shows worse prognosis than IIIC1.[4] However, in clinical practice, it is not clearly defined who should benefit from a systematic para-aortic lymphadenectomy.

Complete systematic pelvic and para-aortic lymphadenectomy has been routinely performed in all operable patients with endometrial cancer in Hokkaido University Hospital, because: (i) nodal status is the most important prognosticator; and (ii) results of lymphadenectomy allow tailoring of postoperative adjuvant treatment.[5,6] Consequently, the retrospective cohort study (SEPAL study) has recently demonstrated that para-aortic lymphadenectomy combined with pelvic node dissection improves survival of endometrial cancer patients with postoperative intermediate risk/high risk for recurrence, but not for patients with low risk for recurrence.[7] Recently, two randomized clinical trials, however, indicated that routine lymphadenectomy provided no survival benefit in endometrial cancer.[8,9] Taken together, we can conclude that no survival benefit of routine lymphadenectomy has been established for patients with postoperative low risk for recurrence. However, survival benefit of lymphadenectomy including para-aortic lymphadenectomy remains controversial for the patients with postoperative intermediate risk/high risk for recurrence, thus prospective study is mandatory to investigate the survival benefit of para-aortic lymphadenectomy shown in the SEPAL study. We are currently proposing a concept and design of a randomized phase III trial investigating the survival effect of para-aortic lymphadenectomy in endometrial cancer. In this article, we would like to discuss the important issues to definitively prove the potential survival advantage associated with lymphadenectomy in endometrial cancer.

Conclusions from Recent Clinical Studies Investigating Therapeutic Role of Lymphadenectomy

Even after the negative results of two randomized trials from Europe (ASTEC trial and Italian study),[8,9] the latest NCCN guideline[1] still recommends systematic pelvic and para-aortic lymphadenectomy for early stage endometrial cancer. In the discussion, they describe the reasons for not changing their guidelines. It is stated that two randomized clinical trials from Europe have reported that lymph node dissection does not improve outcomes in endometrial cancer patients; however, lymphadenectomy did identify those with nodal disease. To avoid over-interpretation of these results, it is important to address the limitations of these randomized studies, including patient selection, extent of lymph node dissection and standardization of postoperative therapy. Other concerns regarding these trials include the lack of central pathology review, the subspecialty of surgeons and inadequate statistical power. It is also stated that there is a high rate of lymphatic metastasis above the inferior mesenteric artery, suggesting a need for systematic pelvic and para-aortic lymphadenectomy. However, in these two European randomized trials, para-aortic lymphadenectomy was performed at the discretion of the surgeon. Clearly, the standardization of surgical effort to include systematic para-aortic lymphadenectomy may be important to definitively prove the potential survival advantage associated with lymphadenectomy.

The SEPAL study has shown that para-aortic lymphadenectomy significantly improved the survival of the endometrial cancer patients at intermediate risk/high risk for recurrence, but not patients at low risk for recurrence.[7] There are several possible reasons for the positive results of the SEPAL study. First, surgeons were familiar with lymphadenectomy, and the lymph node count is high, because the median number of nodes removed was 34 in the pelvic lymphadenectomy alone group, and 82 nodes in the pelvic and para-aortic lymphadenectomy group. However, the SEPAL study also has some limitations; these include the fact that it was a retrospective cohort study, and adjuvant therapy was not uniformly given. Indeed, in the pelvic lymphadenectomy group, 46% received adjuvant radiotherapy, whereas 98% received adjuvant chemotherapy in the pelvic and para-aortic lymphadenectomy group. Comparison of overall survival (OS) among intermediate risk patients receiving adjuvant chemotherapy, para-aortic lymphadenectomy did not significantly improve the survival.

Based on the results obtained from previous clinical studies, we conclude that patients at 'low-risk for lymph node metastasis' should not be included in future prospective trials to investigate the therapeutic role of lymphadenectomy. The survival effect of para-aortic lymphadenectomy should be investigated for the patients at 'risk of lymph node metastasis' by prospective studies based on the positive results obtained from the SEPAL study. Patient selection and quality assurance of lymphadenectomy should be discussed and, finally, when considering the results of phase III trials,[10–12] adjuvant chemotherapy should be uniformly given in future lymphadenectomy trials.

Study Design and End-points

We are proposing a new trial concept and design to prospectively investigate the survival effect of para-aortic lymphadenectomy in endometrial cancer (Fig. 1). This new concept is a randomized phase III trial and patients will be randomly assigned to undergo pelvic lymphadenectomy alone or pelvic and para-aortic lymphadenectomy. Adjuvant chemotherapy will be given to cases at postoperative risk for recurrence. The trial schema is shown in Figure 2. First of all, we will estimate the risk of lymph node metastasis preoperatively. After getting informed consent from all eligible cases, they will be randomly assigned to two arms. For cases at intermediate risk/high risk for recurrence confirmed by postoperative pathological examination, adjuvant chemotherapy will be given. After completing the initial treatment, we will follow-up participants until recurrence. Because we aim to investigate the therapeutic significance of primary treatments, including surgery and adjuvant chemotherapy, the primary end-point could be recurrence-free survival (RFS). In addition, RFS would be a surrogate for OS in endometrial cancer. Secondary end-points include OS, relationship between number of harvested nodes and recurrence rate, concordance rate of pre- and postoperative assessments (e.g. imaging, pathological diagnosis, grade, histology), predictive value of the combination of preoperative risks for lymph node metastasis, intraoperative tumor size and lymph node metastasis, initial failure site, and perioperative, chemotherapy-related adverse events. At the same time, we can create a scoring system to select patients who are at risk of lymph node metastasis and in whom pelvic and/or para-aortic lymphadenectomy can be safely omitted.[13,14]

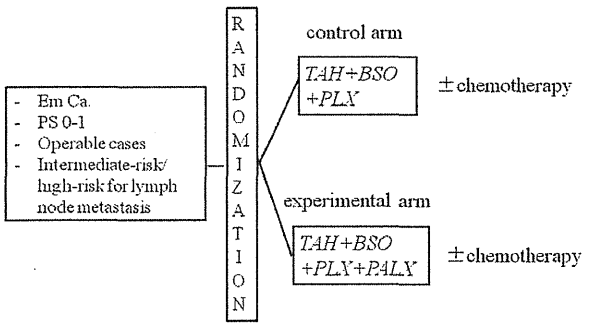

Figure 1 Proposal of a design of a future prospective trial investigating the survival effect of para-aortic lymphadenectomy in endometrial cancer. BSO, bilateral salpingo-oophorectomy; PALX, para-aortic lymphadenectomy; PLX, pelvic lymphadenectomy; PS, performance status; TAH, total abdominal hysterectomy.

Important Issues Which Need to be Discussed to Finalize the New Concept

First, we have to consider what the control arm for lymphadenectomy should be. Should it be pelvic lymphadenectomy alone or pelvic and para-aortic lymphadenectomy? The second point is how to select the study population. We need to estimate risk for lymph node metastasis preoperatively, and need to exclude inappropriate cases. Third, one of the most important issues is quality assurance of lymphadenectomy and, as such, we need to define the adequate extent and appropriate area of lymphadenectomy. Next we must consider which chemotherapeutic regimen should be given.

What is a control arm for lymphadenectomy in surgical treatment for endometrial cancer?

The latest NCCN guidelines[1] and Japanese guidelines[2] both recommend pelvic and para-aortic lymphadenectomy for staging purposes, but not for therapeutic

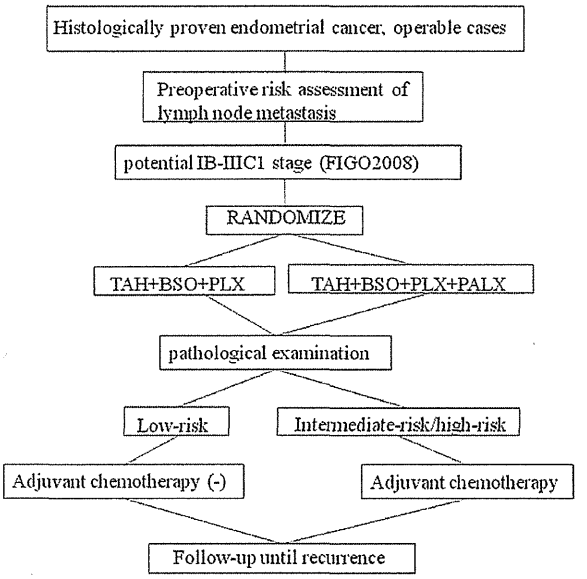

Figure 2 Study schema of a randomized phase III trial investigating the survival effect of para-aortic lymphadenectomy in endometrial cancer. BSO, bilateral salpingo-oophorectomy; PALX, para-aortic lymphadenectomy; PLX, pelvic lymphadenectomy; TAH, total abdominal hysterectomy.

purposes. According to a previous Japanese survey, most Japanese institutions perform pelvic lymphadenectomy routinely, but perform para-aortic lymphadenectomy selectively depending on the risk for lymph node metastasis.[3] Because 'standard' lymphadenectomy has not been established yet, either arm could be a control arm, but pelvic lymphadenectomy alone seems to be a more plausible control arm.

Selection of study population for lymphadenectomy trial in endometrial cancer

To select an appropriate study population, we need to estimate risk for lymph node metastasis preoperatively. We should assess myometrial invasion and cervical invasion with magnetic resonance imaging (MRI), histological subtype and grade by pathological examination, and extrauterine spread with enhanced computed tomography and/or MRI. Based on these evaluations, we should exclude potential FIGO (2008) stage IA cases (myometrial invasion <1/2) with any grade and subtype, which shows extremely low risk for lymph node metastasis (<3%),[15] cases with carcinosarcoma or sarcoma. Potential FIGO (2008) stage IV disease (peritoneal metastasis, bladder/rectum invasion and distant metastasis) by imagings will be excluded because positive node status does not affect their staging and probably survival. Cases with swelling of para-aortic nodes will not be eligible, because they will have great chance of positive nodes in the para-aortic area. Thus, an appropriate study population includes potential FIGO (2008) stage IB, II and III (IIIA, IIIB and IIIC1) disease.

Quality assurance of lymphadenectomy

To investigate the therapeutic role of lymphadenectomy, the extent and area of lymphadenectomy should be defined, because pelvic lymphadenectomy alone does not have any therapeutic role, but para-aortic lymphadenectomy combined with pelvic lymphadenectomy has survival benefits, and systematic dissection, but not sampling, is appropriate for therapeutic purposes. Indeed, when we compare the area and number of lymph nodes removed among the three recent clinical studies, systematic para-aortic lymphadenectomy was performed in the SEPAL study only, and the lymph node count in the SEPAL study was higher than the other two randomized studies, which is one of the main reasons why the SEPAL study could show a survival effect of lymphadenectomy (Table 1). In addition, other retrospective data has also demonstrated that the extent of lymphadenectomy (over 20 nodes removed) significantly improves disease-specific survival for intermediate risk/high risk patients, but not for low risk patients.[16]

To define the appropriate area for lymphadenectomy, we analyzed the distribution of lymph node metastasis sites in node-positive cases at our institution among cases undergoing systematic pelvic and para-aortic lymphadenectomy. We found that for nodal disease, para-aortic nodes above and below the inferior mesenteric artery, common iliac nodes, internal iliac nodes, external iliac nodes and obturator nodes were prevalent (unpubl. data). The most prevalent sites of nodal disease were the obturator nodes followed by the para-aortic nodes below the inferior mesenteric artery, para-aortic nodes above the inferior mesenteric artery up to the level of the renal vein, the internal iliac nodes, the common iliac nodes and the external iliac nodes (unpubl. data).

To define the extent of the lymphadenectomy, a lower limit of lymph nodes to be removed should be set. In addition, photos and/or videos of the dissected area should be submitted and inspected.

Which chemotherapeutic regimen should be given as an adjuvant therapy?

From the view point of clinical practice, adjuvant chemotherapy is frequently used in Japanese institutions, and the paclitaxel and carboplatin (TC) regimen is

Table 1 Comparison of recent clinical studies investigating the therapeutic role of lymphadenectomy in endometrial cancer

	ASTEC trial	Italian study	SEPAL study
Recurrence risk	Low–high	Intermediate/high	Low–high
Area of LNX	Pelvic	Pelvic (PAN)	Pelvic (PAN)
Lymph nodes count (median)	12	PLX, 26; PLX + PALX, 30.	PLX, 34; PLX, 59 + PALX, 23 = 82
Adjuvant therapy	RT	RT or CT	RT or CT
Therapeutic role of pelvic LNX	(–)	(–)	Not determined
Therapeutic role of para-aortic LNX	Not determined	Not determined	Low risk (–); intermediate/high (+)

CT, chemotherapy; LNX, lymphadenectomy; PALX, para-aortic lymphadenectomy; PAN, para-aortic node; PLX, pelvic lymphadenectomy; RT, radiotherapy.

widely given.[17] In the GOG209, randomized phase III trial to compare the efficacy of paclitaxel, adriamycin and cisplatin (TAP) and TC for advanced, recurrent disease, it was shown that TC is not inferior to TAP.[12] Therefore, TC is the current standard regimen of GOG for advanced, recurrent endometrial cancer. We are currently thinking that TC should be given to patients at intermediate risk/high risk for recurrence as an adjuvant chemotherapy in the future clinical trial.

Target Accrual

Concerning target accrual, if we assume that 3-year progression-free survival of the control arm would be 70%, the 3-year progression-free survival of the experimental arm should exceed 10% of that of the control arm, and if we have a significance level of 5% and over 90% statistical power, then 604 cases are necessary. If we estimate a 5% dropout rate, then a sample size of 630 cases (315 in each arm) is necessary.

In the year of 2011, 7273 new cases of endometrial cancer were registered in the Japan Society of Obstetrics and Gynecology. Among them, cases that are classified as FIGO (2008) stage IA (old stage IA and IB) are not eligible for the future clinical trial. Cases that are classified as FIGO (2008) stage IV are also not eligible. Therefore, approximately 40% of all endometrial cancer cases fit the inclusion criteria.

Acknowledgment

We would like to thank Dr Koji Ohba for statistical consideration of target accrual.

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THE JOURNAL OF
Obstetrics and Gynaecology Research

doi:10.1111/jog.12309 J. Obstet. Gynaecol. Res. Vol. 40, No. 2: 317–321, February 2014

Tailoring lymphadenectomy according to the risk of lymph node metastasis in endometrial cancer

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Abstract

It has been strongly suggested that patients with endometrial cancer with low risk of lymph node metastasis do not benefit from lymphadenectomy and that intermediate-risk/high-risk endometrial cancer patients benefit from complete pelvic and para-aortic lymphadenectomy. This hypothesis needs to be validated by prospective studies. For randomized controlled trials (RCT), heterogeneity of intervention compromises internal validity and non-participation of experienced doctors compromises external validity. As these situations easily occur in randomized surgical trials (RST) intended for high-risk patients, the effects of complicated surgery, such as full lymphadenectomy, might be underestimated in RST. In a famous RST, data for all eligible patients implied that survival outcome for the non-randomized group was significantly better than that for the randomized group. One plausible explanation is that physicians' judgment and experience produce better treatment decisions than do random choices. Although two RCT from European countries showed negative results of lymphadenectomy on prognosis, valuing the care of individual patients may be more important than uncritically adopting the results of RCT. In endometrial cancer, lymphadenectomy must be tailored to maximize the therapeutic effect of surgery and minimize its invasiveness and adverse effects. Two strategies are: (i) to remove lymph nodes most likely to harbor disease while sparing lymph nodes that are unlikely to be affected; and (ii) to perform full lymphadenectomies only on patients who can potentially benefit from them. Here, we focus on the second strategy. Preoperative risk assessments used in Japan and Korea to select low-risk patients who would not benefit from lymphadenectomy are discussed.

Key words: cancer of the endometrium, gynecologic imaging, gynecologic oncology, gynecology.

Reasons for Tailor-made Surgery

It is well known that uniform treatment for patients with the same disease is not always appropriate. Although the term 'personalized medicine' was coined in the context of genetics, this notion makes sense also in the context of surgical therapy. In the evidence-based medicine era, results of randomized controlled trials (RCT) tend to be uncritically accepted. In a famous RCT called the Emory Angioplasty versus Surgery Trial (EAST), the outcomes of percutaneous transluminal coronary angioplasty (PTCA) and coronary angioplasty bypass grafting (CABG) surgery were compared.[1] Of the 842 eligible patients, 392 (46.6%) agreed to participate, but 450 (53.4%) were not approached due to the attending or referring physician's refusal to participate ($n = 353$) or refusal by the patient ($n = 97$). Two interesting results were provided by EAST: (i) there was no survival difference between the PTCA group and the CABG group on the basis of data for 392 patients included in the trial; and (ii) survival outcome for the non-randomized group was

Received: August 16 2013.
Accepted: September 26 2013.
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significantly better than that for the randomized group on the basis of data for all 842 eligible patients.[2] Two plausible explanations can be provided to account for the result of the latter. One is that prognosis of patients in the non-randomized group may have been better than that of patients in the randomized group. The other is that physicians' judgment based on experience may be more important for treatment decision-making than a random choice. CABG generally tends to be performed for patients who have three-vessel disease or proximal left anterior descending artery stenosis. Therefore, the right treatment may have been conducted in the right disease status on the basis of physicians' appropriate experience. Valuing the care of individual patients may be more important than uncritically adopting the results of RCT.

Two reports in *The Lancet*[3,4] strongly suggest that pelvic lymphadenectomy (PLX) has no survival benefit for patients with endometrial cancer with low risk of lymph node metastasis and that combined pelvic and para-aortic lymphadenectomy (PLX + PALX) improves survival of patients with intermediate-risk/high-risk endometrial cancer. The former report was based on a randomized controlled trial by A Study in the Treatment of Endometrial Cancer (ASTEC), while the latter report was based on a retrospective cohort study. Some gynecologists seem to have been skeptical about the efficacy of lymphadenectomy in endometrial cancer based on the results of the ASTEC trial. Some physicians have believed that standard surgery for endometrial cancer does not include lymphadenectomy even though many previous reports suggested the efficacy of lymphadenectomy. Such an idea is an overgeneralization of the results of the ASTEC trial because the study population included only a small number of patients with high-risk endometrial cancer. If lymphadenectomy has a survival benefit for high-risk patients and lymphadenectomy is excluded from standard surgery in endometrial cancer, high-risk patients would not be able to receive optimal treatment. On the other hand, full lymphadenectomy was shown to have a survival benefit for patients with intermediate-risk/high-risk endometrial cancer in the Survival Effect of Para-aortic Lymphadenectomy (SEPAL) study.[4] Although omission of lymphadenectomy can be applied to patients with clinical stage I endometrial cancer according to the results of the ASTEC trial, clinical stage I includes not only low-risk patients but also intermediate-risk and high-risk patients. The range of application for omission of lymphadenectomy should probably be limited to patients with low-risk endometrial cancer. Although the results of these two studies in *The Lancet* are referred to as contradictory statements, they can be compatible. We need to deepen discussions regarding tailoring of lymphadenectomy in endometrial cancer.

A Problem Inherent in Surgical Studies in High-risk Cancer

The SEPAL study was based on a retrospective observational study.[4] Another observational study from the Mayo Clinic also showed the effectiveness of full lymphadenectomy for patients with high-risk endometrial cancer.[5] Some physicians have underestimated these results due to the study design inherent in a retrospective cohort study. However, the authors believe that study design is not grounds for underestimating the value of the SEPAL study. Well-designed cohort studies may in fact be more appropriate formats than RCT for assessing optimal surgery in high-risk cases. Special difficulties are encountered in randomized surgical trials intended for high-risk patients. Some physicians would decline participation in a randomized controlled trial in which pelvic lymphadenectomy versus combined pelvic and para-aortic lymphadenectomy is compared for patients with high-risk endometrial cancer because they might be familiar with para-aortic lymphadenectomy and its benefits and would be reluctant to perform pelvic lymphadenectomy alone. Conversely, doctors with limited experience may be assigned the task of performing complicated surgery. However, they might not achieve the optimal desired outcome due to inadequate experience. Both scenarios create a situation where quality control of treatment might be reduced in the para-aortic lymphadenectomy group. The situation easily occurs in randomized surgical trials intended for high-risk patients. It is generally accepted that RCT are internally valid. However, non-participation of experienced doctors is a threat to external validity. Heterogeneity of intervention is also a threat to internal validity. Should we stick to randomized surgical trials intended for high-risk patients? A high-risk group is not suitable for a randomized surgical trial. In my humble opinion, a prospective cohort study is an option for assessing the role of lymphadenectomy in high-risk endometrial cancer because it would promote homogeneity of surgical intervention.

There are two interesting reports published in the *New England Journal of Medicine* in which results of

RCT and those of well-designed observational studies on the same topics were compared.[6,7] Benson *et al.* reviewed 136 reports about 19 diverse treatments, such as calcium channel-blocker therapy for coronary artery disease, and hormone-replacement therapy for osteoporosis, and showed that well-designed observational studies and RCT overall produce similar results.[6] Concato *et al.* reviewed 99 reports published in five major journals (*Annals of Internal Medicine*, *The British Medical Journal*, *The Journal of the American Medical Association*, *The Lancet*, and *The New England Journal of Medicine*) about five clinical topics and showed that results of RCT are inconsistent in some series. In contrast, results of well-designed observational studies are mostly consistent.[7] In view of the reproducibility of study results, observational studies were superior. How can we account for these results? McKee *et al.* pointed out that RCT have been conducted using very small groups and that subjects excluded from an RCT tend to have a poorer prognosis than that of subjects included in the trial.[8] RCT definitely rank at the top of all types of clinical studies because they are internally valid. However, the results of RCT are relevant to just a definable group of patients in a particular setting. Therefore, results of RCT cannot be easily overgeneralized.

Reasons for Preoperative Risk Assessment in Surgical Studies

What should we do in order to maximize the therapeutic effect of surgery and minimize its invasiveness? Two strategies are: (i) to remove lymph nodes most likely to harbor disease and spare lymph nodes that are unlikely to be affected; and (ii) to allocate only patients with potential benefit from lymphadenectomy to full lymphadenectomy. The first strategy includes sentinel lymph node (SLN) mapping surgery[9–11] and circumflex iliac nodes distal to the external iliac nodes (CINDEIN)-sparing surgery.[12–14] The second strategy needs preoperative risk assessment. However, it has not been clarified which patients have potential benefit from lymphadenectomy. In this session, we focus on the second strategy. GOG #33 showed that there was no case with nodal metastasis in the low-risk group defined as having no myometrial invasion, grade 1 endometrioid histology, and no intraperitoneal disease.[15] Mariani *et al.* confirmed a low-risk group with grade 1 to 2 endometrioid histology, depth of invasion of ≤50%, and tumor size of ≤2 cm.[16] They concluded that lymphadenectomy does not benefit patients in the low-risk group (so-called Mayo criteria). Milam *et al.* also demonstrated that these criteria led to a rate of nodal metastasis of only 0.8% in the low-risk group of the Mayo criteria.[17] However, all of these criteria depend on surgicopathologic findings. There have been only a few studies that aimed to establish preoperative risk assessment for predicting lymph node metastasis in endometrial cancer.[18,19] The results of these studies are shown in Table 1. In 2007, Todo *et al.* proposed a low-risk group with grade 1 to 2 endometrioid histology by endometrial biopsy, volume index of ≤36 by magnetic resonance imaging (MRI), and low cancer antigen (CA)-125 level (70 U/mL for patients aged less than 50 years and 28 U/mL for patients aged 50 years or over) before surgery; only 2.1% of the patients in the group had lymph node metastasis at the assumed prevalence of nodal metastasis of 10%.[18] In 2012, Kang *et al.* confirmed a low-risk group with endometrioid histology by endometrial biopsy, <50% myometrial invasion with no extension beyond the corpus and no enlarged lymph nodes by MRI, and CA-125 level ≤35 U/mL before surgery; only 1.3% of the patients in the group had lymph node metastasis when assuming that the prevalence of lymph node metastasis is 10% in the target patient cohort.[19] As many physicians are not familiar with measuring tumor volume of endometrial cancer, volume index could not be easily used as a factor of preoperative risk assessment. On the other hand, myometrial invasion assessment by MRI has a problematic issue, namely, interobserver inconsistency or variability. MRI-based evaluation of deep myometrial invasion in a multi-institutional cooperative study showed sensitivity of 54% and specificity of 89%, indicating that results of previous single institutional studies might have been biased.[20] There would be some occasions where attending physicians have difficulty in judging myometrial invasion using MRI. Although each set of criteria have their merits and demerits, it is possible to reconcile these criteria. When it is difficult to judge myometrial invasion using MRI, volume index could be used as a substitute index. When planning a prospective clinical trial on the therapeutic significance of lymphadenectomy, an adequate population is needed to assess the full benefit of lymphadenectomy. If a population comprises a large proportion of low-risk patients, the significance of lymphadenectomy would be underestimated because low-risk patients do not benefit from lymphadenectomy.

Table 1 Results of preoperative risk assessment for excluding lymph node metastasis in endometrial cancer

Author	Todo *et al.*[18]		Kang *et al.*[19]	
Journal	*Gynecol Oncol* (2007)		*J Clin Oncol* (2012)	
Study design	Retrospective cohort study		Retrospective cohort study	
Study aim	Model Derivation	Validation	Model Derivation	Validation
Cases (*n*)	214	211	360	180
Median age (range)	56 (23–80)	57 (24–77)	53 (29–76)	54 (31–82)
FIGO stage (1988)	I: 68%	I: 64%	I: 71%	I: 76%
	II: 5%	II: 8%	II: 7%	II: 5%
	III/IV: 27%	III/IV: 28%	III/IV: 20%	III/IV: 19%
	Unknown: 0%	Unknown: 0%	Unknown: 2%	Unknown: 0%
Histological subtype	Endometrioid: 97%	Endometrioid: 94%	Endometrioid: 94%	Endometrioid: 94%
	Non-endometrioid: 3%	Non-endometrioid: 6%	Non-endometrioid: 6%	Non-endometrioid: 6%
LNM (rate)	14.5%	17.1%	12.5%	12.8%
PANM (rate)	8.9%	12.3%	NA	NA
Number of lymph nodes harvested (median)	70	77	27	22
Para-aortic node dissection (rate)	99%	100%	61%	51%
Low-risk criteria for LNM	Histologic subtype/grade (endometrial biopsy): endometrioid G1 or G2 Tumor volume (MRI): <36 cm^3 CA-125: <70 U/mL (less than 50 years), <28 U/mL (50years or over)		Histologic subtype (endometrial biopsy): Endometrioid Myometrial invasion (MRI): <1/2 Extension beyond uterine corpus (MRI): none Lymph node size (MRI): <1 cm in short axis CA-125: <35 U/mL	
Proportion of patients in the low-risk group	54%	45%	53%	43%
LNM (false negative) rate in the low-risk group	3.6%	3.2%	1.7%	1.4%
Bayesian-adjusted LNM (false negative) rate in the low-risk group†	2.5%	1.9%	1.4%	1.1%

†Adjusted rate at the prevalence of nodal metastasis of 10%. CA-125, cancer antigen 125; FIGO, International Federation of Gynecology and Obstetrics; LNM, lymph node metastasis; MRI, magnetic resonance imaging; NA, not available; PANM, para-aortic node metastasis.

Disclosure

The author declares no conflicts of interest.

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MicroRNA-106b Modulates Epithelial–Mesenchymal Transition by Targeting TWIST1 in Invasive Endometrial Cancer Cell Lines

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Type II endometrial carcinoma is an aggressive subtype of endometrial cancer (EC). TWIST1, a helix-loop-helix transcription regulator, is known to induce epithelial–mesenchymal transition (EMT) and promote tumor metastasis. MicroRNAs (miRNAs) also serve as important regulators of EMT and metastasis by regulating EMT-related genes. In this study, we sought to explore the role of TWIST1 in inducing EMT in representative type II EC cell lines, and to determine the miRNAs involved in regulating TWIST1 gene expression. Functional analysis suggested that TWIST1 contributes to the EMT phenotypes of EC cells, as evidenced by the acquisition of fibroblast-like properties, enhanced invasiveness, and induction of an EN-switch (downregulation of epithelial marker E-cadherin and upregulation of mesenchymal marker N-cadherin). Conversely, silencing of TWIST1 by siRNA inhibited cell invasion and the mesenchymal phenotype, which was accompanied by a reversion of the EN-switch. We also observed a novel post-transcriptional regulatory mechanism of TWIST1 expression mediated by miR-106b via its direct interaction with TWIST1 mRNAs at the 3′-untranslated region. Our data suggest that TWIST1 is a critical inducer of EMT in invasive EC cells and that miR-106b could suppress EC cell invasion by downregulating TWIST1 expression.

Key words: EMT; endometrial cancer; TWIST1; miRNA

INTRODUCTION

Endometrial cancer (EC) can be classified into two major types, types I and II, based on histopathology, molecular profile and clinical behavior [1]. Type I (endometrioid) EC is usually low-grade, estrogen-related, diagnosed at an early stage, and has a good prognosis. Conversely, type II EC has non-endometrioid histology with a high incidence of deep myometrial invasion and lymph node metastasis [2]. At a molecular level, p53 mutations appear to be the most important genetic alterations in type II EC [3]. However, the molecular and cellular mechanisms involved in type II EC progression are still largely unknown.

Tumor cell invasion is a complex, multistep process that includes cell proliferation, cell migration, and destruction of the extracellular matrix. Epithelial–mesenchymal transition (EMT) describes a transcriptional mechanism that ensures tissue remodeling during embryonic morphogenesis and is viewed to be an important step in cancer cell dissemination and metastasis [4]. TWIST1, a helix-loop-helix transcription regulator, has been shown to promote EMT in human cancer, by directly repressing epithelial markers like E-cadherin and by upregulating mesenchymal markers such as N-cadherin [5]. A previous study showed an association between increased TWIST1 expression and deep myometrial invasion of EC [6]. Moreover, TWIST1 expression was strongly induced in EC cells where EMT was induced by irradiation [7]. Although these findings indicate a role for TWIST1 as a potential EMT and metastasis promoter in aggressive ECs, the detailed functions of this gene during the EMT process and EC cell invasion have not yet been fully investigated.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally control gene expression by base-pairing with the 3′ untranslated region (UTR) of target mRNAs, which triggers either mRNA translation repression or RNA degradation [8]. More than 1000 miRNA genes have been identified in the

Abbreviations: EC, endometrial cancer; EMT, epithelial–mesenchymal transition; miRNAs, microRNAs; UTR, untranslated region; FBS, fetal bovine serum; qRT-PCR, quantitative RT-PCR; WT, wild type.

Contract grant sponsor: Department of Women's Health Educational System; Contract grant sponsor: Ministry of Health, Labour and Welfare of Japan.

Peixin Dong and Masanori Kaneuchi are contributed equally to this work.

Conflicts of interest: None.

Authors' contributions: D.P. designed research. D.P. carried out the molecular genetic studies. D.P., K.M., W.H., S.S., and S.N. analyzed data. D.P. wrote the article. All authors read and approved the final manuscript.

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Received 28 September 2012; Accepted 25 October 2012

DOI 10.1002/mc.21983

Published online 3 September 2013 in Wiley Online Library (wileyonlinelibrary.com).

human genome (miRBase, http://www.mirbase.org) and they play regulatory roles in diverse human diseases and tumorigenesis [9]. Some miRNAs, such as miR-372, miR-373, and miR-520c, have been shown to regulate tumor metastasis by downregulating target genes expression [10,11]. In addition, miR-200 and miR-192 family members have important regulatory roles in EMT and metastasis by regulating expression of EMT-related genes [12]. We previously showed that miR-194 can inhibit EMT and invasion of EC cells by targeting the oncogene BMI-1 [13]. However, the molecular mechanisms of miRNA-mediated TWIST1 gene regulation in type II EC cells remain unclear.

In the present report, we show that TWIST1 is a critical inducer of EMT in type II EC cells and that its expression is negatively regulated by miR-106b.

MATERIALS AND METHODS

Cell Lines and Culture

The endometrial cancer cell lines HEC-50 and HEC-1, which represent the aggressive type II ECs [14,15], were cultured in DMEM/F12 medium (Sigma–Aldrich, Poole, UK) supplemented with 15% fetal bovine serum (FBS). HOUA-I cells (undifferentiated EC cells) were cultured in DMEM/F12 medium (Sigma–Aldrich, UK) containing 20% FBS. All cell lines used were obtained from the RIKEN cell bank (Tsukuba, Japan). The immortalized human endometrial epithelial cell line EM-ER-A [16] was kindly provided by Professor Satoru Kyo (Kanazawa University, Ishikawa, Japan), and maintained in DMEM/F12 medium supplemented with 15% FBS.

Plasmids and Stable Transfection

TWIST1 cDNA in an expression vector was purchased from OriGene Technologies (Rockville, MD). HEC-1 cells were stably transfected as previously described [17]. In brief, upon reaching 80% confluency, cells were transfected with Lipofectamine PLUS Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols and selected in DMEM/F12 medium (Sigma, UK) containing 0.5 mg/mL G418 (Sigma, St. Louis, MO) at 48 h post-transfection. The selected cell clones of cells were then expanded.

Transient Transfection

HEC-50 cells (50% confluence) were transfected with 10 nM TWIST1 siRNA or control siRNA (Ambion, Austin, TX) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h, the cells were used for protein extraction.

Enforced Expression and Knockdown of miR-106b

The precursor miR-106b (miR-106b), negative control precursor miRNA (control miRNA), anti-miR-106b inhibitor (anti-miR-106b), and the negative control of anti-miRNA (control anti-miRNA; Ambion) were transfected into EC cells, using Lipofectamine 2000 (Invitrogen) at a final concentration of 15 or 30 nM. The cells were then harvested for analysis 24 h post-transfection.

Western Blot Analysis

Whole cell lysates were obtained using the M-Per Mammalian Protein Extraction Reagent (Pierce Biotechnology, Woburn, MA). Proteins (30 μg) were separated on 10% SDS–PAGE and transferred to nitrocellulose membranes. Antigen–antibody complexes were detected using the enhanced chemiluminescence (ECL) blotting analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The following antibodies were used: mouse rabbit polyclonal anti-Twist (sc-81417), mouse monoclonal anti-GAPDH (sc-47724; both Santa Cruz Biotechnology, Santa Cruz, CA),. rabbit polyclonal anti-E-cadherin (A01589), and mouse monoclonal anti-N-cadherin (both BD, Transduction, San Jose, CA). Immunoblot images were digitized and quantified using the NIH Image software.

miRNA Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNA containing small RNA was extracted from cell lines using the mirVana miRNA isolation kit (Ambion). qRT-PCR was performed to quantify mature miRNA expression by NCode miRNA qRT-PCR analysis (Invitrogen) according to the manufacturer's protocol. The forward primer used for qRT-PCR was the exact sequence of the mature miR-106b (TAAAGTGCTGACAGTGCAGAT). GAPDH was used for normalization [18]. Quantitative miRNA expression data were acquired and analyzed using an Applied Biosystems 7300 real-time PCR system.

Luciferase Activity Assay

The 3′-UTR vector of TWIST1 containing an intact miR-106b recognition sequence was purchased from OriGene. A pGL3 construct containing TWIST1 3′-UTR with point mutations in the seed sequence was constructed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), using the following primers: 5′-GTGGGGCGCAACCTTAAAA-GAGAAAG-3′ (forward) and 5′-CTTTCTCTTTTAA-GGTTGCGCCCCAC-3′ (reverse). Cells were transfected with 30 nM miR-106b, control miRNA, anti-miR-106b, and control anti-miRNA (Ambion), along with the wild-type or mutant TWIST1 3′-UTR-luciferase constructs. At 48 h after transfection, the luciferase activity was measured using a dual-luciferase assay (Promega, Madison, WI).

In Vitro Cell Invasion Assay

At 24 h post-transfection, HEC-50, HEC-1, or HOUA-I cells (1×10^5) in 500 μL serum-free medium were added to the upper chamber of a transwell

plate while 750 μL medium supplemented with 15% FBS was added to the lower chamber. The cells were allowed to migrate through the intermediate membrane for 24 h at 37°C. Membranes were then fixed with 10% formalin and stained in 1% toluidine blue solution. The cells attached to the lower side of the membrane were counted under a microscope in ten high-power (200×) fields. Assays were performed in triplicate for each experiment with each experiment repeated three times.

qRT-PCRs for EMT Markers

Total RNAs was reverse-transcribed using a Takara PrimeScript RT reagent kit (Takara, Japan). qRT-PCRs were performed with the Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA) using the Takara SYBR Premix Ex Taq II (Takara, Japan). Primers for BMI-1, CK-18, vimentin, and GAPDH were obtained from PrimerBank database (http://pga.mgh.harvard.edu/primerbank/).

Cell Viability Assay

3×10^3 HEC-50 or HOUA-I cells were plated into 96-well plates and transfected with 30 nM of miR-106b, anti-miR-106b or non-specific controls, respectively. After 48 h, 10 μL of MTT solution (cell counting kit-8, Dojindo, Japan) was added into each well and the plates were incubated for additional 4 h at 37°C. The UV absorbance of each sample was then measured in a microplate reader at 450 nm. The experiment was performed in triplicate wells and repeated three times.

Cell Apoptosis Assay

3×10^3 HEC-50 or HOUA-I cells were plated in triplicates in 96-well plates and transfected with30 nM of miR-106b, anti-miR-106b or non-specific controls, respectively. Apoptosis in EC cells was determined using the Caspase-Glo 3/7 assay kit according to the manufacturer's instructions (Promega, Mannheim, Germany). Luminescence was measured after 3 h of incubation with the caspase substrate.

Statistical Analysis

All experiments were performed in triplicate. Statistical analyses were performed using SPSS statistical software and Student's *t*-test. Significance was defined as $P < 0.05$.

RESULTS

TWIST1 Is Up-Regulated in Invasive EC Cell Lines

We first evaluated endogenous TWIST1expression by qRT-PCR and Western blot analysis in three human EC-derived cell lines along with immortalized human endometrial epithelial cells, and asked whether their expression levels correlated with the invasive properties of EC cells. Both the mRNA (Figure 1A) and protein levels (Figure 1B and C) of TWIST1 were higher in EC cell lines compared to the human endometrial epithelial cell line EM-ER-A. Importantly, two highly invasive type II EC cell lines (HEC-50 and HEC-1) expressed higher TWIST1 levels compared to the less invasive HOUA-I cells, suggesting that TWIST1 has possible roles in mediating EC cell invasion.

TWIST1 Contributes to the Invasive Phenotype of EC Cells

To further define whether TWIST1 expression is involved in regulating the invasive properties of EC, we generated HEC-1 cells that stably expressed TWIST1 cDNA. TWIST overexpression was confirmed by immunoblotting (Figure 2A and B). We next performed a cell invasion assay and observed a significant increase in the invasive capacity of TWIST1-expressing cells compared to mock cells transfected with empty vector (Figure 2C). To further investigate whether TWIST1 expression is responsible for increased EC cell invasion, TWIST1-specific siRNA was used to knockdown TWIST1 expression in HEC-50 cells (Figure 2D and E). Transfection of HEC-50 cells with TWIST1 siRNA, but not control siRNA, led to reduced cell invasion (Figure 2F).

TWIST1 Induces EMT in EC Cells

In addition to the effects on cell invasion, we found that modulation of TWIST1 expression corresponded to marked changes in human EC cell morphology. Compared to mock-transfected cells that had a round, tightly packed morphology, the morphology of HEC-1 cells transfected with TWIST1 cDNA was dramatically changed from epithelial-like to a spindle-shaped appearance (Figure 3A). In agreement with this finding, HEC-50 cells where TWIST1 was depleted by siRNA showed tight cell-to-cell contacts and decreased cellular scattering (Figure 3B). We further examined the expression of epithelial and mesenchymal markers by immunoblotting. Up-regulation of TWIST1 in HEC-1 cells induced an EN-switch (Figure 3C and D), which is a process involved in EMT during cancer metastasis [19]. In contrast, when TWIST1 was downregulated by siRNA in invasive HEC-50 cells, they showed increased E-cadherin and decreased N-cadherin expression, respectively (Figure 3E and F). We previously showed that the oncogene BMI-1, which is known to be a downstream effector of TWIST1 [20], is involved in inducing an EMT phenotype in EC cells [13]. Since EMT is often associated with loss of the epithelial marker CK-18 and the gain of the mesenchymal marker vimentin [4], we investigated the mRNA expression of BMI-1, CK-18, and vimentin using qRT-PCR. Ectopic expression of TWIST1 in HEC-1 cells led to increased expression of BMI-1 and Vimentin and decreased expression of CK-18 (Figure 3G). In contrast, transfection of HEC-50 cells

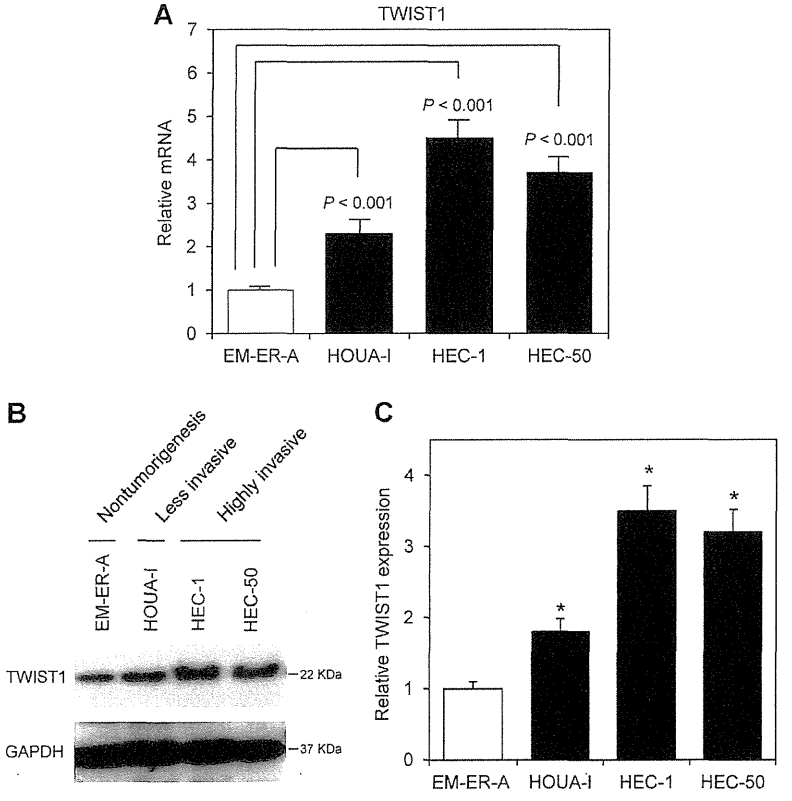

Figure 1. TWSIT1 levels are upregulated in invasive EC cell lines. Expression of TWIST1 in immortalized human endometrial epithelial cells EM-ER-A, less invasive EC cell line HOUA-I, and highly invasive EC cell lines HEC-1 and HEC-50, as detected by qRT-PCR analysis (A) and Western blot analysis (B). All qRT-PCR values were normalized to GAPDH (mean ± SD; $n = 3$). (C) Quantitative analysis of the Western blots shown in B (mean ± SD; $n = 3$; $^*P < 0.05$, normalized to GAPDH).

with TWIST1 siRNA, but not control siRNA, resulted in a significant suppression of BMI-1 and vimentin and upregulation of CK-18 (Figure 3H). Taken together, these data demonstrate the critical role of TWIST1 activation in the induction of EMT and the invasive phenotypes of EC cells.

Inverse Correlation Between TWIST1 and miR-106b Expression Levels

To identify candidate miRNAs that can control EC cell invasion by modulating TWIST1 expression, we used two target-prediction algorithms TargetScan 6.2 (http://www.targetscan.org) and microRNA.org (http://www.microrna.org) to search for miRNA binding sites in the TWIST1 3′-UTR and identified 179 miRNAs that potentially target TWIST1. Among these 179 miRNAs, only 29 were predicted by these two algorithms (Figure 4A). miR-106b was of particular interest, because it was downregulated by mutant p53s in HEC-50 cells [21]. Bioinformatics analysis revealed the predicted binding site of miR-106b within the TWIST1 3′-UTR (Figure 4B), which is conserved among species (Figure 4C), indicating that miR-106b is a potential miRNA targeting TWIST1. We therefore hypothesized that miR-106b is a tumor suppressor gene in EC and that repression of this miRNA could contribute to increased TWIST1 expression in invasive EC cells.

To determine whether there is a relationship between the expression of miR-106b and TWIST1, we examined the endogenous miR-106b expression level by qRT-PCR in EC cell lines (HOUA-I, HEC-1, and HEC-50) and the immortalized endometrial epithelial cell line EM-ER-A. When comparing EM-ER-A cells and the less invasive HOUA-I cells, miR-106b expression was significantly lower in invasive HEC-1 and HEC-50 cells that express higher levels of TWIST1 (Figure 4D), showing that miR-106b levels inversely correlate with TWIST1 expression.

miR-106b Directly Targets the TWIST1 3′-UTR and Reverses the Invasive, EMT Phenotype of EC Cells

To examine the effects of miR-106b on TWIST1 mRNA and protein levels, we performed qPCR and

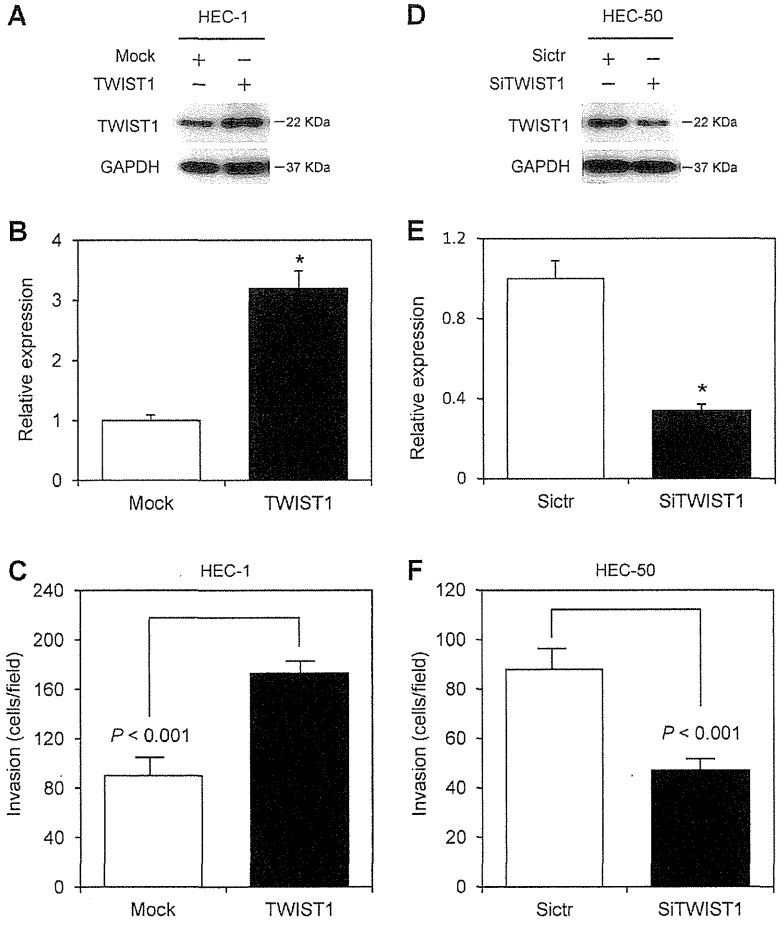

Figure 2. TWIST1 contributes to the invasive phenotype of EC cells. (A) Protein expression of TWIST1 as analyzed by immunoblot in HEC-1 cells transfected with empty vector (mock) or TWIST1 expression vector. (B) Quantitative analysis of the Western blots shown in A (mean ± SD; $n = 3$; $^*P < 0.01$, normalized to GAPDH). (C) Invasion assay of HEC-1 cells following overexpression of TWIST1 (mean ± SD; $n = 3$). (D) Western blot analysis of TWIST1 expression in HEC-50 cells transfected with control siRNA (Sictr) or TWIST1 siRNA (SiTWIST1). (E) Quantitative analysis of the Western blots shown in D (mean ± SD; $n = 3$; $^*P < 0.01$, normalized to GAPDH). (F) Invasion assay of HEC-50 cells transfected with control siRNA or TWIST1 siRNA (mean ± SD; $n = 3$).

Western blot analysis 48 h after miR-106b transfection of HEC-50 cells. Overexpression of synthetic precursors of miR-106b significantly decreased the mRNA (Figure 5A) and protein levels (Figure 5B and C) of TWIST1 in HEC-50 cells compared to control miRNA. In contrast, knockdown of miR-106b with anti-miR-106b in HOUA-I cells led to increased TWIST1 mRNA (Figure 5D) and protein expression (Figure 5E and F). These results verified the repression of TWIST1 by miR-106b.

To confirm whether miR-106 directly targets the 3′-UTR of TWIST1 mRNA, firefly luciferase reporter vectors containing either the wild type (WT) TWIST1 3′-UTR, or TWIST1 3′-UTR with a mutation in the predicted miR-106b target sequence, were co-transfected into HEC-50 cells together with miR-106b or control miRNA. Introduction of miR-106b resulted in marked inhibition of the WT TWIST1 3′-UTR, but had no effect on the mutant TWIST1 3′-UTR (Figure 5G). In contrast, miR-106b inhibition by anti-miR-106b in HOUA-I cells substantially increased the luciferase activities of WT TWIST1 3′-UTR compared to control anti-miRNA (Figure 5H). These data suggest that miR-106b directly targets the 3′-UTR of TWIST1 and represses its expression.

According to our observations, TWIST1 induces EMT to promote EC cell invasion, so we hypothesized that restoration of miR-106b in EC cells via TWIST1downregulation could affect cell invasion. To this end, invasion assays were performed using HEC-50 cells transfected with miR-106b or HOUA-I cells transduced with anti-miR-106b. A significant

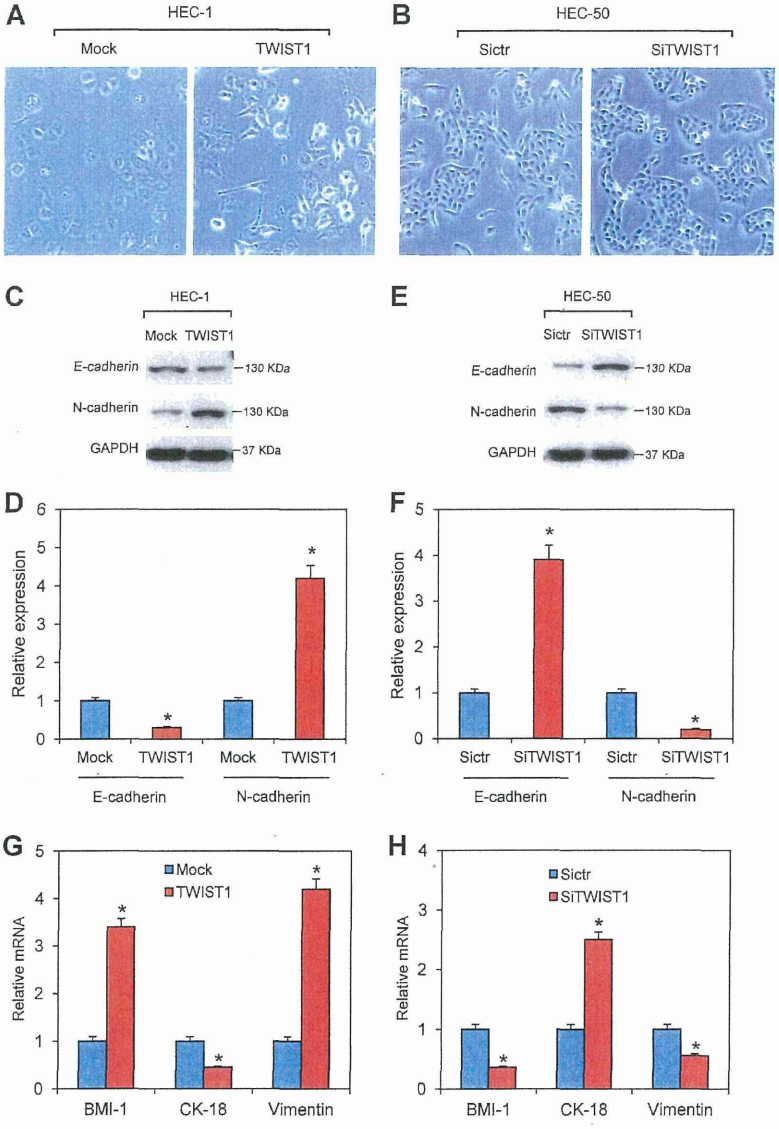

Figure 3. TWIST1 induces EMT in EC cells. (A) Morphology of HEC-1 cells transduced by TWIST1-expressing vector, or empty vector. (B) Images of HEC-50 cells transfected with control siRNA or TWIST1 siRNA. Western blot examined the protein level of epithelial marker E-cadherin and mesenchymal marker N-cadherin after overexpression of TWIST1 in HEC-1 cells (C), or knockdown of TWIST1 in HEC-50 cells (E). (D and F) Quantitative analysis of the Western blots shown in C and E, respectively (mean ± SD; $n = 3$; $^*P < 0.01$, normalized to GAPDH). Relative mRNA expression of epithelial marker CK-18 and mesenchymal markers (BMI-1 and Vimentin) in HEC-1 cells transduced by TWIST1-expressing vector, or empty vector (G), or in HEC-50 cells after TWIST1 silencing by TWIST1 siRNA (H), determined by qRT-PCR (mean ± SD; $n = 3$; $^*P < 0.01$, normalized to GAPDH).

decrease in invasion was observed in miR-106b-transfected HEC-50 cells compared with control cells (Figure 6A) while miR-106b knockdown by anti-miR-106b in HOUA-I cells enhanced cell invasion (Figure 6B).

Transfection of miR-106b, but not control miRNA, consistently induced a loss of the mesenchymal phenotype (Figure 6C) by restoring the expression of epithelial marker E-cadherin and reducing mesenchymal marker N-cadherin expression in HEC-50 cells (Figure 6D and E). In the EC cell line HOUA-I, transfection with anti-miR-106b induced a significant change in cell morphology from an epithelial phenotype to one with a mesenchymal appearance (Figure 6F) and an EN-switch (Figure 6G and H). Similar to TWIST1 siRNA, miR-106b significantly

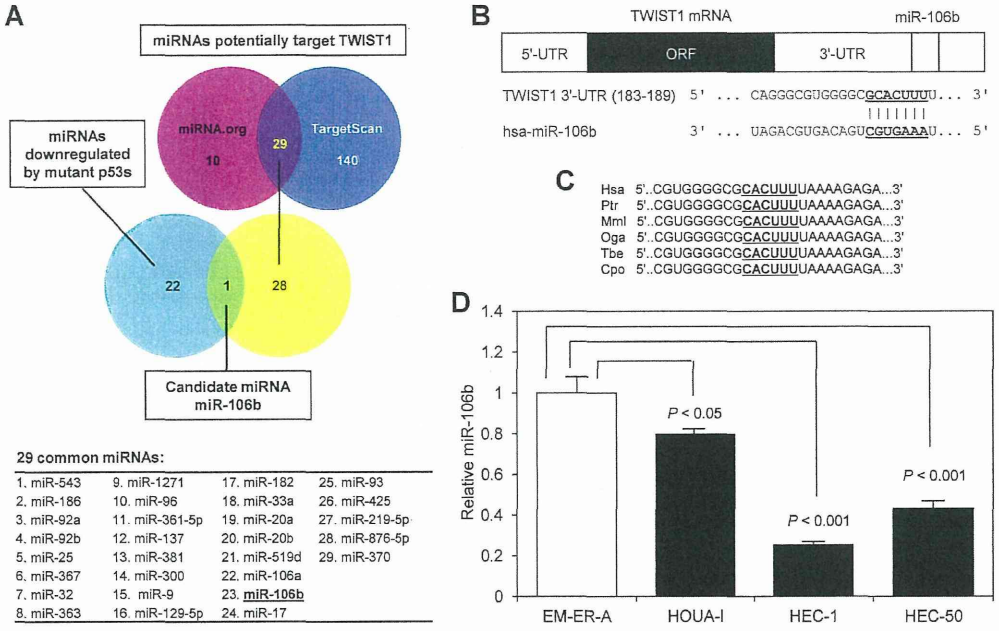

Figure 4. Inverse correlation between TWIST1 and miR-106b expression levels. (A) Summary of the number of miRNAs that were predicted to bind to the 3′-UTR of TWIST1 by TargetScan and microRNA.org (upper panel). The 29 predicted miRNAs were common to these two algorithms. MiR-106b, a miRNA that is downregulated by mutant p53s in EC cells, was highlighted (lower panel). (B) Schematic representation of the 3′-UTR of TWIST1 with the predicted target site for miR-106b. (C) Sequence of mature miR-106b reveals the evolutionary conservation of the target site across six species. (D) Relative miR-106b expression in immortalized human endometrial epithelial cells EM-ER-A and in three EC cell lines (HOUA-I, HEC-1, and HEC-50), as detected by qRT-PCR analysis (mean ± SD; $n = 3$, normalized to GAPDH).

inhibited BMI-1 and vimentin expression, and promoted CK-18 expression in HEC-50 cells (Figure 6I). On the other hand, we observed upregulation of BMI-1 and vimentin with CK-18 downregulation in HOUA-I cells following transfection with anti-miR-106b (Figure 6J), which is consistent with the effects caused by TWIST1 overexpression. Taken together, our results suggest that miR-106b directly targets TWIST1, and thereby suppresses EMT-associated EC cell invasion.

The Effects of miR-106b on EC Cell Viability and Apoptosis

Since changes in cell viability or cell apoptosis might influence tumor cell invasion, we next study the impact of miR-106b on cell viability and apoptosis of EC cells 2 d after transfection. Overexpression or knockdown of miR-106b had no significant effect on cell viability and cell apoptosis in HEC-50 cells or HOUA-I cells, respectively (Figure 7). Our results suggest that the impairment of cell invasion by miR-106b restoration appears not to be due to the loss of viability or increased apoptosis of EC cells.

DISCUSSION

EMT occurs during development and cancer metastasis and results in enhanced cell invasion. Accumulating evidence suggests that TWIST1 has crucial roles in conferring the invasive potential of various human cancers [22]. However, the potential contributions of the TWIST1 gene to the EMT phenotypes and invasion in aggressive EC cells remain unclear. In this work, we performed in vitro gain-of-function (overexpression) and loss-of-function (siRNA) experiments to show that TWIST1 promotes cell invasion by inducing the EMT in invasive EC cells, which supports a role for TWIST1 as a critical regulator of EMT induction in EC cells. These effects were achieved by inducing an EN-switch. Therefore, our data provide new evidence to demonstrate that TWIST1 has a pivotal role in conferring the invasive and EMT potential of type II EC cells.

miRNAs have important roles in modulating tumor metastasis [23]. Our study revealed that miR-106b represses TWIST1 levels by directly targeting TWIST1 mRNA and subsequently causing a decrease in cell invasion. Therefore, miR-106b may serve as a potential therapeutic target for those EC patients who have high TWIST1 levels.

miR-106b was previously reported to be frequently overexpressed in various human tumors including gastric cancer [24], colorectal cancer [25], and hepatocellular carcinoma [26]. Upregulation of miR-106b is also associated with enhanced cancer cell

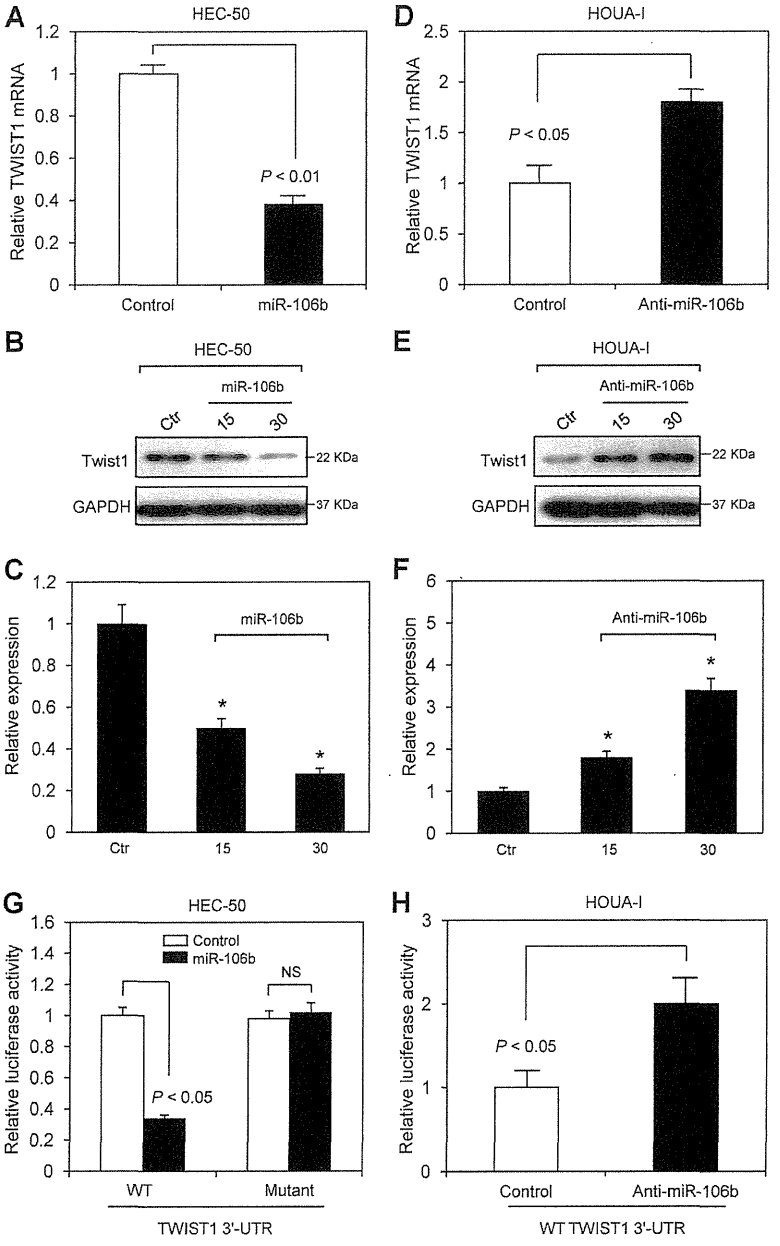

Figure 5. MiR-106b directly targets the TWIST1 3′-UTR. qRT-PCR (A) and Western blotting (B and C) examined the TWIST1 mRNA and protein level in HEC-50 cells transfection with pre-miR-106b or control miRNA. TWIST1 levels were determined by qRT-PCR (D) and Western blotting (E and F) in HOUA-I cells transfected with anti-miR-106b or control anti-miRNA (mean ± SD; $n = 3$; $^*P < 0.01$, normalized to GAPDH). (G) Reporter constructs containing either wild type (WT) TWIST1 3′-UTR, or TWIST1 3′-UTR with mutation at the predicted miR-106b target sequence were co-transfected into HEC-50 cells, along with miR-106b or control miRNA (mean ± SD; $n = 3$). NS, not significant. (H) HOUA-I cells were transfected with WT TWIST1 3′-UTR luciferase vectors, along with anti-miR-106b or control anti-miRNA. Luciferase activity was measured at 48 h after transfection (mean ± SD; $n = 3$).

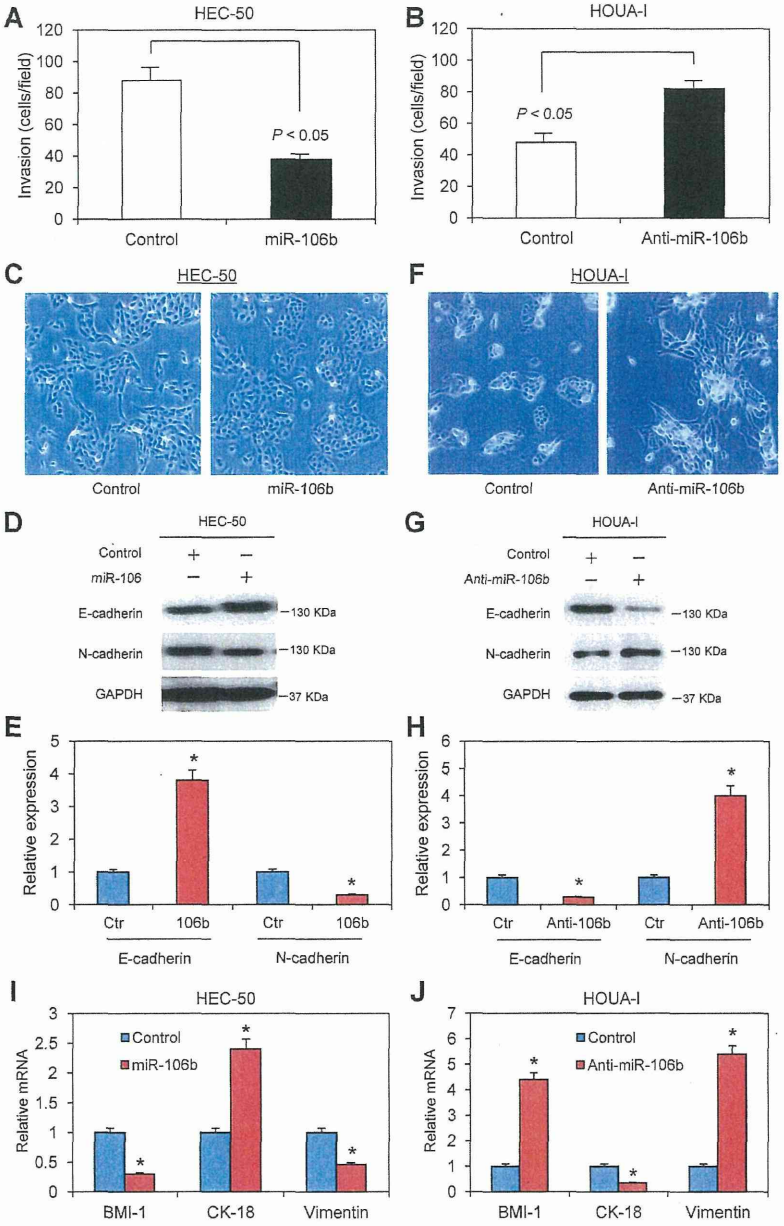

Figure 6. MiR-106b blocks EMT-associated EC cell invasion by silencing TWIST1. (A) Invasion assay of HEC-50 cells transfected with miR-106b or control miRNA (mean ± SD; $n = 3$). (B) The invasive activity of HOUA-I cells transfected with anti-miR-106b or control anti-miRNA (mean ± SD; $n = 3$). Representative images of HEC-50 cells (C) or HOUA-I cells (F) transfected as described above. Immunoblot of epithelial marker E-cadherin and mesenchymal marker N-cadherin after restoration of miR-106b expression in HEC-50 cells (D and E), or after knockdown of miR-106b in HOUA-I cells (G and H, mean ± SD, $n = 3$, $^*P < 0.01$, normalized to GAPDH). Relative mRNA expression of epithelial marker CK-18 and mesenchymal markers (BMI-1 and Vimentin) in HEC-50 cells transfected with miR-106b or control miRNA (I), or in HOUA-I cells transfected with anti-miR-106b or control anti-miRNA (J), as determined by qRT-PCR (mean ± SD; $n = 3$; $^*P < 0.01$, normalized to GAPDH).

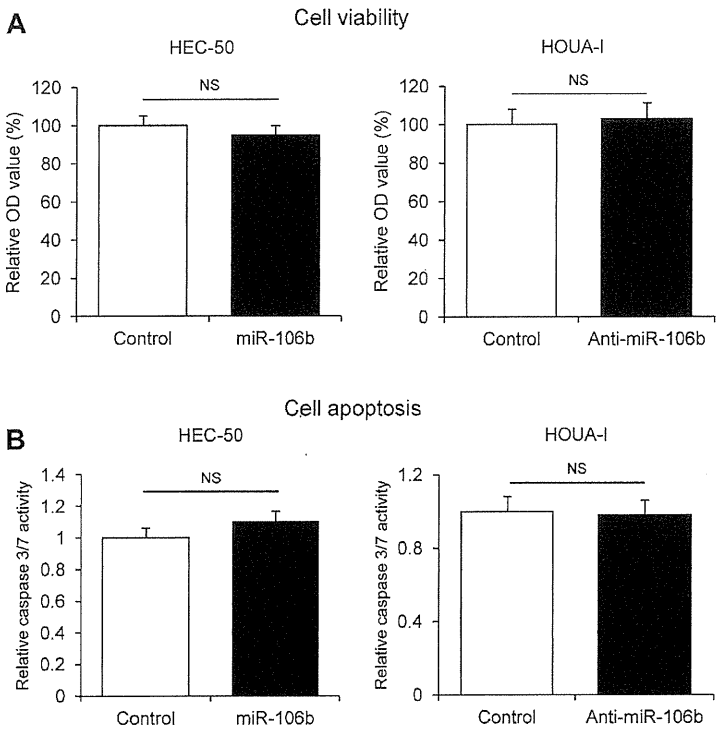

Figure 7. The impacts of miR-106b on EC cell viability and apoptosis. HEC-50 cells or HOUA-I cells were transfected with 30 nM of miR-106b, anti-miR-106b or non-specific controls for 48 h, respectively. Cell viability assay (A) and cell apoptosis assay (B) were performed (mean ± SD; $n = 3$, NS, not significant).

proliferation [27], and invasion [28] in human tumors. However, our experiments suggest that miR-106b is decreased in EC cells with highly invasive properties, and that it can inhibit the expression of TWIST1, a key promoter of the EMT and EC cell invasion. This result is consistent with previous findings showing that the level of miR-106b was significantly lower in renal cell carcinoma patients who developed metastasis, and with higher miR-106b expression levels predicting a better prognosis [29]. At least two possible explanations may account for the contradictory roles of miR-106b in different tumor types: (1) miR-106b may have dual functions both an oncogene and tumor suppressor gene depending on the cancer type and cellular context; and (2) Many miRNAs have been shown to correlate with subtypes of a particular cancer [30], implying that miRNAs may play distinct roles in tumor progression in different cancer subtypes. Since type II EC was shown to have distinct miRNA signatures compared with type I EC [31], we postulate that loss of miR-106b might be characteristic of aggressive EC cells with EMT phenotypes, and that profiling miR-106 expression levels might be helpful for predicting the risk of metastasis in patients with type II ECs.

CONCLUSION

Our results define the tumor suppressor function of miR-106b in regulating EMT and cell invasion by targeting TWIST1 in aggressive EC cells and suggest that restoration of miR-106b might be useful for the clinical management of type II EC metastasis.

ACKNOWLEDGMENTS

We thank Dr. Zhujie Xu for excellent technical assistance.

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