miR-130b impairs cell invasion by targeting ZEB1

To investigate whether miR-130b can control EC cell invasion through the modulation of ZEB1 expression, we used TargetScan and miRviewer to search for miRNA-binding sites in the 3′-UTR of *ZEB1*. The miR-130b was found to possess five evolutionary-conserved binding sites, suggesting that a potential interaction between miR-130b seed region and *ZEB1* mRNA 3′-UTR (Figure 5a). To test if miR-130b binds directly to ZEB1 transcript, we transfected biotin-labeled miR-130b into HEC-50 cells, pulled down mRNAs bound to miR-130b and quantified ZEB1 transcript using qRT–PCRs. We found that the levels of ZEB1 mRNA were highly enriched by miR-130b pull-down, as compared with control transcripts of housekeeping genes 5S rRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Supplementary Figure S7). Real-time PCR and western blot analysis demonstrated that, in HEC-50 cells with high levels of ZEB1, restoration of miR-130b reduced ZEB1 mRNA and protein expression (Figures 5b and c). In contrast, in HEC-1 cells, which express low levels of ZEB1, miR-130b inhibition by transfection with anti-miR-130b increased ZEB1 mRNA and protein levels (Figures 5b and c). These results demonstrate that miR-130b directly interacts with ZEB1 mRNA and represses its expression.

To assess if miR-130b targets *ZEB1* 3′-UTR, reporter constructs containing either the WT *ZEB1* 3′-UTR or *ZEB1* 3′-UTR with mutation at the predicted miR-130b target sequence were co-transfected into HEC-50 cells together with miR-130b, control miRNA, anti-miR-130b or control anti-miRNA. Transduction of miR-130b caused marked inhibition of the WT *ZEB1* 3′-UTR, but had no effect on mutant *ZEB1* 3′-UTR (Figure 5d, compare lane 1 to lanes 2 and 3). In addition, miR-130b inhibition by anti-miR-130b substantially increased luciferase activities of WT *ZEB1* 3′-UTR compared with control anti-miRNA (Figure 5d, compare lane 4 to lane 5). These data together suggest that miR-130b downregulates ZEB1 expression in EC cells by destabilizing the ZEB1 mRNA as well as translational suppression.

To determine the functional effects of *miR-130b*-mediated ZEB1 suppression on cell invasion, a cell invasion assay was performed. We found that elevated expression of miR-130b in HEC-50 cells decreased cell invasion, and knockdown of miR-130b by anti-miR-130b in HEC-1 cells enhanced cell invasion (Figure 5e). In agreement with these findings, transfection of miR-130b, but not control miRNA, significantly reduced the mRNA levels of *BMI-1*, *Snail*, *KLF4*, *NANOG* and *MDR-1*, and increased mRNA expression of *E-cadherin* in HEC-50 cells (Figure 5f). Taken together, these results suggest that miR-130b directly targets *ZEB1*, and as a result reverses EMT-associated EC cell invasion.

The p53 GOF mutants stimulate EMT features through downregulation of *miR-130b*

To further define the involvement of miR-130b in mutant p53-stimulated ZEB1 expression and EMT characteristics, miR-130b was transfected into mutant p53 R175H-expressing HEC-50 cells. Reintroduction of miR-130b abolished the mRNA expression of *ZEB1*, *Snail*, *BMI-1*, *KLF4* and *NANOG*, restored *E-cadherin* expression and markedly diminished p53R175H-induced cell invasion (Figures 6a and b). To further confirm these results, we used HEC-1 cells expressing shRNA against *p53* or control cells to show that transfection with anti-miR-130b was capable of restoring the mRNA levels of *ZEB1*, *Snail*, *BMI-1*, *KLF4* and *NANOG*, as well as decrease the expression of *E-cadherin* and initiate sphere formation (Figures 6c and d). These data demonstrate that a p53 GOF mutant downregulates miR-130b expression, which results in activation of ZEB1, and its downstream pathway and contributes to the induction of EMT and increased EC cell invasion.

Clinical association of miR-130b expression with prognosis of EC patients

The expression of miR-130 was significantly reduced ($P=0.02$) in EC tissues (Figure 7a). Moreover, patients with higher expression levels of miR-130b survived longer ($P=0.05$) than patients with lower expression levels (Figure 7b).

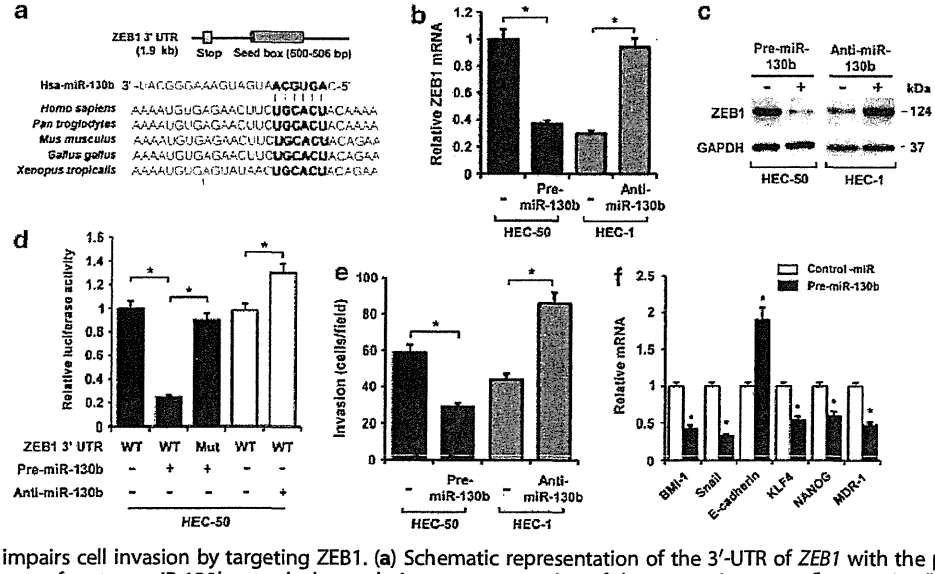

Figure 5. miR-130b impairs cell invasion by targeting ZEB1. (**a**) Schematic representation of the 3′-UTR of *ZEB1* with the predicted target site for miR-130b. Sequence of mature miR-130b reveals the evolutionary conservation of the target site across five species (below). (**b**, **c**, **e**) qRT–PCR (**b**, mean ± s.d.; n = 3; *$P<0.05$), western blotting (**c**) and cell invasion assay (**e**, mean ± s.d.; $n=3$; *$P<0.01$) of HEC-50 or HEC-1 cells transfected with pre-miR-130b or anti-miR-130b, respectively. (**d**) Reporter constructs containing either wild-type *ZEB1* 3′-UTR or *ZEB1* 3′-UTR with mutation at the predicted miR-130b target sequence were co-transfected into HEC-50 cells, along with miR-130b, control miRNA, anti-miR-130b or control anti-miRNA. Relative luciferase activity was assayed (mean ± s.d.; $n=3$; *$P<0.01$). (**f**) Expression of EMT and stemness markers in HEC-50 cells transfected with pre-130b or control miRNA were analyzed by qRT–PCR (mean ± s.d.; $n=4$; *$P<0.01$). All qPCR or luciferase values were normalized to GAPDH or Renilla activity, respectively.

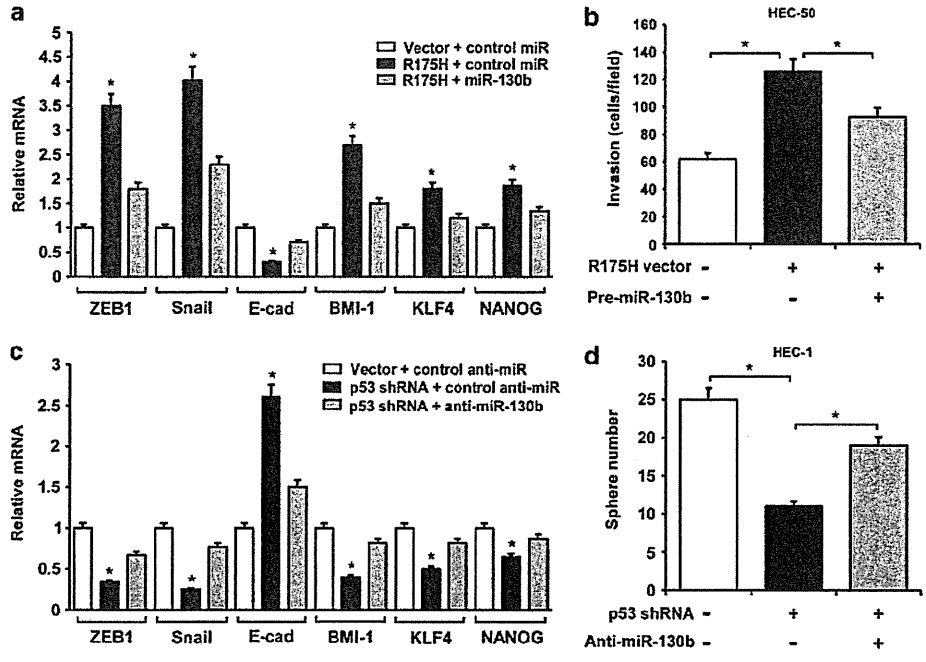

Figure 6. The p53 GOF mutants stimulate EMT features through downregulation of *miR-130b*. (**a**, **c**) qRT–PCR for EMT and stemness markers in HEC-50 cells (**a**) or in HEC-1 cells (**c**) expressing indicated constructs, and pre-miRNAs and anti-miRNAs (mean ± s.d.; $n=3$; $^*P<0.01$). (**b**) Invasion assay of HEC-50 cells expressing indicated vectors and pre-miRNAs (mean ± s.d.; $n=3$; $^*P<0.01$). (**d**) Sphere formation assay of HEC-1 cells expressing indicated vectors and anti-miRNAs (mean ± s.d.; $n=3$; $^*P<0.01$).

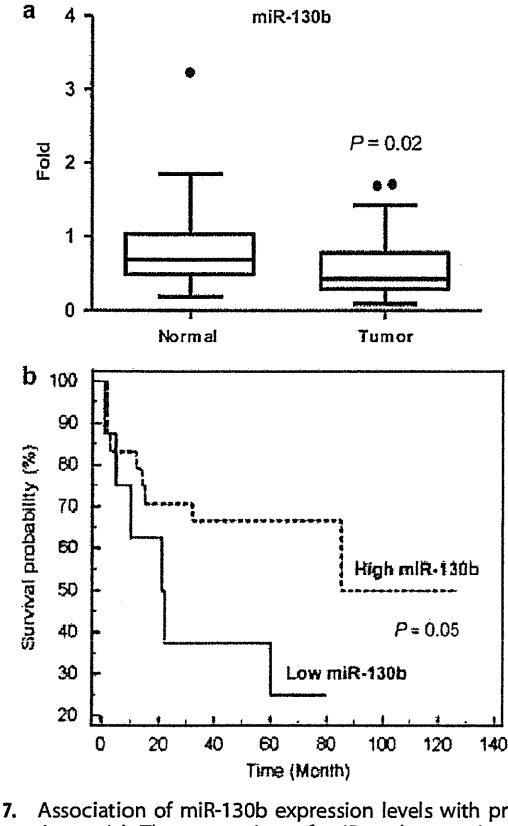

Figure 7. Association of miR-130b expression levels with prognosis of EC patients. (**a**) The expression of miR-130b was significantly reduced in EC patients compared with paired normal specimens. (**b**) Kaplan–Meier overall survival curve according to miR-130b expression levels in EC patients ($P=0.05$).

DISCUSSION

In this study, we have demonstrated that a GOF p53 mutant can induce EMT and increase invasive properties in EC cells by regulating a large set of EMT-associated genes. More importantly, we provide an underlying mechanism for p53 GOF-enhanced metastasis: mutant p53 can bind directly and transrepress the promoter of miR-130b, which is a specific inhibitor of ZEB1, leading to the upregulation of ZEB1 and subsequent activation of the E-cadherin suppressors BMI-1 and Snail (Figure 8). Notably, the effects of mutant p53 on EMT features and cell invasion can be partly abolished by restoration of miR-130b expression. Therefore, re-expression of miR-130b may inhibit tumor metastasis and progression, providing a potential therapeutic use in patients with EC.

Although upregulation of some miRNAs is reported in different tumors,[28] the widespread reduction of miRNA expression has been observed in a range of tumor types and is associated with increased metastatic activity.[29,30] Our findings suggest that the principal consequence of a p53 GOF mutant on miRNA expression is global repression. Thus, the correlation between a p53 GOF mutant and general repression of miRNAs suggests that the overall repression of miRNAs, especially those miRNAs with tumor suppressor function, is involved in p53 GOF mutant-stimulated EC metastasis and progression.

Several reports showed that p53 mutations not only target a set of genes that are different from those controlled by WT p53 such as c-myc,[31] but also differently modulate WT p53-target genes.[32–34] Mechanistically, the transcriptional effects of mutant p53 on WT p53-dependent promoters are mediated by at least four types of molecular interactions: (1) mutant p53 binds to WT p53-target gene promoters in the region distinct from WT p53 response elements.[35] (2) However, ChIP analysis reveals that mutant p53 physically associates with several promoters, such as EGR1[36] and MSP/MST-1,[37] which also interact with WT p53. (3) There is increasing evidence that both WT and mutant p53 can form a complex with sequence-specific transcription factors (Sp1,[38,39] NF-Y,[40,41] or other factors), and be recruited to binding

sites of those factors on chromatin, and modulate their transcriptional activities. (4) In addition, p53 mutants can display the WT p53 conformation due to a shift in the conformational equilibrium,[42] and thereby bind to the consensus sequence.[43] Our data indicate that both mutant p53 and WT p53 are present on the miR-130b promoter to regulate its expression, which is consistent with the reported finding of mutant p53. Future studies will be required to identify the transcriptional factors that specifically interact with mutant p53 and clarify the biological significance of their physical interaction.

Previous studies have shown that miR-130b is downregulated in hepatocellular carcinoma[44] and aggressive papillary thyroid carcinoma.[45] Furthermore, downregulation of miR-130b confers a multidrug-resistant phenotype in ovarian cancer cells.[46] However, other reports also suggest that overexpression of miR-130b in CD133 (+) liver tumor-initiating cells increased their self-renewal capacity and chemoresistance.[47] We found that the transcriptional inactivation of miR-130b by mutant p53 is required for p53 GOF mutant-mediated EMT and invasive phenotypes in EC cells. These results suggest that miR-130b may have a dual function as both a tumor suppressor and oncogene, depending on the cancer type and cellular context.

The HEC-50 cell line (p53 null) was derived from a patient with invasive grade 3 EC. Thus, the inverse relationship between miR-130b and mutant p53 expression observed in these cells, which is a genetic alteration frequently found in aggressive EC, suggests that miR-130b expression is likely reduced in later stages of tumor progression when mutant p53 becomes the main driver of invasion and metastasis.

Some evidence has suggested that miR-194 is a WT p53-responsive miRNA with potent anti-proliferative activity.[48] Interestingly, we have recently shown that miR-194 is able to inhibit EMT and cell invasion of EC cells by targeting oncogene *BMI-1*.[16] Our present study suggests that the expression of miR-194 is negatively regulated by mutant p53 in EC cells. The significance and clinical relevance of miR-130b were further demonstrated in EC patients (Figure 7). Therefore, we postulate that p53 mutations induce EMT and promote EC metastasis, at least in part, through regulating both miR-130b/ZEB1 and miR-194/BMI-1 signaling pathways (Figure 8). Our results open a possibility that multiple molecular mechanisms with different miRNAs are involved in p53 GOF mutant-dependent EMT programming. Further research is clearly needed to understand the mechanisms of p53 mutant-mediated EMT induction and the functional cross-talk between p53 signal pathways and miRNA-modulated gene expression profiles.

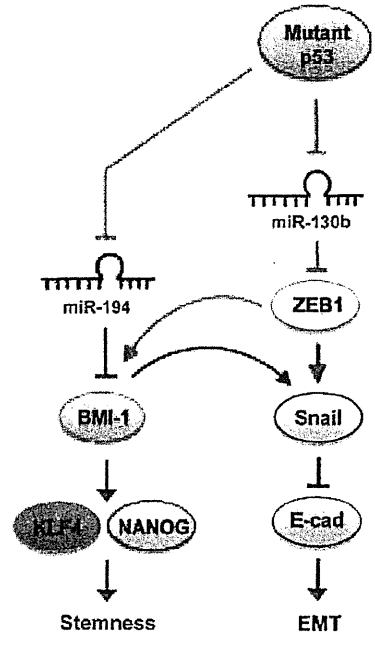

Figure 8. Schematic model indicating proposed mechanisms by which mutant p53 GOF induces EMT. Mutant p53 GOF induces EMT, through direct transrepression of miR-130b, an inhibitor of ZEB1, and subsequent activation of ZEB1-dependent signaling pathway.

MATERIALS AND METHODS

Cell culture

The EC cell lines HEC-50 and HEC-1 were cultured in Eagle's MEM medium (Sigma-Aldrich, Poole, UK) supplemented with 15% fetal bovine serum. The EC cell line HHUA was maintained in Ham's F12 medium containing 15% fetal bovine serum. All cell lines used were obtained from the RIKEN cell bank (Tsukuba, Japan).

Generation of cells overexpressing mutant p53 and knockdown of WT p53

HEC-50 cells at 80% confluency were transfected with vectors containing WT p53, mutant p53 (R273H, R175H, C135Y) or control vector as previously described.[49] The selection of stably transfected clones was achieved using a medium containing 400 μg/ml of G418 (Sigma-Aldrich) in the media. We knocked down p53 expression in HEC-1 and HHUA cells using a pSUPER-p53 vector or pSUPER control vector performed as previously described,[49] and selected cells with 1 μg/ml puromycin (Sigma-Aldrich).

Western blot analysis

Whole-cell lysates were obtained using the M-Per Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA). Proteins (40 μg) were separated on 10% SDS–PAGE and transferred to nitrocellulose membranes. Antigen–antibody complexes were detected using the electrochemiluminesence blotting analysis system (Amersham Pharmacia Biotech, London, UK). The following antibodies were used: mouse monoclonal anti-p53 (DO-7), goat polyclonal anti-ZEB1 (C-20), rabbit polyclonal anti-Twist (sc-81417), goat polyclonal anti-Snail (sc-10432), mouse monoclonal anti-p63 (sc-8431) and mouse monoclonal anti-GAPDH (sc-47724) (Santa Cruz, Santa Cruz, CA, USA). Rabbit polyclonal anti-BMI-1 (ab38295) (Abcam, Cambridge, MA, USA), rabbit polyclonal anti-E-cadherin (A01589), rabbit polyclonal anti-Vimentin antibody (A01189) (GenScript, Edison, NJ, USA) and mouse monoclonal anti-N-cadherin (BD Transduction, San Jose, CA, USA) antibodies were also used. Primary and secondary antibodies were used at 1:1000 and 1:5000 dilutions, respectively.

Molecular cloning of *miR-130b* promoter

The genomic region overlapping the p53-binding site was synthesized with forward primer (5′-ATACGCGTGGGTAAGGGACTCCTGAAGC-3′, *MluI*) and reverse primer (5′-CGAGATCTGAGACAAGGTTTCACCACGTT-3′, *BglII*), and sub-cloned into *MluI/BglII* sites of the pGL3-basic plasmid (Promega, Madison, WI, USA) to produce pGL3-miR-130b (WT pGL3-miR-130b). (The underlined primer sequence indicates the sites for enzyme *MluI*.) Correct insertion was confirmed by gel electrophoresis and DNA sequencing.

ChIP assay and qPCR analysis

The ChIP assay was performed using the Pierce Agarose ChIP kit (Pierce; Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol. Immunoprecipitation was carried out using mouse monoclonal p53 antibody (DO-7) or unrelated rabbit IgG as a negative control. To amplify the potential p53-binding site from nucleotides −3540 to −3520 in the promoter of miR-130b, real-time PCR was performed using the forward primer 5′-TTCATCCGTTCTCACACTGC-3′ and the reverse primer 5′-CAGGCTGGTCTCGAACTCC-3′. The human telomerase (*hTERT*) and *p21* genes were used as positive controls for mutant p53 and WT p53 binding, respectively.[21,25]

Patients and samples

The clinical sample cohort used for this study was approved by the Institutional Review Board of Stony Brook Medicine. Written informed

Table 1. Clinical features of 32 endometrial cancer patients used for microRNA analysis

Characteristics	*Frequency*	*Percentage (%)*
Mean age in years (range)	67 (49–86)	
Histology		
Endometrioid carcinoma	15	48.6
Serous carcinoma	8	25.7
Clear cell carcinoma	5	14.3
Malignant mixed mullerian tumor	3	8.6
Undifferentiated carcinoma	1	2.8
TNM stage		
I	18	60
II	1	2.8
III	5	14.3
IV	8	22.9
Survival (months)		
Mean (range)	52 (1–127)	
0–40	13	44.1
40–80	10	29.4
>80	9	26.5

consent was obtained from all participants involved in the study. Paraffin blocks containing formalin-fixed paraffin-embedded tissue samples were acquired from the archived collections of the Department of Pathology and used for subsequent analyses. The specimens were selected from samples obtained between 1995 and 2010, and each case had up to 15 years of clinical follow-up information. For RNA extraction, tumor samples and the adjacent normal tissues were obtained from 32 EC patients who underwent hysterectomy at Stony Brook Medicine, Stony Brook, New York. The characteristics of these patients are shown in Table 1.

RNA isolation

Using archived containing formalin-fixed paraffin-embedded tissues, separate areas of tumor and normal endometrium were identified from the corresponding hematoxylin and eosin-stained sections, and cores measuring 1.5 mm in diameter and 2 mm in length (~0.005 g) were extracted. The samples were then deparaffinized, hydrated and digested with proteinase K. Subsequently, total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA).

Real-time qRT–PCR analysis of miRNA expression

The miR-130b-specific primers and the internal control *RNU44* gene were purchased from Ambion (Applied Biosystems, Foster City, CA, USA). cDNA synthesis was performed using the High Capacity cDNA Synthesis Kit (Applied Biosystems). qRT–PCR was carried out on an Applied Biosystems 7500 Real time system (ABI 7500HT instrument) using the TaqMan Gene Expression Assay.

Statistical analysis

All experiments were performed in triplicate. All statistical analyses were performed using GraphPad Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS statistical software (SPSS Japan Inc., Tokyo, Japan). A Student's *t*-test was used for analysis, and statistical significance was defined as $P<0.05$. Gene expression ΔC_t values of miR-130b from each sample were calculated by normalizing them to the expression of the RNU44 internal control, and relative quantification values were plotted. The differences between tumor and normal tissues were analyzed using the Wilcoxon matched pairs test. Kaplan–Meier survival curves were generated to evaluate the correlation of miR-130b expression levels with survival rate.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We appreciate the critical review by Ms Sonya R Lorrain. This work was funded by a grant from Women's Health Educational System, a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan, Shanghai Municipal Natural Science Foundation (11ZR1430500) and by Stony Brook University Translational Research Laboratory Start-up fund (J Ju), R01CA155019 (J Ju) and R33CA147966 (J Ju). We thank Dr Zhujie Xu for his technical assistance.

REFERENCES

1 Kang Y, Massagué J. Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell* 2004; **118**: 277–279.
2 Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem* 2008; **283**: 14910–14914.
3 Li Y, VandenBoom 2nd TG, Kong D, Wang Z, Ali S, Philip PA *et al.* Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res* 2009; **69**: 6704–6712.
4 Meng Z, Fu X, Chen X, Zeng S, Tian Y, Jove R *et al.* miR-194 is a marker of hepatic epithelial cells and suppresses metastasis of liver cancer cells in mice. *Hepatology* 2010; **52**: 2148–2157.
5 Kim T, Veronese A, Pichiorri F, Lee TJ, Jeon YJ, Volinia S *et al.* p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. *J Exp Med* 2011; **208**: 875–883.
6 Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW *et al.* p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. *Nat Cell Biol* 2011; **13**: 317–323.
7 Rivlin N, Brosh R, Oren M, Rotter V. Mutations in the p53 tumor suppressor gene: important milestones at the various steps of tumorigenesis. *Genes Cancer* 2011; **2**: 466–474.
8 Scian MJ, Stagliano KE, Anderson MA, Hassan S, Bowman M, Miles MF *et al.* Tumor-derived p53 mutants induce NF-kappaB2 gene expression. *Mol Cell Biol* 2005; **25**: 10097–10110.
9 Xi Y, Formentini A, Chien M, Weir DB, Russo JJ, Ju J *et al.* Prognostic values of microRNAs in colorectal cancer. *Biomark Insights* 2006; **2**: 113–121.
10 Kogan-Sakin I, Tabach Y, Buganim Y, Molchadsky A, Solomon H, Madar S *et al.* Mutant p53 (R175H) upregulates Twist1 expression and promotes epithelial-mesenchymal transition in immortalized prostate cells. *Cell Death Differ* 2011; **18**: 271–281.
11 Fujisawa T, Watanabe J, Kamata Y, Hamano M, Hata H, Kuramoto H. VEGF expression and its reguration by p53 gene transfection in endometrial carcinoma cells. *Hum Cell* 2003; **16**: 47–54.
12 Singh A, Settleman J. EMT cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* 2010; **29**: 4741–4751.
13 Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A *et al.* The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol* 2009; **11**: 1487–1495.
14 Singh M, Spoelstra NS, Jean A, Howe E, Torkko KC, Clark HR *et al.* ZEB1 expression in type I vs type II endometrial cancers: a marker of aggressive disease. *Mod Pathol* 2008; **21**: 912–923.
15 Clapé C, Fritz V, Henriquet C, Apparailly F, Fernandez PL, Iborra F *et al.* miR-143 interferes with ERK5 signaling, and abrogates prostate cancer progression in mice. *PLoS One* 2009; **4**: e7542.
16 Dong P, Kaneuchi M, Watari H, Hamada J, Sudo S, Ju J *et al.* MicroRNA-194 inhibits epithelial to mesenchymal transition of endometrial cancer cells by targeting oncogene BMI-1. *Mol Cancer* 2011; **10**: 99.
17 Forrest AR, Kanamori-Katayama M, Tomaru Y, Lassmann T, Ninomiya N, Takahashi Y *et al.* Induction of microRNAs, mir-155, mir-222, mir-424 and mir-503, promotes monocytic differentiation through combinatorial regulation. *Leukemia* 2010; **24**: 460–466.
18 Bandres E, Bitarte N, Arias F, Agorreta J, Fortes P, Agirre X *et al.* MicroRNA-451 regulates macrophage migration inhibitory factor production and proliferation of gastrointestinal cancer cells. *Clin Cancer Res* 2009; **15**: 2281–2290.
19 Hurst DR, Edmonds MD, Scott GK, Benz CC, Vaidya KS, Welch DR. Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis. *Cancer Res* 2009; **69**: 1279–1283.
20 Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N *et al.* Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 2007; **26**: 731–743.
21 Song B, Wang Y, Kudo K, Gavin EJ, Xi Y, Ju J. miR-192 Regulates dihydrofolate reductase and cellular proliferation through the p53-microRNA circuit. *Clin Cancer Res* 2008; **14**: 8080–8086.

22 Castilla MÁ, Moreno-Bueno G, Romero-Pérez L, Van De Vijver K, Biscuola M, López-García MÁ *et al.* Micro-RNA signature of the epithelial-mesenchymal transition in endometrial carcinosarcoma. *J Pathol* 2011; **223**: 72–80.
23 Cochrane DR, Spoelstra NS, Howe EN, Nordeen SK, Richer JK. MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents. *Mol Cancer Ther* 2009; **8**: 1055–1066.
24 Xi Y, Shalgi R, Fodstad O, Pilpel Y, Ju J. Differentially regulated micro-RNAs and actively translated messenger RNA transcripts by tumor suppressor p53 in colon cancer. *Clin Cancer Res* 2006; **12**: 2014–2024.
25 Scian MJ, Stagliano KE, Deb D, Ellis MA, Carchman EH, Das A *et al.* Tumor-derived p53 mutants induce oncogenesis by transactivating growth-promoting genes. *Oncogene* 2004; **23**: 4430–4443.
26 Oren M, Rotter V. Mutant p53 gain-of-function in cancer. *Cold Spring Harb Perspect Biol* 2010; **2**: a001107.
27 Su X, Chakravarti D, Cho MS, Liu L, Gi YJ, Lin YL *et al.* TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. *Nature* 2010; **467**: 986–990.
28 Navon R, Wang H, Steinfeld I, Tsalenko A, Ben-Dor A, Yakhini Z. Novel rank-based statistical methods reveal microRNAs with differential expression in multiple cancer types. *PLoS One* 2009; **4**: e8003.
29 Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D *et al.* MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834–838.
30 Zhang L, Volinia S, Bonome T, Calin GA, Greshock J, Yang N *et al.* Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. *Proc Natl Acad Sci USA* 2008; **105**: 7004–7009.
31 Frazier MW, He X, Wang J, Gu Z, Cleveland JL, Zambetti GP. Activation of c-myc gene expression by tumor-derived p53 mutants requires a discrete C-terminal domain. *Mol Cell Biol* 1998; **18**: 3735–3743.
32 Sun Y, Cheung JM, Martel-Pelletier J, Pelletier JP, Wenger L, Altman RD *et al.* Wild type and mutant p53 differentially regulate the gene expression of human collagenase-3 (hMMP-13). *J Biol Chem* 2000; **275**: 11327–11332.
33 Chin KV, Ueda K, Pastan I, Gottesman MM. Modulation of activity of the promoter of the human MDR1 gene by Ras and p53. *Science* 1992; **255**: 459–462.
34 Kim E, Deppert W. Transcriptional activities of mutant p53: when mutations are more than a loss. *J Cell Biochem* 2004; **93**: 878–886.
35 Zalcenstein A, Stambolsky P, Weisz L, Müller M, Wallach D, Goncharov TM *et al.* Mutant p53 gain of function: repression of CD95(Fas/APO-1) gene expression by tumor-associated p53 mutants. *Oncogene* 2003; **22**: 5667–5676.
36 Weisz L, Zalcenstein A, Stambolsky P, Cohen Y, Goldfinger N, Oren M *et al.* Transactivation of the EGR1 gene contributes to mutant p53 gain of function. *Cancer Res* 2004; **64**: 8318–8327.
37 Zalcenstein A, Weisz L, Stambolsky P, Bar J, Rotter V, Oren M. Repression of the MSP/MST-1 gene contributes to the antiapoptotic gain of function of mutant p53. *Oncogene* 2006; **25**: 359–369.
38 Gualberto A, Baldwin Jr AS. p53 and Sp1 interact and cooperate in the tumor necrosis factor-induced transcriptional activation of the HIV-1 long terminal repeat. *J Biol Chem* 1995; **270**: 19680–19683.
39 Chicas A, Molina P, Bargonetti J. Mutant p53 forms a complex with Sp1 on HIV-LTR DNA. *Biochem Biophys Res Commun* 2000; **279**: 383–390.
40 Imbriano C, Gurtner A, Cocchiarella F, Di Agostino S, Basile V, Gostissa M *et al.* Direct p53 transcriptional repression: *in vivo* analysis of CCAAT-containing G2/M promoters. *Mol Cell Biol* 2005; **25**: 3737–5371.
41 Di Agostino S, Strano S, Emiliozzi V, Zerbini V, Mottolese M, Sacchi A *et al.* Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. *Cancer Cell* 2006; **10**: 191–202.
42 McLure KG, Lee PW. p53 DNA binding can be modulated by factors that alter the conformational equilibrium. *EMBO J* 1999; **18**: 763–770.
43 Zhang W, Funk WD, Wright WE, Shay JW, Deisseroth AB. Novel DNA binding of p53 mutants and their role in transcriptional activation. *Oncogene* 1993; **8**: 2555–2559.
44 Kutay H, Bai S, Datta J, Motiwala T, Pogribny I, Frankel W *et al.* Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. *J Cell Biochem* 2006; **99**: 671–678.
45 Yip L, Kelly L, Shuai Y, Armstrong MJ, Nikiforov YE, Carty SE *et al.* MicroRNA signature distinguishes the degree of aggressiveness of papillary thyroid carcinoma. *Ann Surg Oncol* 2011; **18**: 2035–2041.
46 Yang C, Cai J, Wang Q, Tang H, Cao J, Wu L *et al.* Epigenetic silencing of miR-130b in ovarian cancer promotes the development of multidrug resistance by targeting colony-stimulating factor 1. *Gynecol Oncol* 2012; **124**: 325–334.
47 Ma S, Tang KH, Chan YP, Lee TK, Kwan PS, Castilho A *et al.* miR-130b promotes CD133 (+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. *Cell Stem Cell* 2010; **7**: 694–707.
48 Braun CJ, Zhang X, Savelyeva I, Wolff S, Moll UM, Schepeler T *et al.* p53-responsive microRNAs 192 and 215 are capable of inducing cell cycle arrest. *Cancer Res* 2008; **68**: 10094–10104.
49 Dong P, Xu Z, Jia N, Li D, Feng Y. Elevated expression of p53 gain-of-function mutation R175H in endometrial cancer cells can increase the invasive phenotypes by activation of the EGFR/PI3K/AKT pathway. *Mol Cancer* 2009; **8**: 103–110.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

IJC
International Journal of Cancer

Incidence risk of cervical intraepithelial neoplasia 3 or more severe lesions is a function of human papillomavirus genotypes and severity of cytological and histological abnormalities in adult Japanese women

Masayoshi Hosaka[1], Hiromasa Fujita[2], Sharon JB Hanley[1,3], Takayuki Sasaki[2], Yozo Shirakawa[2], Mitsuharu Abiko[2], Masataka Kudo[1], Masanori Kaneuchi[1], Hidemichi Watari[1], Kohkichi Kikuchi[4] and Noriaki Sakuragi[1]

[1] Department of Obstetrics and Gynecology, Hokkaido University School of Medicine, Sapporo, Japan
[2] Cytoscreening Center, Hokkaido Cancer Society, Sapporo, Japan
[3] Department of Public Health, Hokkaido University School of Medicine, Sapporo, Japan
[4] Hokkaido Cancer Society, Sapporo, Japan

We examined incidence probabilities of cervical intraepithelial neoplasia 3 (CIN3) or more severe lesions (CIN3+) in 1,467 adult Japanese women with abnormal cytology in relation to seven common human papillomavirus (HPV) infections (16/18/31/33/35/52/58) between April 2000 and March 2008. Sixty-seven patients with multiple HPV infection were excluded from the risk factor analysis. Incidence of CIN3+ in 1,400 patients including 68 with ASCUS, 969 with low grade squamous intraepithelial lesion (LSIL), 132 with HSIL without histology-proven CIN2 (HSIL/CIN2(−)) and 231 with HSIL with histology-proven CIN2 (HSIL/CIN2(+)) was investigated. In both high grade squamous intraepithelial lesion (HSIL)/CIN2(−) and HSIL/CIN2(+), HPV16/18/33 was associated with a significantly earlier and higher incidence of CIN3+ than HPV31/35/52/58 ($p = 0.049$ and $p = 0.0060$, respectively). This association was also observed in LSIL ($p = 0.0002$). The 1-year cumulative incidence rate (CIR) of CIN3+ in HSIL/CIN2(−) and HSIL/CIN2(+) according to HPV genotypes (16/18/33 *vs.* 31/35/52/58) were 27.1% *vs.* 7.5% and 46.6% *vs.* 19.2%, respectively. In contrast, progression of HSIL/CIN2(+) to CIN3+ was infrequent when HPV DNA was undetected: 0% of 1-year CIR and 8.1% of 5-year CIR. All cervical cancer occurred in HSIL cases of seven high-risk HPVs (11/198) but not in cases of other HPV or undetectable/negative-HPV (0/165) ($p = 0.0013$). In conclusion, incidence of CIN3+ depends on HPV genotypes, severity of cytological abnormalities and histology of CIN2. HSIL/CIN2(+) associated with HPV16/18/33 may justify early therapeutic intervention, while HSIL/CIN2(−) harboring these HPV genotypes needs close observation to detect incidence of CIN3+. A therapeutic intervention is not indicated for CIN2 without HPV DNA.

Cervical cancer is the third most commonly diagnosed cancer and the fourth most frequent cause of cancer death in women worldwide, with approximately 529,000 new cases and 275,100 deaths in 2008.[1] Human papillomavirus (HPV) DNA is detected in most cervical cancers.[2] More than 100 types of HPV have been identified, of which approximately 40 can infect the genital area including the cervix. Among them, 13–15 oncogenic types are thought to be responsible for most cases of cervical cancer.[2–4] Cervical intraepithelial neoplasia (CIN) is a premalignant cervical disease caused by HPV infection. CIN contains a spectrum of lesions with different severity. Low-grade disease, CIN1, has minimal potential for progressing to cervical cancer. CIN2 and CIN3 are regarded as high-grade lesions. In the 2006 ASCCP guidelines for the management of women with CIN or adenocarcinoma *in situ*, treatment of CIN2/3 is recommended for adult women but not for adolescents.[5] Because of the high risk of progression for both CIN2/3, and poor reproducibility of histological discrimination between the two CIN grades, CIN2/3 are managed in the same way. According to the guidelines, observation of CIN2/3 with sequential cytology and colposcopy is unacceptable, except in special circumstances such as in adolescent and young women, and treatment of CIN2/3 is not indicated during pregnancy. In adolescent and young women, either treatment or observation of CIN2 and CIN3 is acceptable, provided that the colposcopy result is satisfactory. When CIN2 is specified, observation is preferred. When CIN3 or adenocarcinoma *in situ* is specified, or the colposcopy result is unsatisfactory, treatment is recommended. In

Key words: cervical intraepithelial neoplasia 2 (CIN2), progression, cervical cancer, human papillomavirus, genotyping
Grant sponsor: Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan
DOI: 10.1002/ijc.27680
History: Received 16 Jan 2012; Accepted 23 May 2012; Online 22 Jun 2012
Correspondence to: Noriaki Sakuragi, Department of Obstetrics and Gynecology, Hokkaido University School of Medicine, Sapporo, Japan, Fax: +81-11-706-7711, E-mail: sakuragi@med.hokudai.ac.jp

What's new?

HPV causes a precancerous condition called cervical intraepithelial neoplasia, or CIN. Women with CIN could be on a path to cancer, but accurate assessment of the stage of CIN is important for knowing whether to treat it. Low grade CIN is unlikely to become cancer, and need not be treated, but high grade CIN is harder to assess by cytology alone. Could HPV genotyping help with that assessment? The authors examined HPV genotypes in 1400 Japanese women with abnormal cytology and found that HPV genotyping can predict the risk of more severe lesions developing in women with abnormal cervical cytology and CIN.

Japan, CIN2 lesions are usually followed up with repeated cytology and colposcopy in adult women as well as in adolescent and young women. This practice seems to be acceptable because around 40% of CIN2 regress spontaneously[6] and conization is associated with adverse obstetrical outcomes.[7] Regarding the natural history of CIN2, Ostör reported, on the basis of a literature review, that 20% of CIN2 progresses to CIN3 and 5% of CIN2 progresses to invasive cancer.[8] As far as we could determine in our literature review, only a limited amount of data are available that report the HPV-related progression rate of CIN2 to CIN3 or more severe lesions (CIN3+). Meanwhile, Kataja *et al.* also reported that 20% of HPV-positive CIN2 progressed to CIN3 over a mean follow-up period of 45 months.[9] Therefore, it is crucial to elucidate whether or not HPV genotyping is useful to screen patients with CIN2 for higher progression risk who would benefit from more intensive follow-up or therapeutic intervention. In Japan, this is of concern because the incidence of carcinoma *in situ* (CIS) and that of invasive cervical cancer are rapidly increasing among women aged ≤39 years.[10] The oncogenic potential of HPV has been suggested to differ according to genotype.[11] Persistent infection by oncogenic HPV, especially Type 16, has been shown to be associated with progression of a CIN lesion to a higher grade CIN.[12] Of all oncogenic HPV types, Types 16 and 18 are the two most frequently detected HPVs in cervical cancer.[13] The association between higher incidence of CIN3 in normal cytology or low-grade abnormal cytology (ASCUS/LSIL) with HPV 16 and 18 has been previously reported.[14,15] In contrast, Matsumoto *et al.* found that the incidence of CIN3 in mild abnormal cytology, and low-grade and moderate-grade CINs (LSIL/CIN1-2) for HPV16, −18, −31, −33, −35, −52 and −58 was significantly higher than other high- and low-risk types. However, they did not find a difference in CIN3 incidence probability among these seven oncogenic HPV genotypes.[16] Consequently, in this study we aimed to clarify whether or not HPV genotyping is a useful tool to discriminate CIN2 patients who are at a higher risk for progression to CIN3+.

Material and Methods

Study design

The study protocol was evaluated and approved by the institutional review board of Hokkaido University Hospital. Between April 2000 and March 2008, a total of 1,467 women with abnormal cytology (ASCUS, LSIL and HSIL) and no CIN3+ underwent HPV genotyping at the Cytology Center of Hokkaido Cancer Society. Among them, we found 67 cases with multiple HPV-type infection. The age distribution of the women was as follows: 59 women aged ≤29, 254 aged 30–39, 431 aged 40–49, 385 aged 50–59, 203 aged 60–69 and 68 aged ≥70. KK and HF provided histological diagnosis of biopsied specimens. The age of the women according to cytological abnormality was 50 years at median (range 22–78) for ASCUS, 49 (21–82) for LSIL and 46 (23–84) for HSIL. The follow-up period at median was 26 months and the range was 1–108 months.

Cervical cells were collected using a wooden extended-tip spatula, and cytology specimens were prepared using a conventional, non-liquid-based method. When the presence of CIN3+ was suspected, a biopsy was done under colposcopy. LSIL and HSIL without evidence of CIN3+ were followed up with repeated cytology at 3-month intervals and ASCUS at 6-month intervals. Each woman who needed to be followed up was informed by mail about the time of the next visit. If the woman did not appear for the next cytology, she was contacted by mail or by phone to encourage her to reschedule a visit to the Cytology Center. Histological evidence for the presence of CIN3+ was used as the endpoint of the study. Women who showed no abnormal cytological findings suggesting CIN in two consecutive visits exited the follow-up and returned for routine cytoscreening.

HPV genotyping

After written informed consent had been obtained, DNA was extracted from cells in the cytology specimens as previously described.[17] We examined the seven types of oncogenic HPV (16, 18, 31, 33, 35, 52, 58) that are most frequently found in cervical cancer in Japan.[18] HPV45 is quite rare in Japan, and, therefore, we did not perform genotyping for this HPV type. We used multiplex PCR with type-specific primers designed for the E6 and E7 regions[19] for five of the seven types: Types 16, 18, 31, 52 and 58. Forward primer at E6 was as follows: Type 16, 5′-TGTATGTCTTGTTGCAGATCATCA-3′; Type 18, 5′-CCATTCGTGCTGCAACCG-3′; Type 31, 5′-GTATG-GAACAACATTAGAAAAATTGAC-3′; Type 52, 5′-CTAT-TAGATGTATAATTTGTCAAACG-3′; and Type 58, 5′-ATGTAAAGTGTGCTTACGATTGC-3′. The combined use of these forward primers with the consensus reverse primer at E7 (E7CR3: 5′-TGAGCTGTCGCTTAATTGCTC-3′) enabled the identification of each HPV type from the size of the PCR amplicons after agarose gel electrophoresis. The PCR conditions were as follows: the mixture was denatured for 5 min at 95°C, then alternately cycled for denaturation at 95°C, annealing at 55°C and extension at 72°C for 30 sec

periods over 35 cycles, followed by a final extension at 72°C for 7 min using a GeneAmp 9600 PCR System (Applied Biosystems, Foster City, CA).

We examined Types 33 and 35 by using the PCR-RFLP method. When type-specific PCR was negative, we performed PCR using two types of consensus primer sets designed for the E6 and E7 regions to amplify HPV DNA. The first primer set included pU1-M (forward: 5′-TGTCAAAAACCG TTGTGTCC-3′) and pU2-R (reverse: 5′-GAGCTGTCGCTTA ATTGCTC-3′) reported by Fujinaga *et al.*[19] The second primer set was E6CF4 (forward: 5′-ATTCTGTGTATGGAGA AACATTAGAA-3′) and E7CR3 (reverse: 5′-TGAGCTGTCG CTTAATTGCTC-3′) reported by Yamaguchi *et al.*[17] We performed PCR first with pU-1M and pU-2R. The PCR conditions were as follows: denaturation for 30 sec at 94°C, annealing for 2 min at 55°C and extension for 30 sec at 72°C for 30 cycles followed by a final extension at 72°C for 7 min. We digested the amplicon with Ava I and Ava II. When the amplicon was cut by Ava I, the detected HPV was Type 35; when it was cut by Ava II, it was Type 33. When PCR using pU-1M and pU-2R gave negative results, we then performed PCR using E6CF4 and E7CR3 to confirm the negative results.

We classified the HPV status into three groups. We grouped the seven oncogenic HPV types (16, 18, 31, 33, 35, 52, 58) as prevalent-type (pt)-HPV. We designated any other high-risk HPV DNA for which we could not determine the genotype as un-genotyped (ug)-HPV. If we did not find any HPV DNA, we classified the case as having undetectable/negative-HPV (ud/n-HPV).

Statistical analysis

The Kaplan–Meier method was used to calculate the cumulative incidence rate (CIR) with pathological diagnosis of CIN3+ as the event. Women who were lost to follow-up and who exited the follow-up because of regression were censored at their last visit. The time to progression to or incidence of CIN3+ was compared between the categorized groups using the Mann–Whitney *U*-test. The difference between the cumulative incidence curves was compared using the log-rank test. Hazard risk for incidence of CIN3+ was evaluated using Cox regression analysis. Statistical significance was set at $p < 0.05$. All statistical analyses were carried out using the StatView J (version 5.0, SAS Institute Japan, Tokyo, Japan) software package.

Results

Distribution of HPV genotypes

Regarding the 1,467 cases that included multiple HPV infection, HPV DNA, either pt-HPV or ug-HPV, was detected in 43.4% (637/1,467) of the cases. Multiple HPV DNA were detected in 67 cases (two types of HPV DNA in 65 cases and three types in two cases). The overall detection rates of HPV DNA in ASCUS, LSIL and HSIL were 32.4%, 33.6% and 70.9%, respectively. Regarding each HPV genotype, HPV16 was most frequently detected (11.7%), followed by HPV52 (10.0%), −58 (8.0%), −31 (4.4%), −18 (2.4%), −33 (0.9%) and −35 (0.5%). Each genotype in double and triple HPV infections was counted separately two or three times. ug-HPV was detected in 10.2% of the cases.

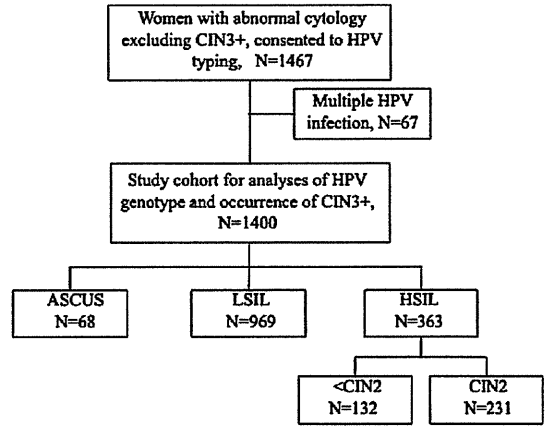

Figure 1. Study design.

HPV genotypes and CIN3+ incidence

To minimize a possible dilution effect when comparing risk factor profiles, we excluded the 67 cases with multiple HPV infection from the risk factor analyses. As a result, the analytical cohort included 1,400 women composed of 68 ASCUS cases, 969 LSIL cases and 363 HSIL cases (Fig. 1). The HSIL cases included 132 cases without histology proven CIN2 (HSIL/CIN2(−)) and 231 cases with histology-proven CIN2 (HSIL/CIN2(+)). The 5-year CIRs of CIN3+ in ASCUS, LSIL, HSIL/CIN2(−) and HSIL/CIN2(+) were 0%, 5.7%, 23.9% and 50.6%, respectively (Table 1). Table 2 shows the result of a Cox multivariate regression analysis of risk factors for incidence of CIN3+. Younger age (≤39 years), severe cytological abnormality (HSIL) and each high-risk HPV type were independent risk factors for the incidence of CIN3+. When we compared the HRs between the two groups of seven pt-HPVs that were divided sequentially in order of the HR magnitude obtained by the Cox multivariate regression analysis, we found that the difference was largest and most statistically significant between the subgroup containing HPV16/18/33 and the subgroup containing HPV31/35/52/58 (HR: 1.6, 95% CI: 1.1–2.2, $p = 0.0066$) (Fig. 2).

Among 969 cases of LSIL, 23 developed CIN3+ and no cervical cancer was observed. The incidence ratio of CIN3 by HPV status was 17/89 (19.1%) in HPV16/18/33, and 5/118 (4.2%) in HPV31/35/52/58. There was only one CIN3 case among 762 ug-HPV or ud/n-HPV cases. CIN3 occurred in the HPV16/18/33 subgroup at a significantly higher CIR than in the HPV31/35/52/58 subgroup in LSIL cases ($p = 0.0002$) (Fig. 3).

Among 363 HSIL cases either with or without histology-proven CIN2, there were 140 CIN3 cases and 11 cases of

Table 1. HPV status and incidence/progression rates to CIN3+ from LSIL, HSIL without histology-proven CIN2, and HSIL with histology-proven CIN2

Cytology/histology and HPV status	No. of cases	No. of incidences of CIN3/CC[1]	Cumulative incidence rate (%)		
			1 Year	3 Year	5 Year
LSIL					
HPV (16/18/33)	89	17/0	7.6	18.0	25.1
HPV (31/35/52/58)	118	5/0	1.0	1.0	3.6
ug-HPV[2]	93	0/0	0	0	0
ud/n-HPV[3]	669	1/0	0	0.1	0.1
total	969	23/0	0.8	2.9	5.7
HSIL without CIN2					
HPV (16/18/33)	20	7/3	27.1	47.0	47.0
HPV (31/35/52/58)	40	11/0	7.5	13.6	28.0
ug-HPV[2]	28	7/0	3.6	20.3	26.0
ud/n-HPV[3]	44	4/0	0	3.2	3.2
total	132	29/3	6.9	17.2	23.9
HSIL with histology-proven CIN2					
HPV (16/18/33)	45	30/4	46.6	69.0	72.1
HPV (31/35/52/58)	93	60/4	19.2	41.6	60.6
ug-HPV[2]	24	17/0	0	26.1	49.6
ud/n-HPV[3]	69	4/0	0	3.0	8.1
total	231	111/8	16.5	34.9	50.6

[1]Cervical cancer. [2]Un-genotyped HPV. [3]Undetectable/negative HPV.

Table 2. Multivariate Cox regression model of risk factors for progression to CIN3+ among 1400 women with ASCUS/LSIL/HSIL

Risk factor	Multivariate analysis	
	Hazard ratio (95% C.I.)[1]	*p*-Value
Age		
over 39 vs. 39 or under	1.5 (1.1–2.1)	0.0095
Cytology		
ASCUS/LSIL vs. HSIL	8.5 (5.4–72.5)	<0.0001
HPV genotype		
HPV16	22.2 (10.8–45.5)	<0.0001
HPV18	12.8 (4.9–33.3)	<0.0001
HPV31	9.3 (4.0–20.8)	<0.0001
HPV33	33.3 (11.0–100.0)	<0.0001
HPV35	N.A.	
HPV52	9.8 (4.7–20.4)	<0.0001
HPV58	8.1 (3.8–17.2)	<0.0001
ug-HPV[2]	5.1 (2.3–11.0)	<0.0001

[1]95% confidence interval. [2]Un-genotyped HPV, N.A.: not available.

cervical cancer, which included 10 microinvasive and one small (<2 cm) stage IB1 invasive cancer during the follow-up period. The incidence rate of CIN3+ in HSIL cases was higher in patients with pt-HPV and ug-HPV than in patients with ud/n-HPV ($p < 0.0001$). Management of CIN2 was our special concern and we wanted to clarify whether HPV genotype is related to progression of CIN2 to CIN3+ or not. Among 231 HSIL/CIN2(+) cases, 119 progressed to CIN3+, 92 regressed, 14 showed no change and 6 were lost to follow-up. The baseline age distribution of 119 patients with CIN2 who developed CIN3+ was 23–77 years (median: 44 years). The number of women aged 39 years or under was 77 out of 231 (33.3%) patients with CIN2. There were 35 incidences of CIN3+ and one incidence of invasive cancer among them. We also observed three incidences of CIN3 in 11 women aged 29 years or under. We compared the risk of progression to CIN3+ for HSIL/CIN2(+) cases between HPV16/18/33 and HPV31/35/52/58. CIN3+ occurred in the HPV16/18/33 subgroup significantly earlier and at a higher CIR than in the HPV31/35/52/58 subgroup with a *p*-value of 0.0060 (Fig. 4). The 1-year CIR from CIN2 to CIN3+ was as high as 46.6% for HPV16/18/33 compared with 19.2% for HPV31/35/52/58 and 0% for ug-HPV (Table 2). Median time for progression from CIN2 to CIN3+ in HPV16/18/33, HPV31/35/52/58 and ug-HPV was 12 months, 28.5 months and 48 months. The HPV16/18/33 subgroup developed CIN3+ significantly earlier than the HPV31/35/52/58 subgroup ($p = 0.0040$). Regression was observed in 39.8% of HSIL/CIN2(+) cases. The regression ratio by HPV status was 7/45 (15.6%) in HPV16/18/33 and 21/93 (22.6%) in HPV31/35/52/58. The regression ratio 61/69 (88.4%) in undetectable/negative-HPV cases was significantly higher than that in HPV16/18/33 and HPV31/35/52/58 ($p < 0.0001$).

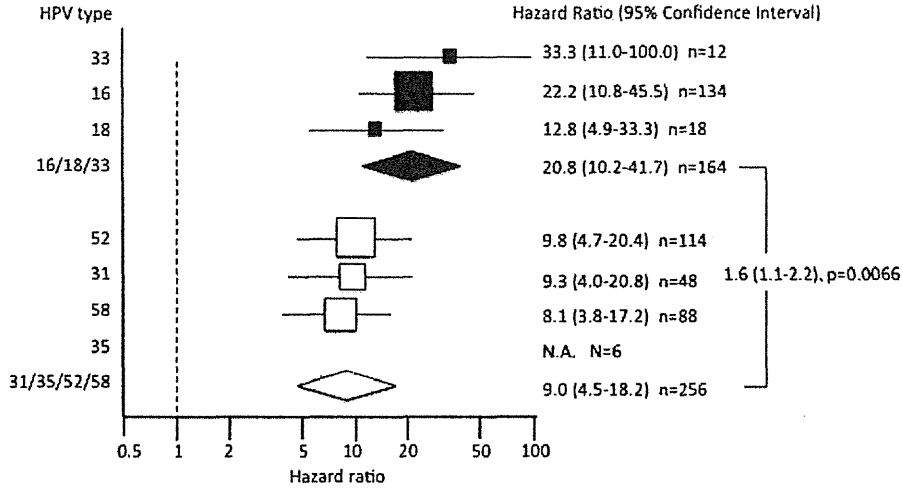

Figure 2. Incidence risk of CIN3+ according to high-risk HPV types.

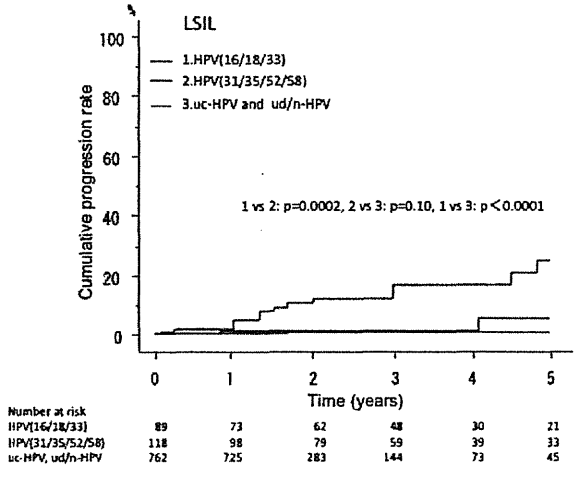

Figure 3. Cumulative incidence curves to CIN3+ in LSIL cases by HPV status. The seven prevalent HPV types were stratified into HPV16/18/33 and HPV31/35/52/58.

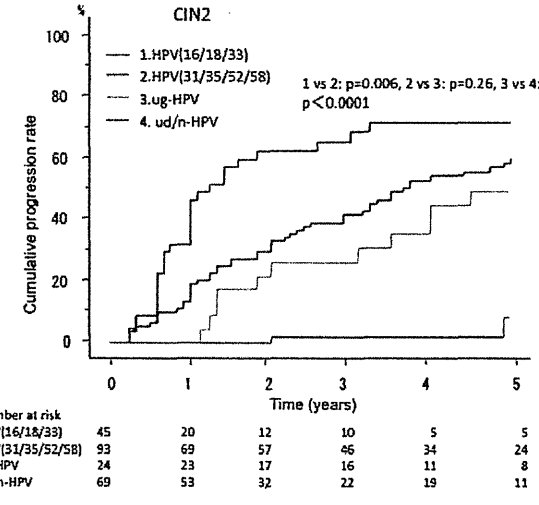

Figure 4. Cumulative progression curves to CIN3+ from HSIL with histology-proven CIN2 by HPV status. The seven prevalent HPV types were stratified into HPV16/18/33 and HPV31/35/52/58.

Even in the absence of histological evidence for CIN2, we frequently observed incidence of CIN3+ in HSIL cases with HPV16/18/33, which showed increased 1- and 5-year CIRs at 27.1% and 47.0%, respectively. There was a significant relationship between incidence of CIN3+ and high-risk HPV subgroups in HSIL/CIN2(−) ($p = 0.049$).

Cervical cancer occurred only in HSIL cases. All cervical cancer occurred in cases of seven high-risk HPV types (11/198) but not in cases of other HPV types or undetectable/negative-HPV (0/165) ($p = 0.0013$). The HPV types associated with cervical cancer were HPV16 (4/50), HPV18 (3/13), HPV52 (2/58) and HPV58 (2/50). Notably, three cases of cervical cancer occurred in HSIL/CIN2(−) when HPV16/18 was positive (one with HPV16 and two with HPV18). HSIL with HPV16/18, even in the absence of histological diagnosis of CIN2, should be observed carefully. The ratio of cervical cancer incidence in cases of HPV16 and 18 (7/63 = 11.1%) was higher than in cases of the other five HPV genotypes (4/135 = 3.7%). There was a statistically significant difference between the two groups ($p = 0.026$).

Discussion

The distribution of oncogenic HPV genotypes in female population-based samples varies among different geographical regions, although HPV16 is the most common type in most continents.[20] It is notable that HPV45 is quite rare in Japan. Onuki *et al.* reported no cases of Type 45 among 342

patients with normal cytology, 281 patients with CIN1, 291 with CIN2/3 or 131 with invasive cervical cancers.[18] Similar findings were reported in Korea and China, where HPV33, −52 and −58 are more common.[18,21,22] Therefore, we did not genotype for HPV45, and the present study is limited by a lack of data on HPV45. Another potential limitation of this study is that the study population did not include women with normal cytology.

The detection rate of high-risk HPV determined by PCR reported in the literature is 17.9%[23] and 53%[24] for ASCUS, 33.3%,[25] 55.5%[24] and 72.4%[26] for LSIL, and 57.1%,[25] 81.7%[26] and 87.5%[24] for HSIL. The detection rate in this study, 33.6% for LSIL and 70.9% for HSIL, seems to be relatively low. The detection rate of high-risk HPV can vary because of differences in the PCR method used[27] and the definition of high-risk HPV. For instance, regarding LSIL cases, a meta-analysis in Japan showed a detection rate of high-risk HPV as high as 72.4%.[26] However, the definition of high-risk HPV in that meta-analysis included 18 HPV types (16/18/26/31/33/35/39/45/51/52/53/56/58/59/66/68/73/82). If the definition is limited to seven prevalent types (16/18/31/33/35/52/58), the detection rate decreases to 43.2%, which is not largely different from the rate in this study. We focused on the seven prevalent types of high-risk HPV in Japan.[18] Our results demonstrated that the presence of a subgroup of prevalent HPV types (16/18/33) indicates a higher risk of incidence/progression to high-grade CIN and cervical cancer than other HPV types (31/35/52/58), which is contrary to a recently published paper from Japan.[16] Matsumoto *et al.* reported that these seven oncogenic HPV types have a higher risk for the incidence of CIN3+ in LSIL/CIN1-2 lesions compared with other oncogenic HPV types in Japan.[16] They observed that the occurrence risk of CIN3 in LSIL cases for HPV16 and 18 was not different from that of other oncogenic HPV types (31, 33, 35, 52, 58). On the contrary, Khan *et al.* reported that HPV16 and −18 in negative, equivocal and mildly abnormal cytology were associated with a higher risk for incidence of CIN3+.[14] The results of the present study suggested that there are differences in risk of earlier incidence of CIN3+ among the high-risk oncogenic HPV types, which indicates that some grouping of oncogenic HPV types might be useful for more effective CIN2 management. We found that HPV16/18/33 were associated with higher risk of CIN3+. However, the actual number of women with HPV18 or −33 infection was only 35 (2.4% of 1,467 cases) and 13 (0.9%), respectively. A low detection rate of these two types of HPV has been previously reported in Japanese women. The reported HPV18 detection rate was 0%,[28] 4.0%[16] and 4.8%[29] in low-grade CIN/LSIL and 2.2%,[29] 2.8%[28] and 4.7%[16] in high-grade CIN/HSIL. The HPV33 detection rate was 1.2%,[28] 1.4%[29] and 1.9%[16] in low-grade CIN/LSIL and 0.7%,[29] 4.1%[16] and 5.6%[28] in high-grade CIN/HSIL. Therefore, our HPV type-specific detection rates correspond with those of previous studies. Because the number of positive cases for each high-risk HPV type was relatively small, the 95% confidence intervals of the CIN3+ incidence HRs were rather wide and comparisons of the HRs between each HPV type hardly found statistical significance. For this reason, we conducted a comparison by categorizing the HPV types into two groups. By doing so, the 95% CIs for the two groups became narrower and we could find a statistically significant difference in the HRs between the groups, as shown in Figure 2. The similar method was used in a recent study.[16]

The role of multiple HPV infection in the progression of CIN and cervical carcinogenesis has been a matter of concern. However, because the effect of multiple HPV infection has not been fully elucidated, we excluded women with multiple HPV type infection from the analyses to avoid a possible dilution effect in the risk factor analysis. Some investigators found a positive relationship between multiple HPV type infection and progression of CIN.[30] However, other investigators could not find a relationship between multiple HPV type infection and CIN or cervical cancer.[16,31] In the current study, multiple high-risk HPV type infection was not related to progression to CIN3+ (data are not shown).

CIN2 diagnosis has poor reproducibility. As such, we need to have a prudent treatment plan for this diagnosis. In Japan, we usually employ expectant management for CIN2 using cytology, colposcopy and biopsy. On the other hand, some other countries often manage CIN2 and CIN3 similarly. Following the ASCCP 2006 consensus guidelines,[5] the American College of Obstetricians and Gynecologists (ACOG) addressed recommendations for the management of CIN, in which non-pregnant women 21 years and older with CIN2 or CIN3 can be treated by either excision or ablation.[32] It was also recommended that for adolescents and pregnant women with CIN2 or CIN3, either observation with colposcopy, or treatment with excision/ablation is acceptable. In treating women of reproductive age with conization, we need to recognize that there exist post-surgical cervical stenosis[33] and obstetrical risks[7] after this intervention. We would be able to construct an individualized algorithm for CIN2 management if the risk of progression to CIN3+ from CIN2 could be predicted by HPV genotyping. The present study has shown that the risk of early progression from CIN2 to CIN3+ is related to HPV genotype. The 1-year cumulative progression rate of CIN2 with HPV16/18/33 was as high as 46.6%, which is remarkable compared with HPV31/35/52/58 and ug-HPV at the rates of 19.2% and 0%, respectively. Because of the high risk of progression to CIN3+ in HPV16/18/33-positive CIN2, this lesion may require earlier surgical intervention than other CIN2. Two additional points are to be noted: (1) any high-risk HPV-positive CIN2 is associated with a significant ultimate risk of CIN3+ at longer observation intervals irrespective of the HPV genotype found; (2) HSIL with HPV16/18/33 is associated with a significant incidence risk of CIN3+ even if a colposcopy-guided biopsy did not reveal histological evidence of CIN2, in which the 1-year CIR of CIN3+ was as high as 27.1%. The former observation seems to correspond to a previous report that the risk of

cervical cancer for any given high-risk HPV type is not different from others. Munoz *et al.* showed that the type-specific odds ratio for cervical cancer seemed different among various high-risk HPV types, but the difference was not statistically significant because of the wide 95% confidence intervals. Because cervical cancer is the ultimate form of progression of cervical epithelial neoplasia, our observation does not contradict their findings, but we would like to emphasize the possibility that there may be type-specific differences in progression speed from mild intraepithelial lesions to higher grade intraepithelial lesions and cervical cancer.

Regarding invasive cervical cancer, the HSIL cases with HPV16/18 developed lesions more frequently compared with the other HPV genotypes in this study. This observation corresponds to the notion proposed by Franceschi and Clifford, which was based on the HPV type-specific prevalence reported in their three systematic reviews.[34] They showed that only HPV16 and −18 are more frequently found in invasive cervical squamous cell carcinoma (SCC) compared with HSIL and LSIL, and HPV33 and −45 are found at approximately equal frequencies in SCC and LSIL. It is likely that the oncogenic HPVs possess similar potency to make cellular changes to HSIL from LSIL, but HPV16 and −18 have a more potent oncogenic property to convert CIN to invasive cancer compared with other oncogenic HPVs. Therefore, although the current study showed that HPV16/18/33 had a higher risk for CIN3+ occurrence, the presence of HPV16 and −18 would be the most serious risk factor for CIN2 progression to invasive cancer.

The proportion of young women included in this study was rather small. Women aged 39 years or under accounted for 22.4% of the cohort. In 1982, the Japanese Government enacted the Health and Medical Service Law for the Aged, which then recommended annual screening for women aged 30 years and over. In 2004, the law was revised, and biannual screening for women aged 20 years and over was initiated. The screening rate for cervical cancer in Japan as of 2010 is reported to be only 24.3%, which is very low compared with other developed countries.[35] Furthermore, only a few young women participate in cervical cancer screening programs: 10.2% of women aged 20–24 years, and 24.2% of those aged 25–29 years,[36] which may explain the increase in incidence and mortality among young women.[10] These situations explain the small proportion (22.4%) of young women aged 39 years or under in this study cohort. Although it is difficult to draw definitive conclusions owing to the small number of young patients in our study, therapeutic intervention may be justified for young women with CIN2 who are positive for HPV16/18/33 because we observed 35 incidences (46.8%) of CIN3 and one cervical cancer in 77 women aged 39 years and under with this condition.

In conclusion, the pace and the cumulative rate of incidence of CIN3+ in women with cytological abnormalities and CIN2 depend on HPV genotype. HPV genotyping is a useful predictor of the risk of CIN3+ incidence in women with abnormal cervical cytology and CIN.

Acknowledgements

The supporting organization played no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript. Authors (M.H., H.F., S.H., T.S., Y.S., M.A., M.K., M.K., H.W., K.K., N.S.) declare that they have no conflict of interest relevant to this article.

References

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. *CA Cancer J Clin* 2011;61:69–90.
2. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 1999; 189:12–19.
3. Bouvard V, Baan R, Straif K, et al.; WHO International Agency for Research on Cancer Monograph Working Group. A review of human carcinogens—Part B: biological agents. *Lancet Oncol* 2009;10:321–2.
4. Smith JS, Lindsay L, Hoots B, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int J Cancer* 2007;121: 621–32.
5. Wright TC, Jr, Massad LS, Dunton CJ, et al. 2006 *Consensus guidelines for the management of* women with cervical intraepithelial neoplasia or adenocarcinoma *in situ*. 2006 American Society for Colposcopy and Cervical Pathology-sponsored Consensus Conference. *Am J Obstet Gynecol* 2007;197:340–5. Review.
6. Castle PE, Schiffman M, Wheeler CM, et al. Evidence for frequent regression of cervical intraepithelial neoplasia-grade 2. *Obstet Gynecol* 2009;113:18–25.
7. Kyrgiou M, Koliopoulos G, Martin-Hirsch P, et al. Obstetric outcomes after conservative treatment for intraepithelial or early invasive cervical lesions: systematic review and meta-analysis. *Lancet* 2006;367:489–98. Review.
8. Ostör AG. Natural history of cervical intraepithelial neoplasia: a critical review. *Int J Gynecol Pathol* 1993;12:186–92. Review.
9. Kataja V, Syrjänen K, Mäntyjärvi R, et al. Prospective follow-up of cervical HPV infections: life table analysis of histopathological, cytological and colposcopic data. *Eur J Epidemiol* 1989;5:1–7.
10. Ioka A, Tsukuma H, Ajiki W, et al. Influence of age on cervical cancer survival in Japan. *Jpn J Clin Oncol* 2005;35:464–9.
11. Gaarenstroom KN, Melkert P, Walboomers JM, et al. Human papillomavirus DNA and genotypes: prognostic factors for progression of cervical intraepithelial neoplasia. *Int J Gynecol Cancer* 1994;4:73–8.
12. Remmink AJ, Walboomers JM, Helmerhorst TJ, et al. The presence of persistent high-risk HPV genotypes in dysplastic cervical lesions is associated with progressive disease: natural history up to 36 months. *Int J Cancer* 1995;61: 306–11.
13. Muñoz N, Bosch FX, Castellsagué X, et al. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004;111:278–85.
14. Khan MJ, Castle PE, Lorincz AT, et al. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J Natl Cancer Inst* 2005;97:1072–9.
15. Xi LF, Hughes JP, Edelstein ZR, et al. Human papillomavirus (HPV) type 16 and type 18 DNA loads at baseline and persistence of type-specific infection during a 2-year follow-up. *J Infect Dis* 2009;200:1789–97.
16. Matsumoto K, Oki A, Furuta R, et al.; for Japan HPV And Cervical Cancer (JHACC) Study Group. Predicting the progression of cervical precursor lesions by human papillomavirus genotyping: a prospective cohort study. *Int J Cancer* 2010;128:2898–910.
17. Yamaguchi A, Hashimoto N, Tsutae W, et al. Detection of human papillomavirus DNA by PCR/microfluorometry for screening of cervical cancer. *Clin Chim Acta* 2002;318:41–9.
18. Onuki M, Matsumoto K, Satoh T, et al. Human papillomavirus infections among Japanese women: age-related prevalence and type-specific risk for cervical cancer. *Cancer Sci* 2009;100: 1312–6.

19. Fujinaga Y, Shimada M, Okazawa K, et al. Simultaneous detection and typing of genital human papillomavirus DNA using the polymerase chain reaction. *J Gen Virol* 1991;72: 1039–44.
20. Clifford GM, Gallus S, Herrero R, et al.; IARC HPV Prevalence Surveys Study Group. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: a pooled analysis. *Lancet* 2005;366:991–8.
21. Zhao R, Zhang WY, Wu MH, et al. Human papillomavirus infection in Beijing, People's Republic of China: a population-based study. *Br J Cancer* 2009;101:635–40.
22. Bao YP, Li N, Smith JS, et al. Human papillomavirus type distribution in women from Asia: a meta-analysis. *Int J Gynecol Cancer* 2008; 18:71–9.
23. Schlecht NF, Platt RW, Duarte-Franco E, et al. Human papillomavirus infection and time to progression and regression of cervical intraepithelial neoplasia. *J Natl Cancer Inst* 2003; 95:1336–43.
24. Evans MF, Adamson CS, Papillo JL, et al. Distribution of human papillomavirus types in ThinPrep Papanicolaou tests classified according to the Bethesda 2001 terminology and correlations with patient age and biopsy outcomes. *Cancer* 2006;106:1054–64.
25. Illades-Aguiar B, Alarcón-Romero Ldel C, Antonio-Véjar V, et al. Prevalence and distribution of human papillomavirus types in cervical cancer, squamous intraepithelial lesions, and with no intraepithelial lesions in women from Southern Mexico. *Gynecol Oncol* 2010;117: 291–6.
26. Miura S, Matsumoto K, Oki A, et al. Do we need a different strategy for HPV screening and vaccination in East Asia? *Int J Cancer* 2006;119: 2713–15.
27. Qu W, Jiang G, Cruz Y, et al. PCR detection of human papillomavirus: comparison between MY09/MY11 and GP5+/GP6+ primer systems. *J Clin Microbiol* 1997;35:1304–10.
28. Yoshikawa H, Nagata C, Noda K, et al. Human papillomavirus infection and other risk factors for cervical intraepithelial neoplasia in Japan. *Br J Cancer* 1999;80:621–4.
29. Sasagawa T, Basha W, Yamazaki H, et al. High-risk and multiple human papillomavirus infections associated with cervical abnormalities in Japanese women. *Cancer Epidemiol Biomarkers Prev* 2001;10:45–52.
30. Spinillo A, Dal Bello B, Gardella B, et al. Multiple human papillomavirus infection and high grade cervical intraepithelial neoplasia among women with cytological diagnosis of atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions. *Gynecol Oncol* 2009;113:115–19.
31. Muñoz N, Bosch FX, de Sanjosé S, et al.; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003;348: 518–27.
32. ACOG Practice Bulletin No. 99: management of abnormal cervical cytology and histology. *Obstet Gynecol* 2008;112:1419–44.
33. Mathevet P, Chemali E, Roy M, et al. Long-term outcome of a randomized study comparing three techniques of conization: cold knife, laser, and LEEP. *Eur J Obstet Gynecol Reprod Biol* 2003;106: 214–18.
34. Franceschi S, Clifford GM. Re: a study of the impact of adding HPV types to cervical cancer screening and triage tests. *J Natl Cancer Inst* 2005;97:938–9.
35. Cancer Statistics in Japan. Center for Cancer Control and Information Services, National Cancer Center of Japan. International Comparisons of Cancer Mortality and Cancer Screening Rates. http://ganjoho.jp/data/public/statistics/backnumber/1isaao000000068m-att/fig21.pdf. Accessed on 22 April 2012.
36. Ministry of Health, Labour and Welfare. Comprehensive Survey of Living Conditions of the People on Health and Welfare 2010. http://www.mhlw.go.jp/stf/houdou/2r9852000001igt0-att/2r98520000001iguh.pdf. Accessed on 22 April 2012.

Journal of Surgical Oncology 2012;106:938–941

New Revised FIGO 2008 Staging System for Endometrial Cancer Produces Better Discrimination in Survival Compared With the 1988 Staging System

TATSUYA KATO, MD,[1] HIDEMICHI WATARI, MD, PhD,[1]* DAISUKE ENDO, MD,[1] TAKASHI MITAMURA, MD,[1] TETSUJI ODAGIRI, MD,[1] YOUSUKE KONNO, MD,[1] MASAYOSHI HOSAKA, MD, PhD,[1] NORIKO KOBAYASHI, MD, PhD,[1] YUKIHARU TODO, MD, PhD,[2] SATOKO SUDO, MD, PhD,[1] MAHITO TAKEDA, MD, PhD,[1] PEIXIN DONG, MD, PhD,[1] MASANORI KANEUCHI, MD, PhD,[1] MASATAKA KUDO, MD, PhD,[1] AND NORIAKI SAKURAGI, MD, PhD[1]

[1]*Department of Obstetrics and Gynecology, Hokkaido University Graduate School of Medicine, Sapporo, Japan*
[2]*Division of Gynecology, Hokkaido Cancer Center, Sapporo, Japan*

Background and Objectives: The aim of this study was to analyze the stage migration and survival of endometrial cancer by the revised FIGO 2008 staging system compared with the 1988 staging system.
Methods: A total of 355 patients with endometrial cancer, who underwent complete surgical staging, were enrolled. We compared the surgical stages and survival by FIGO 1988 staging system with those by FIGO 2008 staging system.
Results: 2008 FIGO staging system resulted in an increase of stage I patients and decrease of stage II and IIIa patients. The 5-year overall survival (OS) rates for patients with 2008 FIGO stage IA and IB disease were 98.2% and 91.9%, respectively ($P = 0.004$). Five-year OS rate of new stage II (82.6%) was significantly worse than that of new stage IA (98.2%, $P = 0.003$). Patients with positive washing cytology alone revealed a 5-year OS rate similar to that of patients with new stage IIIA disease (96.2% vs. 90.9%, respectively; $P = 0.53$). The 5-year OS rate for patients with stage IIIC1 disease was improved compared with that for patients with stage IIIC2 disease (85.7% vs. 63.0%, respectively; $P = 0.08$).
Conclusion: New revised FIGO 2008 staging system for endometrial cancer produced better discrimination in OS outcomes compared with the 1988 system.
J. Surg. Oncol. 2012;106:938–941.

KEY WORDS: **endometrial cancer; surgical staging; revised FIGO 2008 staging system; survival**

INTRODUCTION

The 1988 International Federation of Gynecology and Obstetrics (FIGO) surgical staging has been utilized in the management of endometrial cancer patients. The revised 2008 FIGO staging system for endometrial cancer is a further attempt to refine the surgical staging system [1]. The new system incorporates several changes as follows: Within stage I, 1988 stages IA and IB are combined to form a single group-2008 IA. Therefore, 1988 stage IC, which included patients with >50% myometrial invasion (MI), is staged as 2008 stage IB. Patients with 1988 stage IIA disease (cervical glandular involvement) are classified with stage IA or stage IB disease according to the depth of MI. Stage II includes only patients with cervical stromal involvement. Within stage III, positive pelvic washings will no longer dictate a stage of IIIA and will be noted separately from stage. Stage IIIC was separated into stage IIIC1, which includes patients with positive pelvic nodes, and stage IIIC2, which includes patients with positive para-aortic nodes.

The objective of our study was to validate these changes in our patient cohort who underwent complete surgical staging including pelvic and para-aortic lymphadenectomy by comparing survival outcomes of patients staged according to the 1988 FIGO staging system with outcomes obtained when patients were staged according to the new 2008 FIGO staging system.

METHODS

Patients

A total of 397 patients with endometrial cancer underwent extensive surgery including systematic pelvic and para-aortic lymphadenectomy from 1982 to 2005 at Hokkaido University Hospital, Sapporo, Japan. The patients with concomitant other malignancies and those who underwent hysterectomy and bilateral salpingooophorectomy (BSO) alone were excluded from this study. All subjects underwent modified radical hysterectomy, BSO, and systematic retroperitoneal lymphadenectomy as previously described [2]. Stage IV disease with distant metastasis (liver or lung metastasis) was excluded from this analysis. We, therefore, defined patients with stage IV endometrial cancer showing peritoneal metastasis. The patients with an intermediate or high risk for recurrence [3] were treated with adjuvant chemotherapy of 350 mg/m^2 cyclophosphamide, 40 mg/m^2 adriamycin, and 50–70 mg/m^2 cisplatin (CAP) or paclitaxel 175 mg/m^2, carboplatin AUC5, every 3 weeks for four to six cycles. The following histopathologic prognostic factors were included in this analysis: histologic subtype, depth of MI, architectural grade (AG), nuclear grade (NG), lymphvascular space invasion (LVSI), ovarian metastasis, and lymph node metastasis (LNM). All risk factors were determined as previously described [2].

Conflicts of interest: none.

*Correspondence to: Hidemichi Watari, MD, PhD, Department of Obstetrics and Gynecology, Hokkaido University Graduate School of Medicine, Sapporo, Japan. Fax: +81-11-706-7711.
E-mail: watarih@med.hokudai.ac.jp

Received 22 April 2012; Accepted 1 June 2012

DOI 10.1002/jso.23203

Published online 27 June 2012 in Wiley Online Library (wileyonlinelibrary.com).

Statistics

Correlation between the variables was analyzed using a chi-square test. Patient survival was calculated using the Kaplan–Meier method. The significance of the survival difference was examined using the log-rank test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using the StatView software package (SAS Institute, Inc., Cary, NC).

RESULTS

In total, 355 patients were enrolled in this retrospective analysis. The patients' characteristics are shown in Table I. The median follow-up period was 78 months (range: 1–236 months). The median age of the patients was 56 years (range: 23–77 years).

Migration of Surgical Stages by New FIGO Staging System

We re-categorized surgical stages by FIGO 1988 to those by FIGO 2008. IA (n = 37) and IB (n = 117) by FIGO 1988 staging were re-categorized into new IA (n = 154). IC (n = 52) by FIGO 1988 was categorized as new IB. IIA (n = 9) by FIGO 1988 was reclassified as new IA (n = 5) or IB (n = 4). Old IIB (n = 19) was categorized as new stage II. IIIA with positive peritoneal cytology only (n = 28) was re-categorized as new IA (n = 16), IB (n = 8), or II (n = 4). Tumor invasion to serosa of uterine corpus and/or adnexal metastasis was categorized as new IIIA (n = 24). IIIC (n = 53) by FIGO 1988 was categorized as IIIC1 (n = 28), or IIIC2 (n = 25). Overall, proportion of stage I disease increased from 58.0% (206/355) to 67.3% (239/355) by new staging. Proportion of stage II, and stage IIIA disease decreased from 7.9% (28/355) to 6.5% (23/355), and from 14.7% (52/355) to 6.8% (24/355), respectively (Tables II and III).

TABLE I. Pathological Characteristics of 355 Patients

Factor	N	%
Histology		
Endometrioid	324	91.3
Non-endometrioid	31	8.7
Architectural grade		
G1	168	47.3
G2/3	187	52.7
Nuclear grade		
G1/2	317	89.3
G3	38	10.7
Myometrial invasion		
<1/2	220	62.0
≥1/2	135	38.0
Lymphvascular space invasion		
(−)/(+)	273	76.9
(++)/(+++)	82	23.1
Cervical involvement		
(−)	282	79.4
(+)	73	20.6
Ovarian metastasis		
(−)	312	87.9
(+)	43	12.1
Pelvic lymph node metastasis		
(−)	294	82.8
(+)	61	17.2
Para-aortic lymph node metastasis		
(−)	322	90.7
(+)	33	9.3

TABLE II. Stage Migration by Revised FIGO 2008 Staging

1988 stage	N	2008 stage	N
IA	37	IA	154
IB	117	IB	52
IC	52		
IIA	9	IA	5
		IB	4
IIB	19	II	19
IIIA (*PC positive only)	28	IA	16
		IB	8
		II	4
IIIA (except *PC positive only)	24	IIIA	24
IIIC	53	IIIC1	28
		IIIC2	25

*PC=peritoneal cytology

Survival Analysis

The 5-year estimated OS rates for patients with 1988 stage IA and IB disease were 100% and 97.4%, respectively (Table III; Figs. 1 and 2). There was no significant difference in survival between 1988 stage IA and stage IB disease. There was a significant difference of survival between 1988 stage IB and IC (97.4% vs. 90.0%, $P = 0.02$). When the patients with old stage I disease were reclassified according to 2008 FIGO staging, the 5-year OS rates for stages IA and IB were 98.2% and 91.9%, respectively ($P = 0.004$; Fig. 1). Patients with 1988 stage IIA disease had OS outcomes even better than those of patients with 1988 stage IC disease with 5-year OS rates of 100.0% and 90.0%, respectively. Five-year OS rate of new stage II was 82.6%, which was significantly worse than that of new stage IA (98.2%, $P = 0.003$, Fig. 1). Within old stage IIIA, patients who were staged as IIIA solely for positive washings cytology had a 5-year OS rate similar to that of patients who were staged as new stage IIIA because of involvement of the serosa or adnexa, with a 5-year OS estimate of 96.2% versus 90.9% ($P = 0.53$). The 5-year OS rate for patients with stage IIIC1 disease was better than

TABLE III. Survival by 1988 Staging and 2008 Staging

1988 stage	N (%)	5-YSR (%)	2008 stage	N (%)	5-YSR (%)
Stage I	206 (58.0)	95.9	Stage I	239 (67.3)	96.5
IA	37 (10.4)	100.0	IA	175 (49.3)	98.2
IB	117 (33.0)	97.4	IB	64 (18.0)	91.9
IC	52 (14.6)	90.0			
Stage II	28 (7.9)	89.3	Stage II	23 (6.5)	82.6
IIA	9 (2.5)	100.0			
IIB	19 (5.4)	84.2			
Stage III	105 (29.6)	84.2	Stage III	77 (21.7)	80.1
IIIA	52 (14.7)	93.7	IIIA	24 (6.8)	90.9
IIIB	0 (0)	—	IIIB	0 (0)	—
IIIC	53 (14.9)	75.2	IIIC1	28 (7.9)	85.7
			IIIC2	25 (7.0)	63.0
Stage IV	16 (4.5)	13.3	Stage IV	16 (4.5)	13.3
IVA	0 (0)	—	IVA	0 (0)	—
IVB	16 (4.5)	13.3	IVB	16 (4.5)	13.3
			PC (+) only	28	96.2

PC, pentoneal cytology.

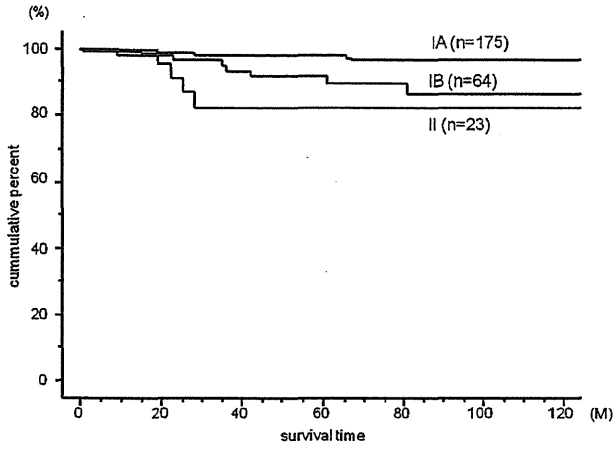

Fig. 1. Kaplan–Meier survival curve of stage I and II disease by FIGO 2008 staging system.

that for patients with stage IIIC2 disease with marginal significance (85.7% vs. 63.0%, respectively; $P = 0.08$, Fig. 2).

DISCUSSION

In this retrospective analysis, we demonstrated that new FIGO 2008 staging system discriminates survival better than FIGO 1988 staging system in endometrial cancer patients who underwent complete staging surgery including systematic pelvic and para-aortic lymphadenectomy. This is the first report on the validation of new 2008 FIGO staging for endometrial cancer from Japanese institutions as far as we know.

As the revised FIGO staging system was introduced in 2008, two groups have analyzed the prognostic significance of the new (FIGO 2008) versus old (FIGO 1988) staging systems for endometrioid adenocarcinomas of the uterine corpus [4,5]. They have analyzed SEER data, and concluded that new revised staging system discriminates patients' survival better than old 1988 staging system. On the contrary, Abu-Rustum et al. [6] reported that the 1988 FIGO classification of stage I endometrial cancer correctly identified three subgroups of patients who had significantly different OS, and

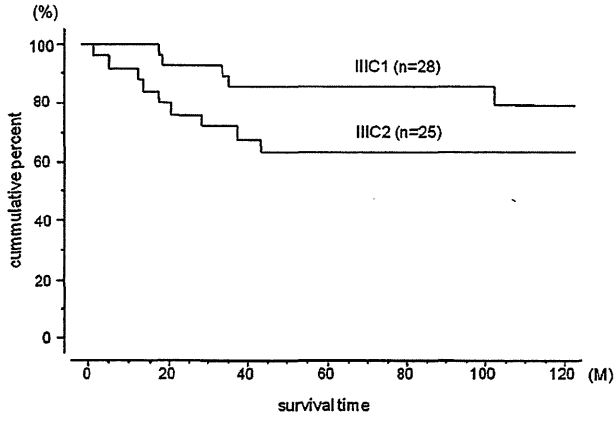

Fig. 2. Kaplan–Meier survival curve of stage IIIC disease by FIGO 2008 staging system.

specifically 1988 FIGO stages IA and IB had distinct oncologic outcomes. They concluded that new FIGO 2008 staging system did not improve its predictive ability over the 1988 system. In our patients' cohort, 1988 stage IA showed excellent 5-year OS (100%), and there was no significant difference of OS compared with that of 1988 stage IB (97.4%). However, to select appropriate patients of endometrial cancer for fertility-sparing treatment with high-dose medroxy progesterone acetate, the concept of 1988 stage IA (no MI) remains important even after adoption of new revised FIGO 2008 system.

Old stage IIIA disease with positive peritoneal cytology only is no longer classified as stage IIIA in revised FIGO 2008 system. This change is reasonable because their 5-year OS rate is excellent (96.2%), which is comparable to new stage I. New stage IIIA disease showed 90.9% of 5-year OS rate in our patients cohort, which is better than stage II without statistically significant difference. This result raises one possibility that new stage IIIA would have better outcome than we expected. Thus, new stage IIIA could be re-categorized as stage I or II disease in further revision in the future.

Substages of nodal disease in new staging system raised one question whether we need to perform systematic pelvic and para-aortic lymphadenectomy for all patients. Prognostically, identifying involved lymph nodes appears to be important; however, practically speaking, there is a lack of consensus regarding the extent and role of lymphadenectomy in patients with endometrial cancer. We demonstrated that 5-year OS with nodal involvement was well discriminated by new staging (85.7% for IIIC1, 63.0% for IIIC2) in this study. Although two reports described that there was significant difference of survival between IIIC1 and IIIC2 using SEER data [4,5], survival difference (approximately 10%) was smaller than ours (57.0–68.2% for IIIC1, 49.4–57.3% for IIIC2). This is in part due to the diversity of surgical approaches in the SEER data set. We uniformly treated nodal disease by systematic pelvic and para-aortic lymphadenectomy, whereas in the SEER data, 45.2% of patients had no lymph nodes examined. Thus, new FIGO staging system discriminates survival of nodal disease better in the patients who underwent systematic lymphadenectomy.

Although some retrospective series have suggested a benefit in terms of survival with the addition of lymphadenctomy [7–10], randomized trials reported that there is not a therapeutic role [11,12]. The role and extent of lymphadenectomy for surgical staging purposes remains under debate. Some surgeons have taken the approach of recommending complete pelvic and para-aortic lymphadenectomy in all patients [13]. Others have identified features that distinguish patient subsets at particularly low risk of lymph node involvement in which lymphadenectomy can be omitted [14,15]. Less than 10% of the patients who were included in the study by Creasman et al. [16] had grossly positive lymph nodes, indicating that palpation of lymph node areas and removal of "suspicious" lymph nodes is unlikely to identify the majority of lymph node metastases. Because we have currently recognized that there are clinical and pathological risks for LNM, it is the most reasonable to individualize lymphadenectomy according to the risk for LNM. Clearly, a more uniform approach to the surgical staging of patients with endometrial cancer needs to be defined to facilitate the adequate assignment of prognosis and the appropriate adjuvant therapy recommendations and to produce more accurate inter-institutional, comparative evaluations. Practically, we need to develop individualized risk-prediction models in endometrial cancer as previously described by us and others [14,15,17].

CONCLUSIONS

In conclusion, recent recommendations for changes to the endometrial cancer staging system were validated in our data set. Revisions within stage I appear appropriate. For patients with positive nodes, positive pelvic lymph nodes portend a better survival

outcome than involvement of the para-aortic nodes. The 2008 FIGO staging system produced better discrimination in OS outcomes compared with the 1988 system.

REFERENCES

1. Pecorelli S: Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. Int J Gynaecol Obstet 2009;105:103–104.
2. Watari H, Todo Y, Takeda M, et al.: Lymph-vascular space invasion and number of positive para-aortic node groups predict survival in node-positive patients with endometrial cancer. Gynecol Oncol 2005;96:651–657.
3. Nagase S, Katabuchi H, Hiura M, et al.: Evidence-based guidelines for treatment of uterine body neoplasm in Japan: Japan Society of Gynecologic Oncology (JSGO) 2009 edition. Int J Clin Oncol 2010;15:531–542.
4. Lewin SN, Herzog TJ, Barrena Medel NI, et al.: Comparative performance of the 2009 International Federation of Gynecology and Obstetrics' Staging System for uterine corpus cancer. Obstet Gynecol 2010;116:1141–1149.
5. Cooke EW, Pappas L, Gaffney DK: Does the revised International Federation of Gynecology and Obstetrics staging system for endometrial cancer lead to increased discrimination in patient outcomes? Cancer 2011;117:4231–4237.
6. Abu-Rustum NR, Zhou Q, Iasonos A, et al.: The revised 2009 FIGO staging system for endometrial cancer: Should the 1988 FIGO stages IA and IB be altered? Int J Gynecol Cancer 2011; 21:511–516.
7. Chan JK, Cheung MK, Huh WK, et al.: Therapeutic role of lymph node resection in endometrioid corpus cancer: A study of 12,333 patients. Cancer 2006;107:1823–1830.
8. Fujimoto T, Nanjyo H, Nakamura A, et al.: Para-aortic lymphadenectomy may inprove disease-related survival in patients with multipositive pelvic lymph node stage IIIc endometrial cancer. Gynecol Oncol 2007;107:253–259.
9. Chang SJ, Kim WY, Yoo SC, et al.: Para-aortic lymphadenectomy improves survival in patients with intermediate to high-risk endometrial carcinoma. Acta Obstet Gynecol Scand 2008;87: 1361–1369.
10. Todo Y, Kato H, Kaneuchi M, et al.: Survival effect of para-aortic lymphadenectomy in endometrial cancer (SEPAL study): A retrospective cohort analysis. Lancet 2010;375:1165–1172.
11. Kitchener H, Swart AM, Qian Q, et al.: Efficacy of systematic pelvic lymphadenectomy in endometrial cancer (MRC ASTEC trial): A randomised study. Lancet 2009;373:125–136.
12. Benedetti Panici P, Basile S, Maneschi F, et al.: Systematic pelvic lymphadenectomy vs no lymphadenectomy in early stage endometrial carcinoma: Randomized clinical trial. J Natl Cancer Inst 2008;100:707–716.
13. Watari H, Mitamura T, Moriwaki M, et al.: Survival and failure pattern of patients with endometrial cancer after extensive surgery including systematic pelvic and para-aortic lymphadenectomy followed by adjuvant chemotherapy. Int J Gynecol Cancer 2009;19:1585–1590.
14. Mariani A, Webb MJ, Keeney GL, et al.: Low-risk corpus cancer: Is lymphadenectomy or radiotherapy necessary? Am J Obstet Gynecol 2000;182:1506–1519.
15. Lee JY, Jung DC, Park SH, et al.: Preoperative prediction model of lymph node metastasis in endometrial cancer. Int J Gynecol Cancer 2010;20:1350–1355.
16. Creasman WT, Morrow CP, Bundy BN, et al.: Surgical pathologic spread patterns of endometrial cancer. A Gynecologic Oncology Group Study. Cancer 1987;60(8 Suppl.):2035–2041.
17. Todo Y, Okamoto K, Hayashi M, et al.: A validation study of a scoring system to estimate the risk of lymph node metastasis for patients with endometrial cancer for tailoring the indication of lymphadenectomy. Gynecol Oncol 2007;104:623–628.

THE JOURNAL OF
Obstetrics and Gynaecology Research

doi:10.1111/j.1447-0756.2011.01727.x J. Obstet. Gynaecol. Res. Vol. 38, No. 2: 420–426, February 2012

Re-consideration of lymphadenectomy for stage Ib1 cervical cancer

Hidenori Kato[1], Yukiharu Todo[1], Yoshihiro Suzuki[1], Youko Ohba[1], Shin-ichiro Minobe[1], Kazuhira Okamoto[1], Katsusige Yamashiro[2] and Noriaki Sakuragi[3]

Divisions of [1]Gynecologic Oncology and [2]Pathology, National Hospital Organization, Hokkaido Cancer Center, and [3]Department of Obstetrics and Gynaecology, Hokkaido University School of Medicine, Sapporo, Japan

Abstract

Aim: Because of less frequent lymph node metastasis and parametrial involvement, patients with stage Ib1 cervical cancer may benefit from a curtailment of surgery. We retrospectively investigated the distribution of lymph node metastasis in stage Ib1 patients. After comparing the data with that of higher stages and sentinel lymph node navigation (SLNN), the appropriate extent of lymphadenectomy (LA) in stage Ib1 disease was newly suggested.

Method: A total of 303 patients underwent a radical hysterectomy with LA and the region-specific rate of node metastasis was obtained. SLNN was performed for 50 patients using ^{99m}Tc phytate injection into the cervix and intra-operative detection by a gamma-probe.

Results: The rate of node metastasis and the average number of nodes removed, respectively, were: 23/189 (12.2%), 65.2 in stage Ib1; 14/47 (29.8%), 70.1 in stage Ib2; 7/20 (35.0%), 78.2 in stage IIa; and 26/47 (55.3%), 69.1 in stage IIb. Lymph node metastasis in stage Ib1 was prevalent in the obturator (Ob) (9.5%), inter-iliac (Ii) (4.9%), superficial common iliac (Sc) (2.3%), cardinal (Cd) (2.2%) and external iliac (Ei) (1.7%) nodes. In patients with upper stage disease, lymph node metastasis could occur in all lymph nodes. In stage Ib1 patients, the sentinel nodes were assigned only to the Ob, Ii, Sc and Ei nodes, being identical with frequent metastatic sites in stage Ib1 (excluding Cd).

Conclusion: The extent of LA can be routinely completed with the removal of Ob, Ii, Ei, Sc and Cd nodes, which may provide a higher quality of life, including the reduction of lymphedema by preventing the removal of the inguinal nodes.

Key words: lymphadenectomy, lymph node metastasis, sentinel lymph node, stage Ib1, uterine cervical cancer.

Introduction

Worldwide, about 500 000 new cases of cervical cancer are diagnosed annually, and 274 000 women die from the disease. This cancer mainly extends to the parametria, vagina, uterine corpus and lymph nodes. Lymph node metastasis is one of the most important factors for prognosis. Treatment outcome of node-negative patients with stage Ib–IIb disease is excellent, with a five-year survival rate of 87.3–95%, while that of pelvic-node-positive patients is poor, with a five-year survival rate of 64–68.2%.[1] The significant benefit of lymph node removal has been shown so far,[1–3] although the therapeutic role of para-aortic node dissection is still controversial.[4] The incidence of lymph node metastasis is apparently in parallel with the International Federation of Gynecology and Obstetrics (FIGO) clinical stage.[1] In patients with stage Ib disease, the incidence of lymph

Received: April 4 2011.
Accepted: August 8 2011.
Reprint request to: Dr Hidenori Kato, Division of Gynecologic Oncology, National Hospital Organization, Hokkaido Cancer Center, 4-2 Kikusui, Shiroishi-ku, Sapporo-shi, Hokkaido 003-0804, Japan. Email: hidekato@sap-cc.go.jp

node metastasis has been reported to be 12–22%. When stage Ib is divided into sub-categories, stage Ib1 has a metastasis rate of 13.6–25.3% and stage Ib2 is 34–50%.[3,5–8] Accordingly, more than 80% of the patients might have little benefit by systemic lymphadenectomy (LA) in stage Ib1, and less aggressive surgery in terms of parametrial removal and lymph node dissection needs to be proposed.

The incidence of early invasive cervical cancer has been reported to be increasing in younger women recently; therefore, for the preservation of quality of life, a reduction in surgical complications is also desirable. Concerning radical hysterectomy, several authors have already proposed the use of less radical surgery, and its feasibility has been supported by Benedetti-Panici *et al.*[9] Regarding lymphadenectomy, most concern has been focused on the sentinel lymph node navigation (SLNN) surgery.[10] In recent years, many reports have supported the usefulness of SLNN for cervical cancer. In most cases, the sensitivity, specificity and false negativity have been reported to be approximately 75–87%, 92–97% and 11%, respectively.[11] However, we have some hurdles to face in order for this technique to become the routine strategy for early invasive cancers. As one of the answers proposed for the resolution of the problems, some reports have indicated great improvement in sensitivity and other parameters by the modification of applicable cases.[12–15] Still, we need to have more data to conclude that the removal of remaining nodes other than sentinel lymph nodes can be safely omitted. In this study, we retrospectively investigated the distribution of lymph node metastasis in stage Ib1 patients with LA. After comparing with the distribution of higher stages, and supported by the data of SLNN, the appropriate extent of LA in stage Ib1 was proposed.

Materials and Methods

Cancer cases

In the period of 2000–2009, 303 patients underwent a radical hysterectomy (Type IV Okabyashi) and systematic lymphadenectomy (LA) at Hokkaido Cancer Center and Hokkaido University Hospital. The number of cases included 189 in stage Ib1, 47 in stage Ib2, 20 in stage IIa and 47 in stage IIb, of which the pathology was found to be squamous cell carcinoma : adeno/adenosquamous/undifferentiated carcinoma/ others in 130:59, 21:26, 16:4 and 23:24, in each stage respectively.

Systemic lymphadenectomy and nomenclature of lymph nodes

Systematic lymphadenectomy in the pelvic cavity was performed by skeletonizing the vessels and dissecting the lymphatic tissues from the point of division of the common iliac artery into the external and internal iliac artery caudally to the level of the deep circumflex iliac vein. Common iliac and para-aortic nodes were removed after the radical hysterectomy. Because of the lack of a uniform, surgically-based classification and nomenclature for each lymph node station, although a standardized system has been proposed,[16,17] the nomenclature in this report has been defined as follows (Fig. 1):

- Para-aortic node, upper inferior mesenteric artery (IMA): between the level of the bilateral renal veins and the IMA

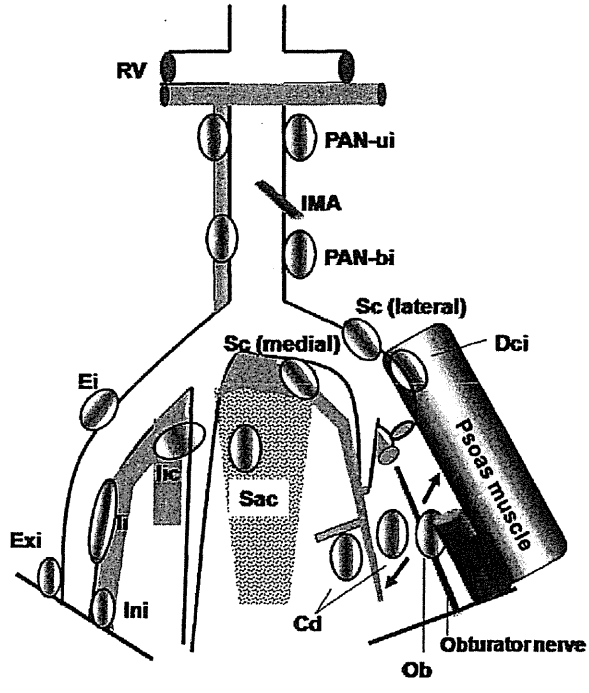

Figure 1 Lymph nodes dissected during an operation for cervical cancer. Cd, cardinal; Dci, deep common iliac; Ei, external inguinal; Exi, external inguinal; Ii, inter-iliac; Iic, internal iliac (distal part was dissected as inter-iliac and proximal part as sacral); IMA, inferior mesenteric artery; Ini, internal inguinal; Ob, obturator; PAN-bi, para-aortic node, below IMA; PAN-ui, para-aortic node, upper IMA; RV, renal vessels; Sac, sacrum; Sc, superficial common iliac. The nomenclature definitions were described in the Materials and Methods section.

- Para-aortic node, below IMA: between the level of the IMA and the bifurcation of the aorta
- Superficial common iliac: lateral and medial (between the right and left common iliac arteries) to the common iliac artery
- Deep common iliac: dorsal to common iliac vessels, located between the vessels and the psoas muscle
- External iliac: lateral to the external iliac artery, bounded ventrally by the origin of the deep circumflex vein; note that the medial nodes are not included here, defined as the obturator and cardinal nodes
- External and internal inguinal: lateral (external) and medial (internal) nodes located at the most peripheral area of the external iliac nodes, just superior to the entry into the femoral canal
- Inter-iliac: between the external iliac artery and vein, distal to the origin of internal iliac artery; often classified as the external iliac nodes, medial group; includes the upper portion of the internal iliac node
- Obturator: bordered superiorly by the caudal wall of the external iliac vein, dorsally by the obturator nerve and medially by the paravesical border; sometimes, this node is included in the external iliac nodes
- Sacral: above the sacral bone, bordered superiorly by the level of the bifurcation of the common iliac vessels, including the lower portion of the internal iliac nodes
- Cardinal: inferior to the level of obturator nerve, bordered superiorly by the beginning of internal iliac vein, along its branches, and superior to the sacral sympathetic nerve plexus
- Parametrial: these nodes were not included in those obtained by LA, because they were removed by radical hysterectomy.

Sentinel lymph node navigation surgery

On the day before surgery (20 h before), ^{99m}Tc phytate (148 MBq) was injected into the subepithelial area of the uterine cervix at the four quadrants (i.e. the 12, 3, 6 and 9 o'clock sites). Three hours later, lymphscintigraphy was performed and the availability of the detection was confirmed beforehand. Intraoperatively, the sentinel lymph node was scanned with a gamma probe (Navigator GPS; Furuno Electric, Nishinomiya, Japan) and nodes with more than 10-fold counts above the background rate were identified.

Results

Incidence of lymph node metastasis

A total of 303 patients underwent a radical hysterectomy with LA during the period 2000–2009 in our hospitals. LA was systematically performed from the inguinal to the para-aortic lesions. The number of cases, incidence of metastasis and average number of dissected nodes in each stage, respectively, were: 189, 12.2%, 65.2 in stage Ib1; 47, 29.8%, 70.1 in stage Ib2; 20, 35.0%, 78.2 in stage IIa; and 47, 55.3%, 69.1 in stage IIb (Fig. 2). A difference in the pathology was not observed. The average number of dissected nodes was far more than in other reported cases,[1] thus the incidence of metastasis can be satisfactory evaluated. The distribution of lymph nodes with metastasis in each stage is shown in Figure 3. In the cases of stage Ib1, mapping of lymph node metastasis was restricted to obturator (Ob), inter-iliac (Ii), superficial (Sc) and deep common iliac (Dci), external iliac (Ei), cardinal (Cd) and para-aortic lymph node (PAN) below IMA. Especially, relatively frequent metastasis was only observed in the Ob (9.5%), Ii (4.9%), Sc (2.3%), Cd (2.2%) and Ei (1.7%) nodes. Metastasis to the Dci and PAN nodes was less frequent, at 0.8% and 1.34%, respectively, but was always associated with other positive pelvic nodes. Because no metastasis to the sacral or either of the inguinal nodes was observed, these nodes can be safely omitted. In the cases of higher stage disease, metastasis was involved in all lymph nodes dissected and the rate of metastasis ranged from 2.7–22.2% in stage Ib2, 5.3–40% in stage IIa, to 2.5–44% in stage IIb (Fig. 3).

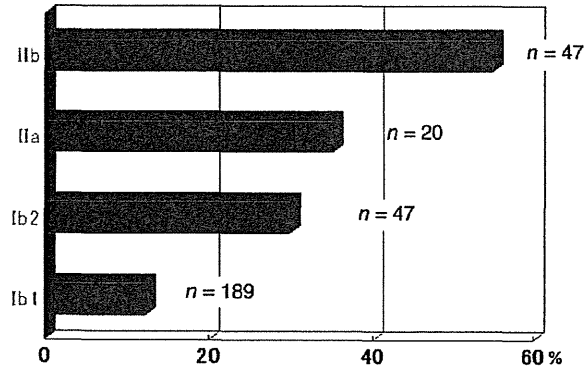

Figure 2 Incidence of lymph node metastasis. The number of cases, incidence of metastasis and average number of dissected nodes, respectively, were 189, 12.2%, 65.2 in stage Ib1; 47, 29.8%,70.1 in stage Ib2; 20, 35.0%, 78.2 in stage IIa; and 47, 55.3%, 69.1 in stage IIb.