to declare.

REFERENCES

1. Zur Hausen H. (2002) Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* **2**: 342–50.
2. De Villiers E.M., Fauquet C., Broker T.R., Bernard H.U., Zur Hausen H. (2004) Classification of papillomaviruses. *Virology* **324**: 17–27.
3. Munoz N., Bosch F.X., De Sanjose S., Herrero R., Castellsague X., Shah K.V., Snijders P.J., Meijer C.J. (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* **348**: 518–27.
4. Coutlee F., Hankins C., Lapointe N. (1997) Comparison between vaginal tampon and cervicovaginal lavage specimen collection for detection of human papillomavirus DNA by the polymerase chain reaction. The Canadian Women's HIV Study Group. *J Med Virol* **51**: 42–7.
5. Gravitt P.E., Peyton C.L., Alessi T.Q., Wheeler C.M., Coutlee F., Hildesheim A., Schiffman M.H., Scott D.R., Apple R.J. (2000) Improved amplification of genital human papillomaviruses. *J Clin Microbiol* **38**: 357–61.
6. De Roda Husman A.M., Walboomers J.M., Van Den Brule A.J., Meijer C.J., Snijders P.J. (1995) The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol* **76**(Pt 4): 1057–62.
7. Kleter B., Van Doorn L.J., Ter Schegget J., Schrauwen L., Van Krimpen, K., Burger M., Ter Harmsel B., Quint W. (1998) Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *Am J Pathol* **153**: 1731–39.
8. Van Den Brule A.J., Pol R., Fransen-Daalmeijer N., Schouls L.M., Meijer, C.J., Snijders P.J. (2002) GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol* **40**: 779–87.
9. Gravitt P.E., Peyton C.L., Apple R.J., Wheeler, C.M. (1998) Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. *J Clin Microbiol* **36**: 3020–27.

10. Kleter B., Van Doorn, L.J., Schrauwen L., Molijn A., Sastrowijoto S., Ter Schegget J., Lindeman J., Ter Harmsel B., Burger M., Quint W. (1999) Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *J Clin Microbiol* **37**: 2508–17.
11. Schmitt M., Bravo I.G., Snijders P.J., Gissmann L., Pawlita M., Waterboer T. (2006) Bead-based multiplex genotyping of human papillomaviruses. *J Clin Microbiol* **44**: 504–12.
12. Oh Y., Bae S.M., Kim Y.W., Choi H.S., Nam G.H., Han S.J., Park C.H., Cho Y., Han B.D., Ahn W.S. (2007) Polymerase chain reaction-based fluorescent Luminex assay to detect the presence of human papillomavirus types. *Cancer Sci* **98**: 549–54.
13. Schmitt M., Dondog B., Waterboer T., Pawlita M. (2008) Homogeneous amplification of genital human alpha papillomaviruses by PCR using novel broad-spectrum GP5+ and GP6+ primers. *J Clin Microbiol* **46**: 1050–59.
14. Nazarenko I., Kobayashi L., Giles J., Fishman C., Chen G., Lorincz A. (2008) A novel method of HPV genotyping using Hybrid Capture sample preparation method combined with GP5+/6+ PCR and multiplex detection on Luminex XMAP. *J Virol Methods* **154**: 76–81.
15. Soderlund-Strand A., Carlson J., Dillner J. (2009) Modified general primer PCR system for sensitive detection of multiple types of oncogenic human papillomavirus. *J Clin Microbiol* **47**: 541–46.
16. Lin C.H., Yeakley J.M., Mcdaniel T.K., Shen R. (2009) Medium- to high-throughput SNP genotyping using VeraCode microbeads. *Methods Mol Biol* **496**: 129–42.
17. Tokarska M., Marshall T., Kowalczyk R., Wojcik J.M., Pertoldi C., Kristensen T.N., Loeschcke V., Gregersen V.R., Bendixen C. (2009) Effectiveness of microsatellite and SNP markers for parentage and identity analysis in species with low genetic diversity: the case of European bison. *Heredity* **103**: 326–32.
18. Mefford H.C., Cooper G.M., Zerr T., Smith J.D., Baker C., Shafer N., Thorland E.C., Skinner C., Schwartz C.E., Nickerson D.A., Eichler E.E. (2009) A method for rapid, targeted CNV genotyping identifies rare variants associated with neurocognitive disease. *Genome Res* **19**: 1579–85.
19. Mori S., Nakao S., Kukimoto I., Kusumoto-Matsuo R., Kondo K., Kanda T. (2011) Biased amplification of human papillomavirus DNA in specimens containing multiple human papillomavirus types by PCR with consensus primers. *Cancer Sci* **102**: 1223–7.
20. Estrade C., Menoud P.A., Nardelli-Haefliger D., Sahli R. (2011) Validation of a low-cost HPV genotyping assay baced on PGMY-PCR and reverse blotting hybridization with reusable membranes. *J Clin Microbiol* **49**: 3474–81.
21. Unger E.R., Dillner J., Zhou T. (2009) *Human Papillomavirus Laboratory Manual*, 1st edition. Geneva, Switzerland: WHO.
22. Kaller M., Hultin E., Zheng B., Gharizadeh B., Wallin K.L., Lundeberg J., Ahmadian A. (2005) Tag-array based HPV genotyping by competitive hybridization and extension. *J Virol Methods* **129**: 102–12.
23. Gheit T., Landi S., Gemignani F., Snijders P.J., Vaccarella S., Franceschi S., Canzian F., Tommasino M. (2006) Development of a sensitive and specific assay combining multiplex PCR and DNA microarray primer extension to detect high-risk mucosal human papillomavirus types. *J Clin Microbiol* **44**: 2025–31.

Published in final edited form as:
Int J Gynecol Pathol. 2011 January ; 30(1): 30–35. doi:10.1097/PGP.0b013e3181e9a319.

Rsf-1 (HBXAP) expression is associated with advanced stage and lymph node metastasis in ovarian clear cell carcinoma

Daichi Maeda, M.D.[a,b,c], **Xu Chen, M.D.**[a], **Bin Guan, Ph.D.**[a], **Shunsuke Nakagawa, M.D., Ph.D.**[d], **Tetsu Yano, M.D., Ph.D.**[d], **Yuji Taketani, M.D., Ph.D.**[d], **Masashi Fukayama, M.D., Ph.D.**[c], **Tian-Li Wang, Ph.D.**[b], and **Ie-Ming Shih, M.D., Ph.D.**[a,b,*]

[a]Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD

[b]Departments of Gynecology and Obstetrics and Oncology, Johns Hopkins Medical Institutions, Baltimore, MD

[c]Department of Pathology, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan

[d]Department of Obstetrics and Gynecology, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan

Summary

Ovarian clear cell carcinoma (CCC) is a unique type of ovarian cancer characterized by distinct clinicopathological and molecular features. CCC is considered to be a highly malignant disease because it is resistant to conventional chemotherapy, and, when presented at advanced stages, has a dismal overall survival. Identifying and characterizing biomarkers associated with its malignant behavior is fundamental toward elucidating the mechanisms underlying its aggressive phenotype. In this study, we performed immunohistochemical analysis on 89 CCCs to assess their expression of *Rsf-1 (HBXAP)*, a chromatin remodeling gene frequently amplified and overexpressed in several types of human cancer. We found that 73 (82%) of 89 CCCs expressed Rsf-1 and most importantly, there was a statistically significant correlation between Rsf-1 immunostaining intensity and two disease parameters: advanced stage ($p = 0.008$) and status of retroperitoneal lymph node metastasis ($p = 0.023$). However, there was no correlation between Rsf-1 expression and patient age, peritoneal tumor dissemination, or overall survival. In conclusion, a higher expression level of Rsf-1 is associated with advanced clinical stage and lymph node metastasis in CCC. Our data suggest that Rsf-1 participates in tumor progression in CCC, and indicates that the contribution of Rsf-1 to disease aggressiveness deserves further study.

Introduction

Ovarian clear cell carcinoma (CCC) represents less than 10% of ovarian cancers in the United States, but occurs more frequently in Asian women (1,2). Multivariate analysis on a large series of CCC shows that women with CCC present at a younger age and at earlier clinical stages as compared to high-grade (conventional) serous carcinoma, the most common and lethal type of ovarian cancer (1). Approximately 50% of CCCs present as stage I diseases (3,4) and, despite being diagnosed at an early stage, are generally considered to be

*Correspondence to Ie-Ming Shih; Johns Hopkins Medical Institutions, 1550 Orleans street, CRB-2, room: 305, Baltimore, Maryland 21231; ishih@jhmi.edu, Tel: 410-502-7774. Fax: 410-502-7943.

highly malignant (5). Morphological and molecular studies have demonstrated that many CCCs develop in a stepwise fashion from endometriosis through atypical endometriosis to overt CCC (6–10). In fact, CCC is the most common ovarian carcinoma associated with endometriosis. There has been increased enthusiasm for identifying markers that are predictive of the clinical outcome in CCC patients. This is because CCC typically presents with stage I or II disease, and prognostic markers could have an impact on clinical decision making in the management of CCC patients, such as administration of adjuvant chemotherapy. For example, IGF2BP3 (IMP3) expression has been reported to be an independent marker of reduced disease-specific survival in CCC, but not in high-grade serous or endometrioid carcinomas of the ovary (11). Similarly, enhanced expression of annexin A4 in CCC and its association with chemoresistance to carboplatin have been recently reported (12).

To further identify markers that are associated with poor prognosis in CCC and to explore the molecular mechanisms that account for the aggressive behavior of CCC, we determined the correlation between immunoreactivity of Rsf-1, also known as HBXAP, and clinical outcome in primary CCCs. We focused on Rsf-1 (HBXAP) because the encoded protein participates in chromatin remodeling, and this gene has been identified as an amplified gene with a tumor-promoting potential in several types of neoplastic diseases including ovarian high-grade serous carcinoma (13,14,15). Our analysis showed that higher expression levels of Rsf-1 (HBXAP) were associated advanced stage disease and retroperitoneal lymph node metastasis. The current study provides new evidence of the biological significance of Rsf-1 expression in CCC.

Materials and methods

Tissue samples

Formalin-fixed and paraffin-embedded CCC tissues were obtained from the Department of Pathology at the University of Tokyo Hospital. A total of 89 cases of primary CCCs were retrieved from the archives, and hematoxylin and eosin (H&E) stained slides were reviewed to confirm the diagnosis based on the most recent criteria of the World Health Organization. The CCC tissues were arranged in tissue microarrays (Beecher Instruments, Silver Spring, MD) with duplicate 2 mm tissue cores obtained from the tumor area in each CCC. The collection of clinical specimens was in compliance of guideline of tissue procurement at the University of Tokyo Hospital.

Clinical information of patients with ovarian clear cell carcinoma

We reviewed the medical records from all 89 CCC patients; data obtained included demographics, age at the time of diagnosis, preoperative diagnosis, clinical stage, and survival time after treatment. None of the patients underwent preoperative chemotherapy or radiotherapy. The correlations of Rsf-1 expression with the following clinical variables were evaluated: age, stage of carcinoma (stage I/II *vs.* stage III/IV), peritoneal dissemination, retroperitoneal lymph node metastasis, and death rate. Stage of carcinoma was assessable in 67 cases in which the appropriate staging procedures were performed; the remaining 22 cases were not included in the staging analysis due either to incomplete surgical procedures or to missing data. Staging was in accordance with the standards of the International Federation of Gynecology and Obstetrics (FIGO). Comprehensive evaluation of peritoneal dissemination that included microscopic examination of the omentum, peritoneal wall and mesentery soft tissues was performed in 79 cases. Retroperitoneal lymph node dissection was performed in 70 cases. Follow-up information included overall survival and cancer-related death. The follow-up interval was calculated from the date of surgery to the date of

death or last clinical evaluation. The mean follow-up interval was 50 months (range 1–196 months).

Immunohistochemistry

The method of immunohistochemistry and scoring of immunoreactivity for Rsf-1 expression were previously described (13,14). Briefly, 4 μm sections were cut from the tissue microarray blocks. Antigen retrieval was performed on deparaffinized sections by steaming them in citrate buffer (pH 6.0). A monoclonal anti-Rsf-1 antibody, clone 5H2/E4 (Upstate, Lake Placid, NY), was used at an optimal dilution of 1:2000 as previously determined (13,14) and a monoclonal anti-NAC1 antibody was used at a dilution of 1:250 (16). The sections were incubated with the antibodies for 2 hours at room temperature, followed by the EnVision+ System (DAKO, Carpinteria, California) using the peroxidase method. An isotype-matched control antibody (MN-4) was used in parallel (17). Our previous studies had shown that the distribution of Rsf-1 immunoreactivity was always homogeneous within a tumor; therefore, we used an intensity score ranging from 0 to 4+ to evaluate Rsf-1 immunoreactivity in tumors as previously described (14). A positive reaction for both Rsf-1 and NAC1 was defined as discrete localization of the chromogen in the nuclei. The tissues were scored in a blinded fashion without the knowledge of clinical information.

Rsf-1 gene knockdown using small hairpin RNA

Ovarian clear cell adenocarcinoma cell lines, ES2 and JHOC5, were used in this study. ES2 was obtained from the American Type Culture Collection (Rockville,, MD, USA); JHOC5 was a kind gift from Dr. Kentaro Nakayama, Shimane University, Japan. Both cell lines used in this study were cultured in RPMI 1640 containing 5% fetal bovine serum.

In order to confirm the specificity of the anti-Rsf-1 antibody used for immunohistochemistry, we performed Rsf-1 knockdown by transduction of two small hairpin RNAs (shRNA) and evaluated the knockdown efficiency by Western blot. The antibody specificity was indicated by reduced protein expression corresponding to Rsf-1 after gene knockdown based on western blot analysis using the same anti-Rsf-1 antibody as used in immunohistochemistry. We used lentivirus carrying the Rsf-1 shRNA sequence templates (CCGGCCAGTTCTGAAC TTTGAAGATCTCGAGATCTTCAAAGTTCAGAACT) and (CCGGCTTCTGAGA CAAAGGGTTCTACTCGAGTAGAACCCTTTGTCTCAGA), and a control shRNA sequence template, which were inserted into the lentiviral plasmid (pLKO.1-puro). Cells were washed and harvested 24 hours after transfection for protein and mRNA extraction.

For Western blot analysis, protein lysates were separated by 4% to 20% Tris-glycine gel electrophoresis and transferred onto polyvinylidene difluoride membranes using a semidry apparatus (Bio-Rad). After blocking, membranes were incubated with the anti-Rsf-1 (clone 5H2/E4) primary antibody at 4°C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Protein bands were detected with Amersham ECL Western blotting detection reagents (GE Healthcare). Antibody reacting to anti-GAPDH was used to evaluate the amount of GAPDH as a loading control. Western blot analysis showed a reduced protein band corresponding to Rsf-1 in cells transfected with Rsf-1 shRNA as compared to control shRNA transfected cells, indicating the specificity of the anti-Rsf-1 antibody (Fig. 1).

Statistical Analysis

Statistical analysis was performed using the χ^2-test. Overall survival of CCC cases was calculated using the Kaplan-Meier method, and statistical analyses were performed using the

log-rank test. Statistical analyses were performed with StatView 5.0 software (SAS Institute, Cary, NC) and $P < 0.05$ was considered statistically significant.

Results

Expression of Rsf-1 in ovarian clear cell carcinomas

Results of Rsf-1 immunohistochemistry in CCCs are summarized in Table 1. Rsf-1 immunoreactivity was detected exclusively in nuclei of almost all tumor cells. Positive immunoreactivity of Rsf-1 was observed in 73 (82%) of 89 cases. Specifically, 16 (18%), 53 (60%), and 19 (21%) of 89 cases had a staining score of 0, 1+, and 2+, respectively. Only one case exhibited intense nuclear staining (3+). Histological features in representative cases with different Rsf-1 immunostaining intensities including 0, 1+, and 2+ are shown in Fig. 2. There was no correlation between Rsf-1 expression and histological pattern and nuclear atypia of the CCC cases.

Correlation of Rsf-1 expression with clinical features

Since Rsf-1 expression has been reported to play a tumor-promoting role in ovarian cancer, we analyzed the possible correlation of Rsf-1 expression with clinical characteristics in CCC (Table 2). Statistically significant correlations were observed between Rsf-1 immunostaining intensity (score > 1) and lymph node involvement ($P = 0.023$). Furthermore, Rsf-1 immunostaining intensity (score > 1) was associated with advanced stage disease (Stage III/IV) ($P = 0.0088$). In fact, none of the Rsf-1 negative cases presented at an advanced stage. The frequency of peritoneal dissemination was higher in Rsf-1 positive cases (12/65), compared to Rsf-1 negative cases (1/14) but the difference was not statistically significant ($P = 0.3$). As a control we also assessed the expression of Nac1, a nuclear protein involved in transcription regulation, in the same set of CCC. We found that there was no significant association of Nac1 expression and any clinical feature including presentation stage or lymph node metastasis status ($p > 0.1$) (data not shown). Kaplan-Meier analyses were performed to determine if there was a correlation between Rsf-1 expression and clinical outcome. We first assessed the association between tumor stage and overall survival in CCCs, and demonstrated that stage III/IV cases ($n = 20$) had a poorer prognosis than stage I/II cases ($n = 47$) ($P < 0.0001$) (Fig. 3). However, Kaplan-Meier analysis did not reveal a significant difference in survival between Rsf-1 positive and negative cases ($P = 0.42$).

Discussion

An increase in DNA copy number at the chromosome 11q13.5 locus containing *Rsf-1* (*HBXAP*) is detected in several types of human cancer including ovarian high-grade serous carcinoma. *Rsf-1* (*HBXAP*) encodes for a cellular nuclear protein that binds to hSNF2H (18), forming a chromatin remodeling protein complex called RSF (Remodeling and Spacing Factor) (19,20). Rsf-1 (HBXAP) has been shown to function as a histone chaperone in the nuclei while its binding partner, hSNF2H, possesses nucleosome-dependent ATPase activity (21). The Rsf-1/hSNF2H complex (RSF complex) mediates ATP-dependent chromatin remodeling, which alters the chromatin structure or positioning of nucleosomes (20). At the cellular level, RSF participates in chromatin remodeling in response to a variety of growth signals and environmental cues. Such nucleosome remodeling is required for transcriptional activation or repression (22,23,24), DNA replication (25), and cell cycle progression (26).

In this study, we used a well characterized anti-Rsf-1 antibody to study the expression pattern of Rsf-1 in CCC, and provided new evidence that expression of Rsf-1 was associated with advanced clinical stages and with the status of lymph node metastasis in CCC. The

findings suggest a biological role for Rsf-1 in disease aggressiveness in this type of ovarian carcinoma. Interestingly, we have previously reported that chromosome 11q13.5 amplification and overexpression in cases of ovarian high-grade serous carcinoma contributes to shorter overall survival compared to cases without amplification. A possible mechanism was thought to be related to the de novo paclitaxel resistance rendered by Rsf-1 overexpression (27). Although Kaplan-Meier survival analysis did not show statistically significant difference between Rsf-1 positive CCC cases and Rsf-1 negative CCC cases, long-term prognosis of Rsf-1 positive cases appears to be slightly worse than Rsf-1 negative cases. However, the number of Rsf-1 negative CCC cases in our series was relatively small, and we believe that analysis in larger series on CCCs is required to conclude if Rsf-1 overexpression predicts worse overall survival in CCCs. Furthermore, our study suggests a potential use of Rsf-1 immunoreactivity as a biomarker that may prove useful for predicting clinical outcomes in primary CCC, including higher clinical stages, and for predicting the risk of developing lymph node metastasis. To this end, several proteins including IGF2BP3 (IMP3) (11) and annexin A4 (12) have been reported as new markers associated with treatment outcomes in CCC. Thus, a panel of different markers including Rsf-1 could be tested in future clinical trials to determine their potential to be used in the management of CCC patients.

In the current report, we observed that, with a single exception, the immunostaining intensity score of Rsf-1 was less than 3+ in all cases analyzed. This finding provides an independent confirmation of our previous observation in another, smaller set of CCC samples in which we demonstrated that the majority of CCCs showed an immunostaining intensity score of 1+ or 2+ (14). In fact, the percentage of Rsf-1 positive and negative cases is very similar between the current and previous reports. Moreover, analysis of SNP arrays performed on affinity-purified CCC specimens did not show an increase in DNA copy number at chromosome 11q13.5, indicating that *Rsf-1* is rarely amplified in CCC (28). The above findings in CCC are in sharp contrast to those in high-grade serous carcinoma (14), and underscore the distinct molecular pathways in developing CCC and high-grade serous carcinoma (reviewed in (29)). It is also noteworthy that endometrioid and mucinous carcinomas of the ovary express Rsf-1 much less frequently as compared to CCCs and high-grade serous carcinoma. Only 49% of endometrioid carcinomas and 48% of mucinous carcinomas were Rsf-1 positive, and the intensity scores of positive cases were mostly 1+ and 2+.

In conclusion, using immunohistochemistry with an Rsf-1 specific antibody we demonstrated that the presence of Rsf-1 immunoreactivity is significantly associated with advanced stage and lymph node metastasis in primary CCCs. Our findings suggest Rsf-1 expression may contribute to disease aggressiveness in CCC, and warrant further study of the biological role of Rsf-1 in progression of CCC.

Acknowledgments

This study was supported by NIH/NCI grant CA129080 and the International Training Program from the Japan Society for the Promotion of Science.

References

1. Chan JK, Teoh D, Hu JM, Shin JY, Osann K, Kapp DS. Do clear cell ovarian carcinomas have poorer prognosis compared to other epithelial cell types? A study of 1411 clear cell ovarian cancers. Gynecol Oncol 2008;109:370–376. [PubMed: 18395777]
2. Ushijima K. Current status of gynecologic cancer in Japan. J Gynecol Oncol 2009;20:67–71. [PubMed: 19590716]

3. Takano M, Kikuchi Y, Yaegashi N, et al. Clear cell carcinoma of the ovary: a retrospective multicentre experience of 254 patients with complete surgical staging. Br J Cancer 2006;94:1369–1374. [PubMed: 16641903]
4. Mizuno M, Kikkawa F, Shibata K, et al. Long-term follow-up and prognostic factor analysis in clear cell adenocarcinoma of the ovary. J Surg Oncol 2006;94:138–143. [PubMed: 16847906]
5. Jenison EL, Montag AG, Griffiths CT, et al. Clear cell adenocarcinoma of the ovary: a clinical analysis and comparison with serous carcinoma. Gynecol Oncol 1989;32:65–71. [PubMed: 2642454]
6. Veras E, Mao TL, Ayhan A, et al. Cystic and adenofibromatous clear cell carcinomas of the ovary: distinctive tumors that differ in their pathogenesis and behavior: a clinicopathologic analysis of 122 cases. Am J Surg Pathol 2009;33:844–853. [PubMed: 19342944]
7. Fukunaga M, Nomura K, Ishikawa E, Ushigome S. Ovarian atypical endometriosis: its close association with malignant epithelial tumours. Histopathology 1997;30:249–255. [PubMed: 9088954]
8. Erzen M, Rakar S, Klancnik B, Syrjanen K. Endometriosis-associated ovarian carcinoma (EAOC): an entity distinct from other ovarian carcinomas as suggested by a nested case-control study. Gynecol Oncol 2001;83:100–108. [PubMed: 11585420]
9. Sato N, Tsunoda H, Nishida M, et al. Loss of heterozygosity on 10q23.3 and mutation of the tumor suppressor gene PTEN in benign endometrial cyst of the ovary: possible sequence progression from benign endometrial cyst to endometrioid carcinoma and clear cell carcinoma of the ovary. Cancer Res 2000;60:7052–7056. [PubMed: 11156411]
10. Marquez RT, Baggerly KA, Patterson AP, et al. Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon. Clin Cancer Res 2005;11:6116–6126. [PubMed: 16144910]
11. Kobel M, Xu H, Bourne PA, et al. IGF2BP3 (IMP3) expression is a marker of unfavorable prognosis in ovarian carcinoma of clear cell subtype. Mod Pathol 2009;22:469–475. [PubMed: 19136932]
12. Aoki D, Oda Y, Hattori S, et al. Overexpression of class III beta-tubulin predicts good response to taxane-based chemotherapy in ovarian clear cell adenocarcinoma. Clin Cancer Res 2009;15:1473–1480. [PubMed: 19228748]
13. Shih IM, Sheu JJ, Santillan A, et al. Amplification of a chromatin remodeling gene, Rsf-1/HBXAP, in ovarian carcinoma. Proc Natl Acad Sci U S A 2005;102:14004–14009. [PubMed: 16172393]
14. Mao TL, Hsu CY, Yen MJ, et al. Expression of Rsf-1, a chromatin-remodeling gene, in ovarian and breast carcinoma. Hum Pathol 2006;37:1169–1175. [PubMed: 16938522]
15. Shih IM, Davidson B. Pathogenesis of ovarian cancer: clues from selected overexpressed genes. Future Oncol 2009;5:1641–1657. [PubMed: 20001801]
16. Nakayama K, Nakayama N, Davidson B, et al. A BTB/POZ protein, NAC-1, is related to tumor recurrence and is essential for tumor growth and survival. Proc Natl Acad Sci U S A 2006 Dec 5;103:18739–18744. [PubMed: 17130457]
17. Shih IM, Nesbit M, Herlyn M, et al. A new Mel-CAM (CD146)-specific monoclonal antibody, MN-4, on paraffin-embedded tissue. Mod Pathol 1998;11:1098–1106. [PubMed: 9831208]
18. Sheu JJ, Choi JH, Yildiz I, et al. The Roles of Human Sucrose Nonfermenting Protein 2 Homologue in the Tumor-Promoting Functions of Rsf-1. Cancer Res 2008;68:4050–4057. [PubMed: 18519663]
19. LeRoy G, Loyola A, Lane WS, Reinberg D. Purification and characterization of a human factor that assembles and remodels chromatin. J Biol Chem 2000;275:14787–14790. [PubMed: 10747848]
20. Loyola A, Huang J-Y, LeRoy G, et al. Functional Analysis of the Subunits of the Chromatin Assembly Factor RSF. Mol Cell Biol 2003;23:6759–6768. [PubMed: 12972596]
21. Aihara T, Miyoshi Y, Koyama K, et al. Cloning and mapping of SMARCA5 encoding hSNF2H, a novel human homologue of Drosophila ISWI. Cytogenet Cell Genet 1998;81:191–193. [PubMed: 9730600]
22. Shamay M, Barak O, Shaul Y. HBXAP, a novel PHD-finger protein, possesses transcription repression activity. Genomics 2002;79:523–529. [PubMed: 11944984]

23. Shamay M, Barak O, Doitsh G, Ben-Dor I, Shaul Y. Hepatitis B virus pX interacts with HBXAP, a PHD finger protein to coactivate transcription. J Biol Chem 2002;277:9982–9988. [PubMed: 11788598]
24. Vignali M, Hassan AH, Neely KE, Workman JL. ATP-dependent chromatin-remodeling complexes. Mol Cell Biol 2000;20:1899–1910. [PubMed: 10688638]
25. Flanagan JF, Peterson CL. A role for the yeast SWI/SNF complex in DNA replication. Nucleic Acids Res 1999;27:2022–2028. [PubMed: 10198436]
26. Cosma MP, Tanaka T, Nasmyth K. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. Cell 1999;97:299–311. [PubMed: 10319811]
27. Choi JH, Sheu JJ, Guan B, et al. Functional analysis of 11q13.5 amplicon identifies Rsf-1 (HBXAP) as a gene involved in paclitaxel resistance in ovarian cancer. Cancer Res 2009;69:1407–1415. [PubMed: 19190325]
28. Kuo K, Mao T, Feng Y, et al. DNA copy number profiles in affinity-purified ovarian clear cell carcinoma. Clin Cancer Res. 2010 April 1; 2010 issue.
29. Cho KR, Shih IM. Ovarian cancer. Annu Rev Pathol Mech Dis 2009;4:287–313.

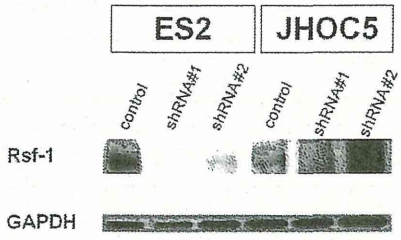

Fig. 1.

Rsf-1 expression in ovarian clear cell carcinoma cell lines, ES2 and JHOC5

Western blot analysis showed a reduced protein band corresponding to Rsf-1 protein in Rsf-1 specific shRNA transfected cells as compared to control shRNA transfected cells, indicating the specificity of the anti-Rsf-1 antibody.

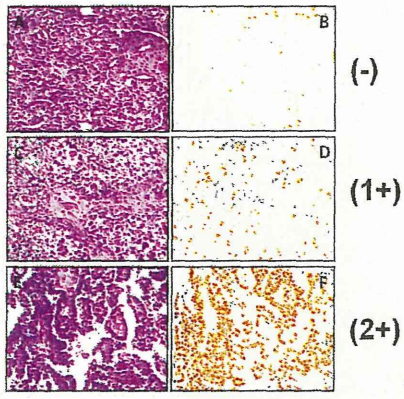

Fig. 2.

Rsf-1 immunoreactivity in representative ovarian clear cell carcinomas

(A, B) Microscopic view of an ovarian clear cell carcinoma showing negative immunoreactivity for Rsf-1. (C, D) A case of ovarian clear cell carcinoma with 1+ immunostaining intensity for Rsf-1. (E, F) A clear cell carcinoma with 2+ immunoreactivity for Rsf-1. A, C and E: hematoxylin and eosin stained sections; B, D and F: Rsf-1 stained sections.

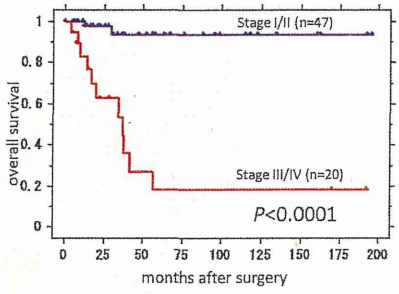

Fig. 3.

Kaplan-Meier survival curve analysis shows that patients with stage III/IV ovarian clear cell carcinoma have a significantly worse overall survival rate than those with stage I/II ovarian clear cell carcinoma ($P< 0.0001$).

Table 1

Rsf-1 expression in ovarian clear cell carcinoma

Immunostaining intensity score	Number of cases	%
0	16	18
1+	53	60
2+	19	21
3+	1	1
4+	0	0
Total	89	100

Table 2

Correlation of Rsf-1 expression with clinical characteristics in ovarian clear cell carcinomas

Clinical characteristics	Rsf-1 (HBXAP) expression		
	Positive	Negative	*P*
Age (n=89)			
≥ 50	46 (79%)	12 (21%)	
< 50	27 (87%)	4 (13%)	0.36
Stage (n = 67)			
I, II	34 (72%)	13 (28%)	
III, IV	20 (100%)	0 (0%)	0.0088*
Peritoneal dissemination (n = 79)			
Negative	53 (80%)	13 (20%)	
Positive	12 (92%)	1 (8%)	0.30
Lymph node metastasis (n = 70)			
Negative	40 (73%)	15 (27%)	
Positive	15 (100%)	0 (0%)	0.023*
Survival status (n = 89)			
Alive	58 (81%)	14 (19%)	
Deceased	15 (88%)	2 (12%)	0.46

* statistically significant

Med Oncol
DOI 10.1007/s12032-011-9878-z

LETTER TO THE EDITOR

Second-line chemotherapy with docetaxel and carboplatin in paclitaxel and platinum-pretreated ovarian, fallopian tube, and peritoneal cancer

Takahide Arimoto · Shunsuke Nakagawa · Katsutoshi Oda · Kei Kawana · Toshiharu Yasugi · Yuji Taketani

Abstract We retrospectively evaluated the efficacy and toxicity of docetaxel and carboplatin in patients with platinum and paclitaxel-pretreated recurrent ovarian, fallopian tube, and peritoneal cancer. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four patients had measurable disease. The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, 34 weeks, and the median overall survival time was 94, 224, 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes (8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months.

T. Arimoto (✉) · S. Nakagawa · K. Oda · K. Kawana · T. Yasugi · Y. Taketani
Department of Obstetrics and Gynecology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
e-mail: tarimoto-tky@umin.ac.jp

Published online: 06 March 2011

Keywords Docetaxel · Carboplatin · Chemotherapy · Early progression · Recurrent ovarian cancer

The standard regimen as second-line chemotherapy in recurrent ovarian cancer has not been established, especially in the patients with a short progression-free interval from the previous treatment. Docetaxel is an active drug as second-line chemotherapy for recurrent ovarian cancer as well as pegylated liposomal doxorubicin, irinotecan, topotecan, gemcitabine, and etoposide [ ].

The purpose of this study was to evaluate activity and toxicity of the combination of docetaxel and carboplatin retrospectively in patients with paclitaxel and platinum resistant (progression-free interval less than 6 months) and partially resistant (progression-free interval of 6–12 months) ovarian, fallopian tube, and peritoneal cancers. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four (81%) patients had measurable disease. Twenty-six (62%) patients had experienced progression of disease within less than 6 months of their last treatment, whereas 16 patients (38%) within 6–12 months. The median number of courses of treatment per patient was 4.5 (range: 1–8 courses). The median follow-up period was 107 weeks (range: 9–373 weeks). The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, and 34 weeks, and the median overall survival time was 94, 224, and 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes

(8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. On the other hand, grade 2/3 neuropathy was observed only in two (4.8%) patients.

Several chemotherapeutic agents such as pegylated liposomal doxorubicin, topotecan, irinotecan, gemcitabine, and etoposide have been used in the treatment of platinum-resistant disease with response rates in the range 10–15% [–]. The results from our study about overall response rate are in line with other chemotherapeutic agents. Notably, our data about median time to tumor progression and overall survival are longer than the previously reported data of other regimens.

The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months. However, chemotherapy with docetaxel and carboplatin may improve time to tumor progression and overall survival time in these cases; this regimen can be an alternative in patients whose hematological toxicity is relatively weak at their previous treatment.

Conflict of interest None.

References

1. Sugiyama T. Second-line chemotherapy for recurrent ovarian cancer. Gan To Kagaku Ryoho. 2005;32:28–32.
2. Swisher EM, Mutch DG, Rader JS, Elbendary A, Herzog TJ. Topotecan in platinum- and paclitaxel-resistant ovarian cancer. Gynecol Oncol. 1997;66:480–6.
3. Gordon AN, Tonda M, Sun S, Rackoff W. Long-term survival advantage for women treated with pegylated liposomal doxorubicin compared with topotecan in a phase 3 randomized study of recurrent and refractory epithelial ovarian cancer. Gynecol Oncol. 2004;95:1–8.
4. Mutch DG, et al. Randomized phase III trial of gemcitabine compared with pegylated liposomal doxorubicin in patients with platinum-resistant ovarian cancer. J Clin Oncol. 2007;25:2811–8.
5. Ferrandina G, et al. Phase III trial of gemcitabine compared with pegylated liposomal doxorubicin in progressive or recurrent ovarian cancer. J Clin Oncol. 2008;26:890–6.