厚生局長先進医療受理通知書

関厚発　0512　第48号
平成　22年　5月12日

埼玉医科大学国際医療センター
開　設　者　様

関東信越厚生局長

先進医療の届出に係る施設基準について

記

貴医療機関から届出のあった標記について、厚生労働省保険局医療課より、連絡があったので、下記のとおり通知します。

記

1　受　理　番　号　厚生労働省発保０４２６第１号
2　算定開始年月日　平成２２年　５月　１日
3　先進医療技術名：上皮性卵巣癌・卵管癌・腹膜原発癌に対するパクリタキセル毎週静脈内投与併用カルボプラチン３周毎複腔内投与

1　受　理　番　号　厚生労働省発保０４２６第２号
2　算定開始年月日　平成２２年　５月　１日
3　先進医療技術名：再発卵巣癌、原発性腹膜癌、卵管癌に対する標準化学療法とベバシズマブの併用療法およびベバシズマブ独独の維持療法

保険医療機関名：埼玉医科大学国際医療センター

厚生労働省発保０４２６第１号

医療機関名　埼玉医科大学国際医療センター
所　在　地　埼玉県日高市山根１３９７－１
開設者氏名　丸　木　　清　浩

下記の医療については、厚生労働大臣の定める評価療養及び選定療養（平成18年厚生労働省告示第495号）第１条第１号の規定に基づく厚生労働大臣の定める先進医療の評価を「適」とする。

平成２２年４月２６日

厚生労働大臣　　長　　妻　　昭

記

先進医療技術名：上皮性卵巣癌・卵管癌・腹膜原発癌に対するパクリタキセル毎週静脈内投与併用カルボプラチン３週毎腹腔内投与

厚生労働省発保０４２６第２号

医療機関名　埼玉医科大学国際医療センター
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記

先進医療技術名：再発卵巣癌、原発性腹膜癌、卵管癌に対する標準化学療法とベバシズマブの併用療法およびベバシズマブ単独の維持療法

V. 文 献

REVIEW

Accuracy of the Papanicolaou Test in Screening for and Follow-up of Cervical Cytologic Abnormalities: A Systematic Review

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Purpose: To evaluate the accuracy of conventional and new methods of Papanicolaou (Pap) testing when used to detect cervical cancer and its precursors.

Data Sources: Systematic search of English-language literature through October 1999 using MEDLINE, EMBASE, other computerized databases, and hand searching.

Study Selection: All studies that compared Pap testing (conventional methods, computer screening or rescreening, or monolayer cytology) with a concurrent reference standard (histologic examination, colposcopy, or cytology).

Data Extraction: Two reviewers independently reviewed selection criteria and completed 2 × 2 tables for each study.

Data Synthesis: 94 studies of the conventional Pap test and three studies of monolayer cytology met inclusion criteria. No studies of computerized screening were included. Data were organized by cytologic and histologic thresholds used to define disease. For conventional Pap tests, estimates of sensitivity and specificity varied greatly in individual studies. Methodologic quality and frequency of histologic abnormalities also varied greatly between studies. In the 12 studies with the least biased estimates, sensitivity ranged from 30% to 87% and specificity ranged from 86% to 100%.

Conclusions: Insufficient high-quality data exist to estimate test operating characteristics of new cytologic methods for cervical screening. Future studies of these technologies should apply adequate reference standards. Most studies of the conventional Pap test are severely biased: The best estimates suggest that it is only moderately accurate and does not achieve concurrently high sensitivity and specificity. Cost-effectiveness models of cervical cancer screening should use more conservative estimates of Pap test sensitivity.

Ann Intern Med. 2000;132:810-819.

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Since the implementation of widespread screening with the Papanicolaou (Pap) test, rates of cervical cancer in the United States have decreased from 14.2 per 100 000 in 1973 to 7.8 per 100 000 in 1994. Nevertheless, cervical cancer is still the ninth-leading cause of cancer deaths among U.S. women (1). Most of these deaths occur in women who have never had a Pap test, but some occur in women who recently received negative test results. Approximately two thirds of false-negative results are caused by sampling error, and the rest are caused by detection error.

Sampling error occurs when abnormal cells are not collected or are not transferred to the Pap slide, and detection error occurs when abnormal cells on the Pap slide are missed or misinterpreted. The most common sampling error is lack of cells from the cervical transformation zone. To reduce sampling error, an endocervical cytobrush and a spatula can be used instead of a cotton swab. However, a recent meta-analysis found that the Pap test did not differ in sensitivity or specificity when different sampling devices were used (2). The Food and Drug Administration (FDA) has approved another potential solution: liquid-based monolayer preparation (ThinPrep, Cytyc Corp., Boxborough, Massachusetts). With this technique, the sample is collected as in the conventional Pap test, but cells are then placed in a fixative solution. The cells are dispersed, collected onto a filter, and transferred to a microscopic slide for interpretation. Because samples are fixed immediately after collection, fewer cellular morphologic artifacts occur. Fewer cells on the slide are obscured because the process reduces the amounts of other sampled material, such as blood and mucus, and deposits cells on the slide in a monolayer.

To reduce detection error, some researchers advocate rescreening slides initially reported to be normal. The Clinical Laboratory Improvement Amendments of 1988 mandate rescreening of a 10% random sample of normal slides as a quality assurance measure. Rescreening can also be performed on a higher proportion of slides by using computerized technologies. The FDA has approved

two such systems, one that is algorithm-based (AutoPap QC System, TriPath Imaging, Inc., Redmond, Washington), and one that uses neural networks (PAPNET, Neuromedical Systems, Inc., Suffern, New York). PAPNET uses neural network computerized imaging of Papanicolaou smear slides to identify cells or clusters of cells that require review; it then displays up to 128 images per slide that are likely to contain abnormalities. A cytotechnologist reviews these images and decides whether to review the actual slide using light microscopy. AutoPap uses its algorithm-based decision-making technology to identify slides that exceed a certain threshold for the likelihood of abnormal cells. The laboratory can select different thresholds, corresponding to 10%, 15%, or 20% review rates. In contrast to random rescreening, AutoPap selects a sample of slides that is enriched with abnormalities, thereby including most of the slides that contain abnormalities missed by manual screening.

Another approach to reducing detection error is improving the sensitivity of the initial screening step. The FDA has recently approved a new method (AutoPap Primary Screening System, TriPath Imaging) for this indication. AutoPap Primary Screening System uses proprietary computerized algorithms to identify slides that exceed a certain threshold for the likelihood of abnormal cells. A cytotechnologist then reviews these slides. The system allows laboratories to concentrate on the 75% of slides that most likely contain abnormal cells while immediately archiving the remainder.

Sampling and detection errors are reduced when Pap test screening is repeated frequently. However, cost-effectiveness analyses have concluded that if persons are screened more than every 3 years, cost-effectiveness ratios exceed $50 000 per life-year saved (3, 4).

Precise estimates of cytologic test sensitivity and specificity are important because they may be used to determine policy decisions, such as recommendations for optimal frequency of screening, management of mild abnormalities, and use of newer methods. Our primary objective was to systematically review the operating characteristics of conventional and new methods (computer screening and monolayer slide preparation) of Pap testing in the detection of cervical cancer and its precursors. We also evaluated test performance among women with previous cytologic abnormalities. The Agency for Healthcare Research and Quality (AHRQ), under contract to Duke University (Durham, North Carolina), funded the study. An AHRQ-approved advisory panel assisted in the design, conduct, and reporting of this work, and the evidence report on which this manuscript is based was reviewed by an external peer review panel (5).

Table 1. Search Strategy

1. Vaginal smears/
2. ((Pap or Papan$) and (smear$ or test$)).tw.
3. (PAPNET or autoPap or ThinPrep).tw.
4. 1 or 2 or 3
5. exp Cervix neoplasms/
6. Cervix dysplasia/
7. Cervical Intraepithelial Neoplasia/
8. dyskaryo$.tw.
9. 5 or 6 or 7 or 8
10. exp "Sensitivity and Specificity"/
11. (sensitivity or specificity).tw.
12. exp Diagnostic errors/
13. 4 and (10 or 11 or 12)
14. 13 and 9
15. limit 14 to (human and English language)
16. Papillomavirus, Human/
17. 15 not 16

Methods

Data Sources

Data sources, including MEDLINE (from 1966), EMBASE (from 1980), HealthStar (from 1975), CancerLit (from 1983), and CINAHL (from 1983) were searched through October 1999 by using a strategy developed with a medical librarian (**Table 1**). Searches were limited to English-language studies in humans. We manually searched newly published relevant journal issues, bibliographies of included studies, and recent systematic reviews (6–9). To locate unpublished studies, we also contacted relevant professional societies and manufacturers of cytologic devices.

Study Selection

We identified studies of conventional Pap testing (with or without manual rescreening), Pap testing using monolayer slide preparation (ThinPrep), Pap testing with primary computer screening (AutoPap or PAPNET), and Pap testing with computer rescreening (AutoPap or PAPNET). Other recently developed methods, the AutoCyte PREP System and the AutoCyte SCREEN system (TriPath Imaging, Inc., Burlington, North Carolina), had not been approved by the FDA at the time of our review and were not evaluated in this study.

Study samples included women undergoing Pap testing for primary screening and those undergoing evaluation for previous cytologic abnormalities. The main outcome measures were the sensitivity and specificity of the cytologic test for detecting "cases." Cytologic abnormality was defined by one of three thresholds: atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL), and high-grade squamous intraepithelial lesions (HSIL). "Cases" were defined as histologic diagnosis of cervical intraepithelial neoplasia (CIN), grades I to III, or carcinoma.

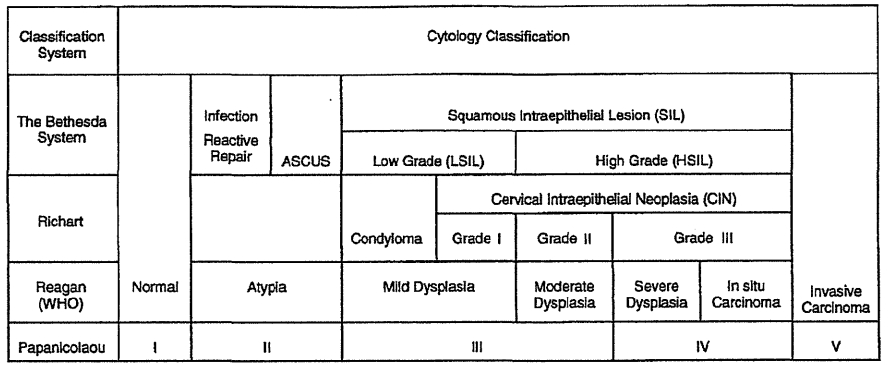

Figure. Map of classification schemes for cervical cytology. ASCUS = atypical squamous cells of undetermined significance; WHO = World Health Organization.

Equivalent categories in other classification schemes (10–14) were also used (**Figure**).

We included studies of the conventional Pap test if a reference standard of histologic examination or colposcopy was reasonably concurrent to the cytologic screening test (within 3 months) and if sufficient data were reported to complete all four cells of a 2 × 2 table. Comparison with such a reference standard provides a more relevant outcome for clinical decision makers because colposcopic or histologic diagnoses form the basis of most clinical management decisions.

Only one study of ThinPrep (15) provided enough information to allow us to extract data on sensitivity and specificity compared with a gold standard of histologic examination or colposcopy. We therefore used a separate set of screening criteria for studies of the new methods, based on cytology society guidelines (16, 17) and FDA documents (18): 1) The study must prospectively compare screening tests or test and reference standard on the same set of patients or slides; 2) if cytologic examination is the reference standard, discordant results from the two study tests must be adjudicated by an independent panel of experienced cytology professionals; 3) at least 50% of patients testing positive for HSIL must be verified by histologic examination or colposcopy; and 4) the study design must allow for separate analyses of sensitivity (or relative true-positive rate) and specificity (or relative false-positive rate).

Data based on a cytologic reference standard cannot be integrated with data based on a histologic reference standard (19–23). However, when negative test results are not verified with the reference standard, information about incremental characteristics of test performance may be obtained by directly comparing independently applied conventional and new tests (21). In this case, both tests must be applied independently to all patients, and all positive results on either test must be verified with the reference standard. A relative true-positive rate and a relative false-positive rate, which can be used to determine relative estimates of the performance of the new test, can then be calculated.

Two investigators independently screened each study. Differences of opinion were reconciled by consensus. The title and abstract of each citation were screened first, and the full report was screened second. Of the 1193 bibliographic references we reviewed, 761 (approximately 64%) were excluded on the basis of title or abstract. We reviewed the full reports of 346 studies of the conventional Pap test and 86 studies of the new methods (18 on AutoPap, 42 on PAPNET, and 26 on ThinPrep).

We developed a numeric quality score to evaluate included citations. Nine members of the study's working group (6 clinicians, 2 economists, and 1 health policy analyst) initially identified more than 12 evaluation criteria on the basis of previously reported criteria (6, 24, 25). We used a consensus process to narrow this list to 7. Blinded to the rest of the group, each participant then independently assigned numerical weights to the criteria. The means of these "votes" were calculated. Each participant received a copy of his or her responses, depicted graphically in relation to the mean for each criterion, and was requested to confirm or reconsider his or her responses. The means of the revised responses for each criterion were then calculated and used as the assigned weights.

Only 94 studies of conventional Pap testing permitted estimation of both sensitivity and specificity. The most common reasons for excluding an article on the conventional Pap test were lack of histologic examination or colposcopy as a reference standard and lack of sufficient data to complete all four cells of a 2 × 2 table.

Only three studies of ThinPrep (15, 26, 27) permitted estimation of both sensitivity and specificity. No studies of PAPNET, the AutoPap 300 QC, or the AutoPap Primary Screening System permitted

Table 2. Quality Evaluation of 94 Studies of the Conventional Papanicolaou Test

Quality Criterion (Points)	Studies, *n* (%)
Reference standard	
Histologic examination (2)	67 (71)
Histologic examination or negative colposcopy or colposcopy (1)	27 (29)
Independence of assessments	
Blinded (2)	23 (24)
Not blinded (0)	71 (76)
Verification	
All positive and negative test results verified (2)	48 (51)
Positive test results and random fraction of negative test results verified (1)	1 (1)
Positive test results and selected sample of negative test results verified (0)	45 (48)
Study sample	
Consecutive or random (2)	77 (82)
Other (0)	17 (18)
Spectrum of disease/nondisease	
Defined (1)	80 (85)
Not defined (0)	14 (15)
Publication type	
Paper (1)	94 (100)
Abstract (0)	0 (0)
Industry relation	
Not performed or supported by a manufacturer (1)	91 (97)
Supported by a manufacturer (0.5)	1 (1)
Performed by a manufacturer (0)	2 (2)

estimates of sensitivity or specificity. Studies that applied manual screening followed by computerized rescreening could not be evaluated because rescreening is conditional on a negative initial screen; thus, the two tests are not applied independently. The other studies of the new methods were excluded because they were not two-armed prospective studies or because they failed to verify at least 50% of all patients who tested positive for HSIL on histologic examination or colposcopy

Data Extraction

Two reviewers independently completed 2 × 2 tables for each study. Where available, we abstracted data at four different combinations of cytologic and histologic thresholds: ASCUS/CIN-I, LSIL/CIN-I, LSIL/CIN-II–III, and HSIL/CIN-II–III. Cases that were indeterminate because of uninterpretable cytologic results or lack of reference standard confirmation were documented but were excluded from the calculation of sensitivity and specificity. If more than one study sample was included in a single report and data for each sample were provided separately, we treated the samples as if they had been presented in individual studies.

Data Synthesis

Three studies described the accuracy of thin-layer cytology relative to histologic examination or conventional Pap testing. Bolick and Hellman (15) compared ThinPrep Pap smear diagnoses of LSIL or higher to a histologic reference standard of CIN-II–III or higher, permitting direct estimation of 94.2% sensitivity and 57.7% specificity. Conventionally prepared Pap smears achieved a sensitivity of 84.6% and a specificity of 37.0% according to the same thresholds. Most negative test results in this study were not verified with histologic examination (15). Both Roberts and colleagues (26) and Hutchinson and coworkers (27) compared conventional and ThinPrep slides prepared with a split sample technique and used a combination of cytologic and histologic examination as the reference

Table 3. Summary Statistics for Studies of the Conventional Papanicolaou Test*

Threshold	Studies, *n*	Sensitivity			Specificity			Positive Likelihood Ratio			Negative Likelihood Ratio			Prevalence		
		Minimum	50%	Maximum	Minimum	50%	Maximum	Minimum	50%	Maximum	Minimum	50%	Maximum	Minimum	50%	Maximum
ASCUS/CIN-I																
Overall	37	0.18	0.74	0.98	0.17	0.68	0.99	0.96	1.93	81.9	0.08	0.47	1.18	0.02	0.51	0.94
Verification																
All or random	21	0.31	0.68	0.92	0.17	0.75	0.99	0.96	2.38	81.9	0.11	0.50	1.18	0.02	0.36	0.91
Some or unclear	16	0.18	0.78	0.98	0.20	0.60	0.92	1.11	1.70	3.20	0.08	0.40	0.90	0.20	0.73	0.94
LSIL/CIN-I																
Overall	71	0.17	0.69	0.99	0.09	0.81	1.0	1.0	2.90	1301.8	0.03	0.44	0.99	0.02	0.64	0.95
Verification																
All or random	38	0.18	0.62	0.98	0.09	0.90	1.0	1.08	4.63	1301.8	0.13	0.49	0.86	0.02	0.43	0.94
Some or unclear	33	0.17	0.75	0.99	0.18	0.71	0.95	1.0	1.80	8.60	0.03	0.39	0.99	0.33	0.72	0.95
LSIL/CIN-II–III																
Overall	54	0.23	0.83	1.0	0.06	0.66	0.99	0.78	1.92	52.6	0.01	0.32	2.76	0.01	0.32	0.91
Verification																
All or random	31	0.23	0.81	0.99	0.06	0.77	0.99	0.78	2.27	52.6	0.01	0.37	2.76	0.01	0.24	0.91
Some or unclear	23	0.44	0.87	1.0	0.08	0.46	0.97	1.07	1.64	18.9	0.01	0.30	0.79	0.02	0.47	0.91
HSIL/CIN-II–III																
Overall	43	0.06	0.58	1.0	0.21	0.92	1.0	1.27	4.46	289.0	0.01	0.52	0.95	0.02	0.41	0.91
Verification																
All or random	25	0.18	0.53	0.92	0.64	0.96	1.0	2.10	9.95	289.0	0.12	0.53	0.84	0.02	0.28	0.91
Some or unclear	18	0.06	0.62	1.0	0.21	0.78	0.99	1.27	2.85	15.4	0.01	0.50	0.95	0.17	0.54	0.91

* ASCUS = atypical squamous cells of undetermined significance; CIN-I–III = cervical intraepithelial neoplasia, grades I–III. LSIL = low-grade squamous intraepithelial lesion; HSIL = high-grade squamous intraepithelial lesion.

Table 4. Studies of the Conventional Papanicolaou Test in Screening Samples without Verification Bias*

Study (Reference)	Year	Patients and Methods	Study Design and Characteristics	Location and Time Period	Outcomes
University of Zimbabwe/ JHPIEGO Cervical Cancer Project (115)†	1999	Consecutive women undergoing primary screening. Phase I included 8731 women (verification: some or unclear); phase II included 2203 women, but only 2147 had complete data (all verified). Mean age, 33 y.	Diagnostic test evaluation of visual inspection with acetic acid and Pap smear	Zimbabwe, 10/95–8/97	Pap smear reported as ≥LSIL; reference standard: histology or negative colposcopy, reported as ≥LSIL, HSIL
Davison and Marty (59)	1994	200 consecutive nonpregnant premenopausal women undergoing screening (196 had complete data). Women with previous abnormal smear were excluded. All included patients were verified.	Diagnostic test evaluation of screening colposcopy and Pap smear	United States, years not specified	Pap smear reported as ≥mild dysplasia; reference standard: histology or negative colposcopy, reported as ≥CIN-I
Giles et al. (79)	1988	200 predominantly middle-class women presenting for screening (24 smears were unsatisfactory). Women with previous cervical abnormalities were excluded. Mean age, 39 y. All included patients were verified.	Diagnostic test evaluation of screening colposcopy and Pap smear	United Kingdom, years not specified	Pap smear reported as ≥mild dyskaryosis; reference standard: histology or negative colposcopy, reported as ≥CIN-I (koilocytosis regarded as negative)
Baldauf et al. (70)‡	1995	1539 consecutive women undergoing routine prenatal or gynecologic examination. Mean age, 36 y. 10% random verification of test-negative patients.	Diagnostic test evaluation of cervicography and Pap smear	France, 1/91–12/92	Pap smear reported as ≥atypical cells; reference standard: histology or negative colposcopy, reported as ≥CIN-I
Guerra et al. (113)	1998	3658 consecutive pregnant women presenting for prenatal care. Mean age, 29 y. All patients were verified.	Diagnostic test evaluation of screening colposcopy and Pap smear	Italy, 2/92–3/93	Pap smear reported as ≥ASCUS, LSIL, HSIL, or microinvasion; reference standard: colposcopy, reported as ≥abnormal transformation zone with minor and major changes
Hockstad (47)	1992	73 consecutive women presenting without previous abnormal Pap smear, pelvic symptoms, or hysterectomy (2 declined and 1 did not follow-up). Age range, 15–39 y. All patients were verified.	Diagnostic test evaluation of Pap smear, HPV test, and colposcopy	United States, 1988–1989	Pap smear reported as ≥atypical or condylomatous changes; reference standard: histology or negative colposcopy, reported as ≥mild dysplasia. If women had negative screening colposcopy but positive Pap smear or HPV test, second colposcopy with biopsy was performed.
Garutti et al. (103)	1994	200 nonpregnant women referred to clinic for screening. Mean age, 41 y. All included patients were verified.	Diagnostic test evaluation of Pap smear and cervicography	Italy, 1/90–9/90	Pap smear reported as ≥HPV; reference standard: histology or negative colposcopy reported as ≥HPV
Loiudice et al. (108)	1998	3342 consecutive nonpregnant women undergoing primary screening (42 protocol violations excluded). Women who were menstruating; had previous abnormal Pap smears, HPV, or HIV; or were immunocompromised were excluded. Mean age, 33 y. All included patients were verified.	Diagnostic test evaluation of Pap smear and speculoscopy	Italy, 2/95–11/95	Pap smear reported as ≥LSIL; reference standard: histology or negative colposcopy, reported as ≥LSIL, high-grade
Kesic et al. (117)	1993	418 asymptomatic women referred for screening (23 had defective cervicograms and were excluded). All patients were verified.	Diagnostic test evaluation of Papanicolaou smear and cervicography	Yugoslavia, 1/88–8/89	Pap smear reported as ≥ class III; reference standard: histology or negative colposcopy, reported as ≥CIN-I, CIN-II–III
Londhe et al. (110)	1997	500 consecutive sexually active nonpregnant women presenting to gynecology clinic (only 372 were included in the study). All included patients were verified.	Diagnostic test evaluation of Pap smear and visual inspection with acetic acid	India, years not specified	Pap smear reported as ≥positive; reference standard: histology or negative colposcopy, reported as ≥LSIL, HSIL
Mannino (114)	1998	3049 consecutive women presenting for screening. Women with previous abnormal Pap smears, vaginitis, or menopause were excluded. All patients were verified.	Diagnostic test evaluation of Pap smear and screening colposcopy	United States, unspecified 10-year period	Pap smear reported as ≥LSIL; reference standard: histology or negative colposcopy, reported as ≥koilocytosis/CIN-I, CIN-II–III
Mann et al. (71)	1993	243 women presenting for screening. Women with previous cervical or vaginal pathologic characteristics were excluded. Mean age, 29 y. All patients were verified.	Diagnostic test evaluation of Pap smear and speculoscopy	United States, 1989–1992	Pap smear reported as ≥ atypia with condylomatous features; reference standard: histology or negative colposcopy, reported as ≥HPV/CIN-I, CIN-II–III

* ASCUS = atypical squamous cells of undetermined significance; CIN-I–III = cervical intraepithelial neoplasia, grades I–III; HPV = human papillomavirus; HSIL = high-grade squamous intraepithelial lesion; JHPIEGO = Johns Hopkins Program for International Education in Gynecology and Obstetrics; LSIL = low-grade squamous intraepithelial lesion; NS = not specified; Pap = Papanicolaou.
† Results of this study are given for phase II only.
‡ Results of this study were estimated from 10% of the patients whose negative test results were verified.

Table 4—*Continued*

Study Results			Quality Evaluation							
Prevalence	Sensitivity	Specificity	Quality Score	Reference Standard	Blinded	Verification	Consecutive	Spectrum	Publication	Industry
CIN-I: 487/2092 (0.23) CIN-II–III: 201/2092 (0.10)	LSIL/CIN-I: 0.30 LSIL/CIN-II–III: 0.44	LSIL/CIN-I: 0.92 LSIL/CIN-I: 0.91	10	1	2	2	2	1	1	1
CIN-I: 30/196 (0.15)	LSIL/CIN-I: 0.53	LSIL/CIN-I: 1.0	10	1	2	2	2	1	1	1
CIN-I: 17/176 (0.10)	LSIL/CIN-I: 0.58	LSIL/CIN-I: 0.95	10	1	2	2	2	1	1	1
CIN-I: 62/1343 (0.05)	ASCUS/CIN-I: 0.56	ASCUS/CIN-I: 0.98	9	1	2	1	2	1	1	1
CIN-I: 72/3658 (0.02) CIN-II–III: 55/3658 (0.02)	ASCUS/CIN-I: 0.90 LSIL/CIN-I: 0.87 LSIL/CIN-II–III: 0.96 HSIL/CIN-II–III: 0.88	ASCUS/CIN-I: 0.97 LSIL/CIN-I: 0.98 LSIL/CIN-II–III: 0.98 HSIL/CIN-II–III: 1.0	8	1	0	2	2	1	1	1
CIN-I: 7/70 (0.10)	ASCUS/CIN-I: 0.31	ASCUS/CIN-I: 0.96	8	1	0	2	2	1	1	1
CIN-I: 72/200 (0.36)	LSIL/CIN-I: 0.42	LSIL/CIN-I: 0.86	8	2	2	2	0	0	1	1
CIN-I: 267/3300 (0.08) CIN-II–III: 25/3300 (0.01)	LSIL/CIN-I: 0.40 LSIL/CIN-II–III: 0.75	LSIL/CIN-I: 0.96 LSIL/CIN-II–III: 0.93	8	1	0	2	2	1	1	1
CIN-I: 27/395 (0.07) CIN-II–III: 19/395 (0.05)	LSIL/CIN-I: 0.52 LSIL/CIN-II–III: 0.53	LSIL/CIN-I: 0.94 LSIL/CIN-II–III: 0.93	7	1	2	2	0	0	1	1
CIN-I: 98/372 (0.26) CIN-II–III: 23/372 (0.06)	NS/CIN-I: 0.14 NS/CIN-II–III: 0.23	NS/CIN-I: 0.96 NS/CIN-II–III: 0.95	7	1	0	2	2	0	1	1
CIN-I: 904/3049 (0.30) CIN-II–III: 60/3049 (0.02)	LSIL/CIN-I: 0.30 LSIL/CIN-II–III: 0.99	LSIL/CIN-I: 1.0 LSIL/CIN-II–III: 0.93	7	1	0	2	2	0	1	1
CIN-I: 29/243 (0.12) CIN-II–III: 6/243 (0.02)	LSIL/CIN-I: 0.32 LSIL/CIN-II–III: 0.64	LSIL/CIN-I: 0.99 LSIL/CIN-II–III: 0.97	6	1	0	2	0	1	1	1

standard. Although sensitivity and specificity could not be calculated directly, the performance of ThinPrep and conventional Pap smears could be estimated and compared. In the study by Roberts and colleagues (26), any positive result on either test was verified cytologically or histologically; histologic verification was obtained on a majority of HSIL samples. The relative true-positive rate was 1.13, indicating that ThinPrep had higher sensitivity, and the relative false-positive rate was 1.12, indicating that ThinPrep had slightly lower specificity (26). In the study by Hutchinson and coworkers (27), final reference diagnoses were made by using a combination of cytologic and histologic examination, but histologic verification was obtained for more than 90% of Pap smears that showed HSIL or cancer. The relative true-positive rate was 1.19, indicating higher sensitivity for ThinPrep, and the relative false-positive rate was 2.05, indicating lower specificity for ThinPrep (27).

Only 94 studies of the conventional Pap test met the inclusion criteria (**Tables 2** and **3**) (28–121). Sample sizes ranged from 9 to 22 412 (median, 202). Most of these studies were conducted in samples of women who were referred for previous cytologic abnormalities, had visible cervical lesions, or were at high risk for cervical cancer (for example, immunocompromised patients). Few studies evaluated low-prevalence screening samples. Most studies used histologic examination as a reference standard, but only 51% obtained verification of all or a random fraction of patients whose test results were negative. Few studies independently assessed the test and reference standard. Although most studies used adequate sample selection procedures, 15% did not provide adequate information on the spectrum of disease in their sample. All included studies were published in full-length reports; no abstracts identified in the screening process provided enough data to meet the inclusion criteria.

Data on sensitivity and specificity were available at four different combinations of test and reference standard thresholds: ASCUS/CIN-I (37 studies), LSIL/CIN-I (71 studies), LSIL/CIN-II–III (54 studies), and HSIL/CIN-II–III (43 studies). Most studies allowed construction of 2 × 2 tables using more than one combination of test and reference standard threshold. For ASCUS/CIN-I, sensitivity estimates ranged from 18% to 98% and specificity estimates ranged from 17% to 99% (**Table 3**). For studies with data at the LSIL/CIN-I threshold, sensitivity ranged from 17% to 99% and specificity ranged from 9% to 100%. As expected, when a higher disease threshold of CIN-II–III was used with the same test threshold, sensitivity was higher and specificity was lower.

Our primary objective was to obtain the best estimates of Pap test performance that were applicable to a low-prevalence screening sample. However, only 12 studies identified low-risk patients undergoing screening Pap smears and also verified all or a random fraction of patients with negative test results (**Table 4**). For the 9 studies that provided data at the LSIL/CIN-I threshold, sensitivity ranged from 30% to 87% (mean, 47%) and specificity ranged from 86% to 100% (mean, 95%). In 8 of these 9 studies, sensitivity was less than 60%. For the LSIL/CIN-II–III threshold, sensitivity was higher (range, 44% to 99%) and specificity was lower (range, 91% to 98%).

Discussion and Conclusions

Thin-layer cytology (ThinPrep), the computerized rescreening device (PAPNET), and the algorithmic classifier (AutoPap) have all received regulatory approval from the FDA. However, because of three deficiencies in methods, most studies of these technologies were excluded from our review. First, many studies did not apply the new technology and conventional Pap testing prospectively to the same sample of women. Although this allows comparison of detection rates in separate samples, it does not directly compare results in individual patients; therefore, even relative sensitivity and specificity cannot be calculated. Second, almost all studies of thin-layer cytology or computer screening or rescreening failed to verify the disease status of women who had negative test results on cytologic screening tests. Most studies applied the reference standard (adjudicated cytologic or histologic examination) only to cases in which diagnoses differed between conventional and new methods. Concordant positive and concordant negative test results are assumed to be true-positives and true-negatives but may actually be concordant false results. This study design has a consistent underlying bias that can be expected to overestimate the sensitivity and specificity of the new test (122). When the conventional tests and the new tests are conditionally dependent—that is, when tests may have similar problems with sample collection or interpretation of mild disease—this bias can be substantial. Third, little evidence is available with which to assess the effects of thin-layer cytology or computer screening or rescreening on specificity.

Conventional Pap testing is less efficient at discriminating between women who have disease and those who do not than is generally believed. However, the conventional Pap test is still the only screening test that has definitively been shown to reduce the incidence and mortality rates of cervical cancer. Because cervical cancer is usually a slow-growing disease and many low-grade lesions regress

spontaneously, serial testing with Pap smears is effective. Decision analyses have shown that Pap testing every 3 to 5 years is valuable because abnormalities missed during one screening interval will probably be detected during the next (3, 5). Most women who develop cervical cancer do so because of lack of screening rather than errors in cytodiagnosis.

Many studies of the conventional Pap test are biased. Some studies were conducted in patients from colposcopy clinics who were referred for previous cytologic abnormalities. In many of these studies, colposcopy or histologic examination was compared with the original cytologic results, all of which were abnormal. Therefore, only two cells of the 2 × 2 table could be filled in because no negative test results were verified. Such studies were excluded from our analysis. Other investigators repeated the Pap smear at the time of colposcopy and compared colposcopy or histologic examination with these repeated smears, some of which were negative. Although this design allowed completion of a 2 × 2 table, it probably biased the spectrum of disease because the study sample was taken from a population that was referred because of abnormalities on an initial Pap test (123). Women with subsequent normal Pap smears must have had an initial false-positive smear or a false-negative smear on repeated testing. We assumed the former, but if our assumption was untrue, we could have underestimated the accuracy of the test.

Our data included studies with several gold standards: histologic examination with cone biopsy, hysterectomy, or punch biopsy, and colposcopy. These reference standards are themselves subject to inaccuracy, and sensitivity and specificity may be underestimated when a diagnostic test is compared with an imperfect reference standard (124). In this case, the sensitivity and specificity of the test may vary with frequency of disease. In the included studies, the proportion of patients with disease ranged from 0.02 to 0.94.

When disease is preferentially verified among women with abnormalities but not among all women, the study sample is further biased. Many studies performed colposcopy only on women with abnormalities (on the Pap test or another test, such as human papillomavirus testing or cervicography) or those with condyloma or abnormal-appearing cervixes. By selecting such patients for verification of results, a high frequency of histologic abnormalities are included in the study sample. This is an example of verification (workup) bias, which can lead to elevated estimates of sensitivity and lowered estimates of specificity (125). In our analysis, studies that verified some or an unclear proportion of patients who tested negative had higher sensitivity and lower specificity than those that verified all patients or a random sample (**Table 3**). This finding was consistent across all cytologic and histologic thresholds.

In a previous systematic review of the accuracy of the Pap test, Fahey and colleagues (6) identified 59 studies, many of which did not meet our strict inclusion criteria. We included 58 additional studies, and many were published after the earlier review. However, Fahey and colleagues (6) found that cytologic methods had a mean sensitivity of 58% and a mean specificity of 69% in screening samples, results that are generally consistent with those of our study.

The few studies of Pap screening that were conducted in low-prevalence samples and avoided verification bias provided the best estimates of sensitivity and specificity. Although specificity was high, the sensitivity estimates are much lower than generally believed. Future decision models, cost-effectiveness studies, and health policy decisions should consider these lower sensitivity estimates in their analyses.

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Disclaimer: This article is based on an evidence report prepared by Duke University under contract with the Agency for Healthcare Research and Quality (AHRQ contract 290-97-0014). The authors are responsible for its content, including any clinical or treatment recommendations. No statement in this article should be construed as an official position of the Agency for Healthcare Research and Quality or the U.S. Department of Health and Human Services.

Acknowledgments: The authors thank Jean Slutsky of the Agency for Healthcare Research and Quality for overall project guidance, Jane Kolimaga for organization and management of the project, Ruth Goslin and Rebecca Gray for assistance with manuscript preparation, and the Advisory Panel and peer reviewers of the Evidence Report for many helpful suggestions throughout the entire process.

Grant Support: Dr. Nanda was a Veterans Affairs Women's Health Fellow at the Durham Veterans Affairs Medical Center while conducting this study.

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References

1. **Landis SH, Murray T, Bolden S, Wingo PA.** Cancer statistics, 1999. CA Cancer J Clin. 1999;49:8-31.
2. **Buntinx F, Brouwers M.** Relation between sampling device and detection of abnormality in cervical smears: a meta-analysis of randomised and quasi-randomised studies. BMJ. 1996;313:1285-90.
3. **Eddy DM.** Screening for cervical cancer. Ann Intern Med. 1990;113:214-26.
4. **Fahs MC, Mandelblatt J, Schechter C, Muller C.** Cost effectiveness of cervical cancer screening for the elderly. Ann Intern Med. 1992;117:520-7.
5. Duke University. Evaluation of Cervical Cytology. Evidence Report/Technology Assessment No. 5. Prepared by Duke University. Rockville, MD: Agency for Health Care Policy and Research; 1999.
6. **Fahey MT, Irwig L, Macaskill P.** Meta-analysis of Pap test accuracy. Am J Epidemiol. 1995;141:680-9.
7. **Brown AD, Garber AM.** The cost-effectiveness of three new technologies to enhance Pap testing. Chicago: Blue Cross Blue Shield Association Technical Evaluation Center Assessment Program; 1998.
8. **Abulafia O, Sherer DM.** Automated cervical cytology: meta-analyses of the performance of the AutoPap 300 QC System. Obstet Gynecol Surv. 1999; 54:469-76.
9. **Abulafia O, Sherer DM.** Automated cervical cytology: meta-analyses of the performance of the PAPNET system. Obstet Gynecol Surv. 1999;54:253-64.
10. The Bethesda system for reporting cervical/vaginal cytologic diagnoses: revised after the second National Cancer Institute Workshop, April 29-30, 1991. Acta Cytol. 1993;37:115-24.
11. **Richart R.** Cervical intraepithelial neoplasia. Pathol Annu. 1973;8:301-28.
12. **Reagan J, Fu Y.** Histologic types and prognosis of cancers of the uterine cervix. Radiation Oncology Biology Physics. 1979;5:1015-20.
13. **Nyirjesy I.** Atypical or suspicious cervical smears. An aggressive diagnostic approach. JAMA. 1972;222:691-3.
14. **Kurman RJ, Henson DE, Herbst AL, Noller KN, Schiffman MH.** Interim guidelines for management of abnormal cervical cytology. The 1992 National Cancer Institute Workshop. JAMA. 1994;271:1866-9.
15. **Bolick DR, Hellman DJ.** Laboratory implementation and efficacy assessment of the ThinPrep cervical cancer screening system. Acta Cytol. 1998;42: 209-13.
16. Proposed guidelines for primary screening instruments for gynecologic cytology. Intersociety Working Group for Cytology Technologies. Acta Cytol. 1997;41:924-33.
17. Proposed guidelines for secondary screening (rescreening) instruments for gynecologic cytology. Intersociety Working Group for Cytology Technologies. Acta Cytol. 1998;42:273-6.
18. Food and Drug Administration. Points to consider for cervical cytology devices, version 7/25/94. Washington, DC: Food and Drug Administration Center for Devices and Radiological Health; 1994.
19. **Giard RW, Hermans J.** The evaluation and interpretation of cervical cytology: application of the likelihood ratio concept. Cytopathology. 1993;4:131-7.
20. **Schulzer M, Anderson DR, Drance SM.** Sensitivity and specificity of a diagnostic test determined by repeated observations in the absence of an external standard. J Clin Epidemiol. 1991;44:1167-79.
21. **Chock C, Irwig L, Berry G, Glasziou P.** Comparing dichotomous screening tests when individuals negative on both tests are not verified. J Clin Epidemiol. 1997;50:1211-7.
22. **Joseph L, Gyorkos TW, Coupal L.** Bayesian estimation of disease prevalence and the parameters of diagnostic tests in the absence of a gold standard. Am J Epidemiol. 1995;141:263-72.
23. **Phelps CE, Hutson A.** Estimating diagnostic test accuracy using a "fuzzy gold standard." Med Decis Making. 1995;15:44-57.
24. **Reid MC, Lachs MS, Feinstein AR.** Use of methodological standards in diagnostic test research. Getting better but still not good. JAMA. 1995;274: 645-51.
25. **Irwig L, Tosteson AN, Gatsonis C, Lau J, Colditz G, Chalmers TC, et al.** Guidelines for meta-analyses evaluating diagnostic tests. Ann Intern Med. 1994;120:667-76.
26. **Roberts JM, Gurley AM, Thurloe JK, Bowditch R, Laverty CR.** Evaluation of the ThinPrep Pap test as an adjunct to the conventional Pap smear. Med J Aust. 1997;167:466-9.
27. **Hutchinson ML, Zahniser DJ, Sherman ME, Herrero R, Alfaro M, Bratti MC, et al.** Utility of liquid-based cytology for cervical carcinoma screening: results of a population-based study conducted in a region of Costa Rica with a high incidence of cervical carcinoma. Cancer. 1999;87:48-55.
28. **Ferenczy A, Robitaille J, Franco E, Arseneau J, Richart RM, Wright TC.** Conventional cervical cytologic smears vs. ThinPrep smears. A paired comparison study on cervical cytology. Acta Cytol. 1996;40:1136-42.
29. **Rasbridge SA, Nayagam M.** Discordance between cytologic and histologic reports in cervical intraepithelial neoplasia. Results of a one-year audit. Acta Cytol. 1995;39:648-53.
30. **Soost HJ, Lange HJ, Lehmacher W, Ruffing-Kullmann B.** The validation of cervical cytology. Sensitivity, specificity and predictive values. Acta Cytol. 1991;35:8-14.
31. **Okagaki T, Zelterman D.** Information, discrimination and divergence in cytology. II. Total discrimination as a measure of performance. Acta Cytol. 1991;35:25-9.
32. **Lozowski MS, Mishriki Y, Talebian F, Solitare G.** The combined use of cytology and colposcopy in enhancing diagnostic accuracy in precinical lesions of the uterine cervix. Acta Cytol. 1982;26:285-91.
33. **Davis JR, Hindman WM, Paplanus SH, Trego DC, Wiens JL, Suciu TN.** Value of duplicate smears in cervical cytology. Acta Cytol. 1981;25:533-8.
34. **Johansen P, Arffmann E, Pallesen G.** Evaluation of smears obtained by cervical scraping and an endocervical swab in the diagnosis of neoplastic disease of the uterine cervix. Acta Obstet Gynecol Scand. 1979;58:265-70.
35. **Naslund I, Auer G, Pettersson F, Sjovall K.** Evaluation of the pulse wash sampling technique for screening of uterine cervical carcinoma. Acta Radiol Oncol. 1986;25:131-6.
36. **Ritter DB, Kadish AS, Vermund SH, Romney SL, Villari D, Burk RD.** Detection of human papillomavirus deoxyribonucleic acid in exfoliated cervicovaginal cells as a predictor of cervical neoplasia in a high-risk population. Am J Obstet Gynecol. 1988;159:1517-25.
37. **Wetrich DW.** An analysis of the factors involved in the colposcopic evaluation of 2194 patients with abnormal Papanicolaou smears. Am J Obstet Gynecol. 1986;154:1339-49.
38. **Jones BA, Novis DA.** Cervical biopsy-cytology correlation. A College of American Pathologists Q-Probes study of 22 439 correlations in 348 laboratories. Arch Pathol Lab Med. 1996;120:523-31.
39. **Coibion M, Autier P, Vandam P, Delobelle A, Huet F, Hertens D, et al.** Is there a role for cervicography in the detection of premalignant lesions of the cervix uteri? Br J Cancer. 1994;70:125-8.
40. **Skehan M, Soutter WP, Lim K, Krausz T, Pryse-Davies J.** Reliability of colposcopy and directed punch biopsy. Br J Obstet Gynaecol. 1990;97:811-6.
41. **Giles JA, Deery A, Crow J, Walker P.** The accuracy of repeat cytology in women with mildly dyskaryotic smears. Br J Obstet Gynaecol. 1989;96:1067-70.
42. **DiBonito L, Falconieri G, Tomasic G, Colautti I, Bonifacio D, Dudine S.** Cervical cytopathology. An evaluation of its accuracy based on cytohistologic comparison. Cancer. 1993;72:3002-6.
43. **Ronco G, Montanari G, Aimone V, Parisio F, Segnan N, Valle A, et al.** Estimating the sensitivity of cervical cytology: errors of interpretation and test limitations. Cytopathology. 1996;7:151-8.
44. **Parham DM, Wiredu EK, Hussein KA.** The cytological prediction of cervical intraepithelial neoplasia in colposcopically directed biopsies. Cytopathology. 1991;2:285-90.
45. **Baldauf JJ, Dreyfus M, Ritter J, Philippe E.** Colposcopy and directed biopsy reliability during pregnancy: a cohort study. Eur J Obstet Gynecol Reprod Biol. 1995;62:31-6.
46. **Kwikkel HJ, Quaak MJ, de With C.** Predictive value of the abnormal PAP smear: a retrospective analysis of error rates. Eur J Obstet Gynecol Reprod Biol. 1986;21:101-12.
47. **Hockstad RL.** A comparison of simultaneous cervical cytology, HPV testing, and colposcopy. Fam Pract Res J. 1992;12:53-60.
48. **Fung Kee Fung M, Senterman M, Eid P, Faught W, Mikhael NZ, Wong PT.** Comparison of Fourier-transform infrared spectroscopic screening of exfoliated cervical cells with standard Papanicolaou screening. Gynecol Oncol. 1997;66:10-5.
49. **Del Priore G, Maag T, Bhattacharya M, Garcia PM, Till M, Lurain JR.** The value of cervical cytology in HIV-infected women. Gynecol Oncol. 1995; 56:395-8.
50. **Smith JJ, Cowan L, Hemstreet G 3d, Smith ML, Pippitt CH Jr, Doggett R, et al.** Automated quantitative fluorescent image analysis of cervical cytology. Gynecol Oncol. 1987;28:241-54.
51. **Pang JC, Hsu C, Kwan-Chau KM.** Cytology and colposcopy in the diagnosis of cervical neoplasia. Gynecol Oncol. 1977;5:134-41.
52. **Kealy WF.** Correlation of cervical cytodiagnosis and histopathology—an exercise in quality control. Ir J Med Sci. 1986;155:381-8.
53. **Korach J, Menczer J, Lusky A.** A comparison between referral and hospital cervical cytology laboratory reports. Isr J Med Sci. 1995;31:352-5.
54. **Bolick DR, Hellman DJ.** Laboratory implementation and efficacy assessment of the ThinPrep cervical cancer screening system. Acta Cytol. 1998;42: 209-13.
55. **Mayeaux EJ Jr, Harper MB, Abreo F, Pope JB, Phillips GS.** A comparison of the reliability of repeat cervical smears and colposcopy in patients with abnormal cervical cytology. J Fam Pract. 1995;40:57-62.
56. **Frisch LE, Milner FH, Ferris DG.** Naked-eye inspection of the cervix after acetic acid application may improve the predictive value of negative cytologic screening. J Fam Pract. 1994;39:457-60.
57. **Lederer H, Lambourne A.** The results of screening by cervical cytology and of histological examination of gynaecological operation specimens. J Obstet Gynaecol Br Commonw. 1973;80:67-71.
58. **Gonzalez D, Hernandez E, Anderson L, Heller P, Atkinson BF.** Clinical significance of a cervical cytologic diagnosis of atypical squamous cells of undetermined significance. Favoring a reactive process or low grade squamous intraepithelial lesion. J Reprod Med. 1996;41:719-23.
59. **Davison JM, Marty JJ.** Detecting premalignant cervical lesions. Contribution of screening colposcopy to cytology. J Reprod Med. 1994;39:388-92.
60. **Ramirez EJ, Hernandez E, Miyazawa K.** Cervical conization findings in women with dysplastic cervical cytology and normal colposcopy. J Reprod Med. 1990;35:359-61.
61. **Wheelock JB, Kaminski PF.** Value of repeat cytology at the time of colposcopy for the evaluation of cervical intraepithelial neoplasia on Papanicolaou smears. J Reprod Med. 1989;34:815-7.
62. **Morrison BW, Erickson ER, Doshi N, Russo JF.** The significance of atypical cervical smears. J Reprod Med. 1988;33:809-12.
63. **Fahim HI, Faris R, Arab SE, Sammour MB.** An epidemiologic study of Papanicolaou smear data at Ain-Shams University hospitals. J Egypt Public Health Assoc. 1991;66:97-111.
64. **Korn AP, Autry M, DeRemer PA, Tan W.** Sensitivity of the Papanicolaou smear in human immunodeficiency virus-infected women. Obstet Gynecol. 1994;83:401-4.
65. **Germain M, Heaton R, Erickson D, Henry M, Nash J, O'Connor D.** A comparison of the three most common Papanicolaou smear collection techniques. Obstet Gynecol. 1994;84:168-73.

66. **Koonings PP, Dickinson K, d'Ablaing G 3d, Schlaerth JB.** A randomized clinical trial comparing the Cytobrush and cotton swab for Papanicolaou smears. Obstet Gynecol. 1992;80:241-5.
67. **Andrews S, Hernandez E, Miyazawa K.** Paired Papanicolaou smears in the evaluation of atypical squamous cells. Obstet Gynecol. 1989;73:747-50.
68. **Wright TC, Sun XW, Koulos J.** Comparison of management algorithms for the evaluation of women with low-grade cytologic abnormalities. Obstet Gynecol. 1995;85:202-10.
69. **Spitzer M, Krumholz BA, Chernys AE, Seltzer V, Lightman AR.** Comparative utility of repeat Papanicolaou smears, cervicography, and colposcopy in the evaluation of atypical Papanicolaou smears. Obstet Gynecol. 1987;69:731-5.
70. **Baldauf JJ, Dreyfus M, Lehmann M, Ritter J, Philippe E.** Cervical cancer screening with cervicography and cytology. Eur J Obstet Gynecol Reprod Biol. 1995;58:33-9.
71. **Mann W, Lonky N, Massad S, Scotti R, Blanco J, Vasilev S.** Papanicolaou smear screening augmented by a magnified chemiluminescent exam. Int J Gynaecol Obstet. 1993;43:289-96.
72. **Wright TC Jr, Ellerbrock TV, Chiasson MA, Van Devanter N, Sun XW.** Cervical intraepithelial neoplasia in women infected with human immunodeficiency virus: prevalence, risk factors, and validity of Papanicolaou smears. New York Cervical Disease Study. Obstet Gynecol. 1994;84:591-7.
73. **Cox JT, Schiffman MH, Winzelberg AJ, Patterson JM.** An evaluation of human papillomavirus testing as part of referral to colposcopy clinics. Obstet Gynecol. 1992;80:389-95.
74. **Tawa K, Forsythe A, Cove JK, Saltz A, Peters HW, Watring WG.** A comparison of the Papanicolaou smear and the cervigram: sensitivity, specificity, and cost analysis. Obstet Gynecol. 1988;71:229-35.
75. **Cecchini S, Bonardi R, Mazzotta A, Grazzini G, Iossa A, Ciatto S.** Testing cervicography and cervicoscopy as screening tests for cervical cancer. Tumori. 1993;79:22-5.
76. **Hellberg D, Axelsson O, Gad A, Nilsson S.** Conservative management of the abnormal smear during pregnancy. A long-term follow-up. Acta Obstet Gynecol Scand. 1987;66:195-9.
77. **Cox JT, Lorincz AT, Schiffman MH, Sherman ME, Cullen A, Kurman RJ.** Human papillomavirus testing by hybrid capture appears to be useful in triaging women with a cytologic diagnosis of atypical squamous cells of undetermined significance. Am J Obstet Gynecol. 1995;172:946-54.
78. **Haddad NG, Hussein I, Livingstone JR, Smart GE.** Colposcopy in teenagers [Letter]. BMJ. 1988;297:1128.
79. **Giles JA, Hudson E, Crow J, Williams D, Walker P.** Colposcopic assessment of the accuracy of cervical cytology screening. Br Med J (Clin Res Ed). 1988;296:1099-102.
80. **Chomet J.** Screening for cervical cancer: a new scope for general practitioners? Results of the first year of colposcopy in general practice. Br Med J (Clin Res Ed). 1987;294:1326-8.
81. **Gundersen JH, Schauberger CW, Rowe NR.** The Papanicolaou smear and the cervigram. A preliminary report. J Reprod Med. 1988;33:46-8.
82. **Jones MH, Jenkins D, Cuzick J, Wolfendale MR, Jones JJ, Balogun-Lynch C, et al.** Mild cervical dyskaryosis: safety of cytological surveillance. Lancet. 1992;339:1440-3.
83. **Bigrigg MA, Codling BW, Pearson P, Read MD, Swingler GR.** Colposcopic diagnosis and treatment of cervical dysplasia at a single clinic visit. Experience of low-voltage diathermy loop in 1000 patients. Lancet. 1990; 336:229-31.
84. **Singh P, Pang M, Ratnam SS.** Selective cervical conization following colposcopy: a critical evaluation of 107 cases. Singapore Med J. 1985;26:289-94.
85. **Syrjanen KJ, Mantyjarvi R, Vayrynen M, Yliskoski M, Syrjanen SM, Saarikoski S, et al.** Cervical smears in assessment of the natural history of human papillomavirus infections in prospectively followed women. Acta Cytol. 1987;31:855-65.
86. **Morrison EA, Goldberg GL, Hagan RJ, Kadish AS, Burk RD.** Self-administered home cervicovaginal lavage: a novel tool for the clinical-epidemiologic investigation of genital human papillomavirus infections. Am J Obstet Gynecol. 1992;167:104-7.
87. **Jones DE, Creasman WT, Dombroski RA, Lentz SS, Waeltz JL.** Evaluation of the atypical Pap smear. Am J Obstet Gynecol. 1987;157:544-9.
88. **Stafl A.** Cervicography: a new method for cervical cancer detection. Am J Obstet Gynecol. 1981;139:815-25.
89. **Tay SK, Jenkins D, Singer A.** Management of squamous atypia (borderline nuclear abnormalities): repeat cytology or colposcopy? Aust N Z J Obstet Gynaecol. 1987;27:140-1.
90. **Soutter WP, Wisdom S, Brough AK, Monaghan JM.** Should patients with mild atypia in a cervical smear be referred for colposcopy? Br J Obstet Gynaecol. 1986;93:70-4.
91. **Tait IA, Alawattegama AB, Rees E.** Screening for cervical dysplasia in department of genitourinary medicine. Genitourin Med. 1988;64:255-8.
92. **Maggi R, Zannoni E, Giorda G, Biraghi P, Sideri M.** Comparison of repeat smear, colposcopy, and colposcopically directed biopsy in the evaluation of the mildly abnormal smear. Gynecol Oncol. 1989;35:294-6.
93. **Oyer R, Hanjani P.** Endocervical curettage: does it contribute to the management of patients with abnormal cervical cytology? Gynecol Oncol. 1986; 25:204-11.
94. **Upadhyay SN, Jha RS, Sinha TK, Mishra NK.** Accuracy of cytology in screening for cervical cancer. Indian J Med Res. 1984;80:457-62.
95. **Walker EM, Dodgson J, Duncan ID.** Does mild atypia on a cervical smear warrant further investigation? Lancet. 1986;2:672-3.
96. **Parker A, Ueki M.** A comparison of preoperative exfoliative cervical cytology with subsequent histology. N Z Med J. 1986;99:414-6.
97. **Sheets EE, Constantine NM, Dinisco S, Dean B, Cibas ES.** Colposcopically directed biopsies provide a basis for comparing the accuracy of ThinPrep and Papanicolaou smears. J Gynecol Tech. 1995;1:27-34.
98. **Kaufman RH, Adam E, Icenogle J, Reeves WC.** Human papillomavirus testing as triage for atypical squamous cells of undetermined significance and low-grade squamous intraepithelial lesions: sensitivity, specificity, and cost-effectiveness. Am J Obstet Gynecol. 1997;177:930-6.
99. **Spinillo A, Capuzzo E, Tenti P, De Santolo A, Piazzi G, Iasci A.** Adequacy of screening cervical cytology among human immunodeficiency virus-seropositive women. Gynecol Oncol. 1998;69:109-13.
100. **Herrington CS, Evans MF, Hallam NF, Charnock FM, Gray W, McGee JD.** Human papillomavirus status in the prediction of high-grade cervical intraepithelial neoplasia in patients with persistent low-grade cervical cytological abnormalities. Br J Cancer. 1995;71:206-9.
101. **Ferris DG, Wright TC, Litaker MS, Richart RM, Lorincz AT, Sun XW, et al.** Triage of women with ASCUS and LSIL on Pap smear reports: management by repeat Pap smear, HPV DNA testing, or colposcopy? J Fam Pract. 1998;46:125-34.
102. **Glenthoj A, Rank F, Peen U, Bostofte E.** Diagnostic efficiency of brush cytology from the uterine cervix. In: Goerttler K, Feichter G, Witte S, eds. New Frontiers in Cytology: Modern Aspects of Research and Practice. Berlin, NY: Springer-Verlag; 1988:421-4.
103. **Garutti P, Immovilli S, Chiari U, Segala V, Mollica G.** Cervical screening for detection of cervical HPV. The Cervix and the Lower Female Genital Tract. 1994;12:31-4.
104. **Slawson DC, Bennett JH, Simon LJ, Herman JM.** Should all women with cervical atypia be referred for colposcopy: a HARNET study. Harrisburg Area Research Network. J Fam Pract. 1994;38:387-92.
105. **Beeby AR, Keating PJ, Wagstaff TI, Wilson EMJ, Sims PF, Manning PJ, et al.** The quality and accuracy of cervical cytology: a study of five sampling devices in a colposcopy clinic. Journal of Obstetrics and Gynaecology. 1993; 13:276-81.
106. **Young NA, Naryshkin S, Bowman RL.** Value of repeat cervical smears at the time of colposcopic biopsy. Diagn Cytopathol. 1993;9.646-9.
107. **Sankaranarayanan R, Wesley R, Somanathan T, Dhakad N, Shyamalakumary B, Amma NS, et al.** Visual inspection of the uterine cervix after the application of acetic acid in the detection of cervical carcinoma and its precursors. Cancer. 1998;83:2150-6.
108. **Loiudice L, Abbiati R, Boselli F, Cecchini G, Costa S, Grossi E, et al.** Improvement of Pap smear sensitivity using a visual adjunctive procedure: a co-operative Italian study on speculoscopy (GISPE). Eur J Cancer Prev. 1998; 7:295-304.
109. **Recio FO, Sahai Srivastava BI, Wong C, Hempling RE, Eltabbakh GH, Piver MS.** The clinical value of digene hybrid capture HPV DNA testing in a referral-based population with abnormal pap smears. Eur J Gynaecol Oncol. 1998;19:203-8.
110. **Londhe M, George SS, Seshadri L.** Detection of CIN by naked eye visualization after application of acetic acid. Indian J Cancer. 1997;34:88-91.
111. **Manos MM, Kinney WK, Hurley LB, Sherman ME, Shieh-Ngai J, Kurman RJ, et al.** Identifying women with cervical neoplasia: using human papillomavirus DNA testing for equivocal Papanicolaou results. JAMA. 1999; 281:1605-10.
112. **Shulman LP, Klett CC, Phillips OP.** Preliminary comparison of papanicolaou smear and speculoscopy for assessing cervical cancer precursor lesions in pregnant adolescents. J Pediatr Adolesc Gynecol. 1999;12:78-82.
113. **Guerra B, De Simone P, Gabrielli S, Falco P, Montanari G, Bovicelli L.** Combined cytology and colposcopy to screen for cervical cancer in pregnancy. J Reprod Med. 1998;43:647-53.
114. **Mannino JR.** Natural history of false-negative papanicolaou smears: a prospective study using screening colposcopy in addition to cytology. J Am Osteopath Assoc. 1998;98:542-6.
115. Visual inspection with acetic acid for cervical-cancer screening: test qualities in a primary-care setting. University of Zimbabwe/JHPIEGO Cervical Cancer Project. Lancet. 1999;353:869-73.
116. **Eskridge C, Begneaud WP, Landwehr C.** Cervicography combined with repeat Papanicolaou test as triage for low-grade cytologic abnormalities. Obstet Gynecol. 1998;92:351-5.
117. **Kesic VI, Soutter WP, Sulovic V, Juznic N, Aleksic M, Ljubic A.** A comparison of cytology and cervicography in cervical screening. International Journal of Gynecological Cancer. 1993;3:395-8.
118. **Cruickshank ME, Buchan S, Melvin WT, Kitchener HC.** Human papillomavirus type 16 and 18 detection in the management of mild dyskaryosis. Br J Obstet Gynaecol. 1999;106:969-76.
119. **Carta G, Di Stefano L, Catellani Perelli A, Toro G, Moscarini M.** Colposcopy, cytology and histology in the diagnosis of squamous intraepithelial lesions of the cervix. Clin Exp Obstet Gynecol. 1999;26:60-6.
120. **Giraud J, Coiffic J, Poulain P, Kerisit J.** High prevalence of cervical intra-epithelial neoplasia in women treated for pelvic inflammatory disease. Eur J Obstet Gynecol Reprod Biol. 1998;81:51-4.
121. **Maiman M, Fruchter RG, Sedlis A, Feldman J, Chen P, Burk RD, et al.** Prevalence, risk factors, and accuracy of cytologic screening for cervical intraepithelial neoplasia in women with the human immunodeficiency virus. Gynecol Oncol. 1998;68:233-9.
122. **Miller WC.** Bias in discrepant analysis: when two wrongs don't make a right. J Clin Epidemiol. 1998;51:219-31.
123. **Ransohoff DF, Feinstein AR.** Problems of spectrum and bias in evaluating the efficacy of diagnostic tests. N Engl J Med. 1978;299:926-30.
124. **Boyko EJ.** Re: "Meta-analysis of Pap test accuracy" [Letter]. Am J Epidemiol. 1996;143:406-7.
125. **Feinstein AR.** Clinical Epidemiology: The Architecture of Clinical Research. Philadelphia: WB Saunders; 1985:434.

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Oncogene (2012), 1–10

www.nature.com/onc

ORIGINAL ARTICLE

Mutant p53 gain-of-function induces epithelial–mesenchymal transition through modulation of the miR-130b–ZEB1 axis

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The tumor suppressor gene p53 has been implicated in the regulation of epithelial–mesenchymal transition (EMT) and tumor metastasis by regulating microRNA (miRNA) expression. Here, we report that mutant p53 exerts oncogenic functions and promotes EMT in endometrial cancer (EC) by directly binding to the promoter of miR-130b (a negative regulator of ZEB1) and inhibiting its transcription. We transduced p53 mutants into p53-null EC cells, profiled the miRNA expression by miRNA microarray and identified miR-130b as a potential target of mutant p53. Ectopic expression of p53 mutants repressed the expression of miR-130b and triggered ZEB1-dependent EMT and cancer cell invasion. Loss of an endogenous p53 mutation increased the expression of miR-130b, which resulted in reduced ZEB1 expression and attenuation of the EMT phenotype. Furthermore, re-expression of miR-130b suppressed mutant p53-induced EMT and ZEB1 expression. Importantly, the expression of miR-130 was significantly reduced in EC tissues, and patients with higher expression levels of miR-130b survived longer. These data provide a novel understanding of the roles of p53 gain-of-function mutations in accelerating tumor progression and metastasis through modulation of the miR-130b–ZEB1 axis.

Oncogene advance online publication, 30 July 2012; doi:10.1038/onc.2012.334

Keywords: EMT; cancer; gain-of-function; miRNA; p53 mutation

INTRODUCTION

Epithelial–mesenchymal transition (EMT) is a transcriptional process that has a key role in regulating embryonic morphogenesis and cancer metastasis. During EMT, epithelial cells lose their polarization and homotypic cell adhesion, resulting in a more motile, spindle-like morphology with increased invasiveness.[1] At the molecular level, EMT occurs as a result of the activity of several transcriptional factors, such as ZEB1/2, Twist, BMI-1, Snail, and Slug, which suppress expression of the epithelial marker E-cadherin and induce the mesenchymal genes *N-cadherin* and *Vimentin*.[1] However, the mechanisms and pathways that drive EMT programs are not fully understood.

Non-coding microRNAs (miRNAs), including miR-200 and miR-194/192 family members, have been identified as negative regulators of EMT and metastasis by repressing the expression of ZEB1/2.[2] The overexpression of miR-200 and let-7b in gemcitabine-resistant pancreatic cancer cells induces the mesenchymal–epithelial transition, which is the reverse process of EMT.[3] Moreover, miR-194 is critical for maintaining the hepatic epithelial cell phenotype and inhibits metastasis by targeting several EMT activator genes.[4]

Recently, a regulatory connection between p53 signaling and miRNA-mediated EMT has been demonstrated. Wild-type (WT) p53 directly activates the transcription of miR-200c and miR-192 family members, which leads to ZEB1/2 downregulation and repression of EMT.[5] Furthermore, mutation of p53 can promote EMT and the aggressive potential of tumor cells by inhibiting WT p53-miR-200c pathways through dominant-negative effects on WT p53.[6] However, besides the dominant-negative effects upon WT p53, increasing evidence suggests that p53 mutations acquire additional oncogenic functions, such as a gain-of-function (GOF), which actively drive cells toward invasion and metastasis[7] through transactivation or transrepression of a large set of genes involved in regulation of cell adhesion, migration and proliferation.[8] In agreement with these findings, previous studies have found that overexpression of miRNAs (miR-181b and miR-200c) is associated with either p53 mutations or shorter patient survival in human colon cancer,[9] indicating that mutant p53 may exert GOF activities and promote EMT by modulating miRNAs.

Here, we identified a novel mechanism by which mutant p53 demonstrates GOF effects to facilitate EMT and cancer cell invasion by repressing miR-130b, an inhibitor of ZEB1. We further demonstrated that the expression of miR-130 was significantly reduced in endometrial cancer (EC) tissues, and patients with higher expression levels of miR-130b survived longer. Thus, these data suggest that restoration of miR-130b may have therapeutic value in tumors expressing mutant p53.

RESULTS

Mutant p53 GOF contributes to EMT in EC cells

Although mutant p53 GOF has been shown to promote EMT by upregulating Twist in prostate cancer cells,[10] to date, the role of mutant p53 GOF in initiating EMT during EC progression remains unknown. To explore this issue, we used p53-null HEC-50 cells[11] stably transduced with vectors encoding p53 mutations R273H,

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Received 28 February 2012; revised 1 June 2012; accepted 20 June 2012

R175H or C135Y, as well as an empty vector. Polyclonal cell lines were generated to omit clonal variation. The p53 protein level was verified using the anti-p53 (DO-7) antibody, which recognizes both WT and mutant p53.

Enforced expression of these p53 mutants induce a shift in cell morphology from a paved stone epithelial appearance to more mesenchymal phenotypes, with loss of cell-to-cell contact and increased cell spreading (Figure 1a). These morphological changes were accompanied by the upregulation of mesenchymal genes, including *Twist*, *ZEB1*, *BMI-1*, *Snail*, *N-cadherin* and *Vimentin*, and decreased expression of the epithelial marker E-cadherin (Figure 1b). To examine whether overexpression of mutant p53 can promote cell invasion, we next performed a cell invasion assay and observed a significant increase in the invasive capacity of mutant p53-expressing cells compared with empty vector-transfected control cells (Figure 1c). These findings were supported by concomitantly enhanced expression of metastatic-associated genes *osteopontin*, *MMP-2* and *MMP-9* in HEC-50 cells containing the p53 mutants (Supplementary Figure S1A).

Recently, EMT has been shown to have critical roles in modulating the cancer stem-like cell phenotype and conferring increased drug resistance of cancer cells.[12] To test the roles of mutant p53 GOF in acquiring stemness and drug-resistant properties in EC cells, we investigated the effects of stable expression of mutant p53 R175H on the self-renewal potential of cells using a sphere formation assay. We also assessed the chemosensitizing properties of this cell line after treatment with paclitaxel using the Cell Counting Kit-8. We found that transfection of this mutant, but not empty vector, enabled HEC-50 cells to form floating spheres in a serum-free medium (Figure 1d) and became more resistant to paclitaxel treatment (Figure 1e). To further explore the mechanisms of mutant p53 GOF-mediated cancer stemness and drug resistance, Quantitative reverse transcription (qRT–PCR) was performed to show that the mRNA levels of well-characterized stem cell markers (*CD133*, *KLF4* and *NANOG*) and chemoresistance-related genes (*MDR-1* and *MRP-1*) was highly enhanced in R175H-expressing cells (Figure 1f).

Similar to the findings obtained from stable transfection experiments, transient transfection of a vector encoding mutant p53 R248Q, but not empty vector, promoted cell invasion (Supplementary Figure S2C). In addition, this mutant also promoted EMT-like changes, including enhanced expression of ZEB1, BMI-1, N-cadherin and Vimentin, as well as repression of E-cadherin in HEC-50 cells (Supplementary Figure S2A). Taken together, these observations suggested a crucial role of mutant p53 GOF in driving EMT and invasive phenotypes of EC cells.

Knockdown of mutant p53 in EC cells causes a reversal of EMT and inhibition of cell invasion ability

To further examine whether loss of endogenous mutant p53 can inhibit EMT features, we performed shRNA-mediated knockdown of mutant p53 in HEC-1 cells, which express endogenous mutant

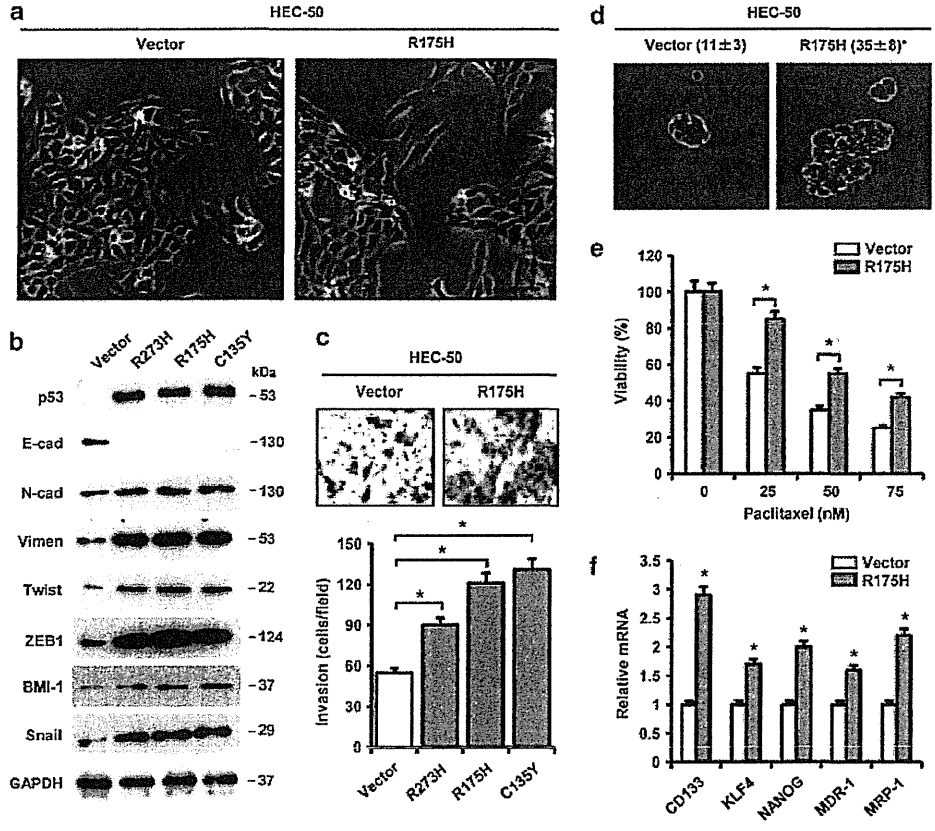

Figure 1. Mutant p53 GOF contributes to EMT in EC cells. (**a**) Morphology of endometrial cancer HEC-50 cells containing a control vector or mutant p53 R175H. Scale bars represent 100 μm. (**b**) Protein expression of p53 and EMT markers as analyzed by immunoblot. (**c**) Invasion of HEC-50 cells following overexpression of mutant p53s (mean ± s.d.; $n=3$; $^*P<0.01$). Representative images of invaded cells are shown. (**d**) Images indicate mammosphere formation in HEC-50 cells expressing the indicated constructs. The number of spheres obtained from 1000 cells at 12 days after plating (scale bar = 50 μm; mean ± s.d.; $n=3$; $^*P<0.01$). (**e**) Mutant R175H- or empty vector-transfected HEC-50 cells were treated with paclitaxel (0, 25, 50 and 75 nmol/l) for 48 h. Cell viability were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (mean ± s.d.; $n=3$; $^*P<0.01$). (**f**) Relative mRNA expression of stemness markers (normalized to GAPDH) in HEC-50 cells transfected with control or R175H vector, determined by qRT–PCR (mean ± s.d.; $n=4$; $^*P<0.01$).

p53 R248Q. Silencing of mutant p53 resulted in significant changes in cell morphology, and the scattered, mesenchymal-like HEC-1 cells began to exhibit a more epithelial-like cobblestone appearance (Figure 2a). Downregulation of this p53 mutant increased the expression of epithelial marker E-cadherin and repressed the expression of mesenchymal markers Twist, ZEB1, BMI-1, Snail, N-cadherin and Vimentin (Figure 2b). In agreement with these findings, knockdown of mutant p53 markedly reduced cell invasion (Figure 2c) and reduced the expression of osteopontin, MMP-2 and MMP-9 (Supplementary Figure S1B). To investigate if reduction of mutant p53 expression can suppress cancer stem-like and drug resistance properties, a sphere formation assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay were used. We found that HEC-1 cells transfected with *p53* shRNA displayed decreased sphere formation (Figure 2d) and were more sensitive to paclitaxel (Figure 2e). These results were supported by qRT–PCR experiments in which the mRNA expression of *CD133*, *KLF4*, *NANOG*, *MDR-1* and *MRP-1* were significantly attenuated following knockdown of endogenous p53 using shRNA (Figure 2f). Collectively, these results demonstrate that knockdown of mutant p53 can reverse the EMT phenotype and rescue cell invasion of EC cells.

ZEB1 is a key downstream mediator in p53 GOF mutant-induced EMT

Previous studies have shown that ZEB1 has a crucial role in the promotion of EMT and cancer stem cell properties in human cancer cells.[13] Overexpression of ZEB1 has been detected in aggressive EC.[14] Therefore, induction of ZEB1 expression by a p53 GOF mutant and reduction of its expression after mutant p53 silencing allowed us to postulate that ZEB1 may be essential for p53 GOF mutant-induced EMT in EC cells. Transfection of HEC-50 cells with *ZEB1* siRNA inhibited mutant p53 R175H-induced BMI-1 and Snail expression, restored E-cadherin expression (Supplementary Figure S3A) and greatly impaired p53 R175H-mediated cell invasion (Supplementary Figure S3B). However, this treatment did not significantly affect the protein level of Twist. Following transfection with *ZEB1* siRNA in HEC-1 cells, the mRNA expression of *BMI-1* and *Snail* was suppressed and *E-cadherin* was elevated (Supplementary Figure S3C). These data indicate that ZEB1 acts as an important downstream effector of these p53 mutants to mediate the EMT process in EC cells.

The p53 GOF mutants contribute to global repression of miRNA expression

To identify miRNAs mediated by the p53 GOF mutants, we performed array-based miRNA profiling of HEC-50 cells transduced with either p53 mutants or empty vector. Of 188 human miRNAs assayed, 23 miRNAs were expressed above background levels. Ectopic overexpression of mutant p53 R273H, R175H and C135Y in HEC-50 cells led to a global downregulation of all these miRNAs (Supplementary Figure S4A). We further validated the microarray results using qRT–PCR (Figure 3b). Notably, the expression of several miRNAs with known tumor suppressor activity, including let-7b,[3] miR-143,[15] miR-194,[16] miR-424,[17] miR-451,[18] and miR-

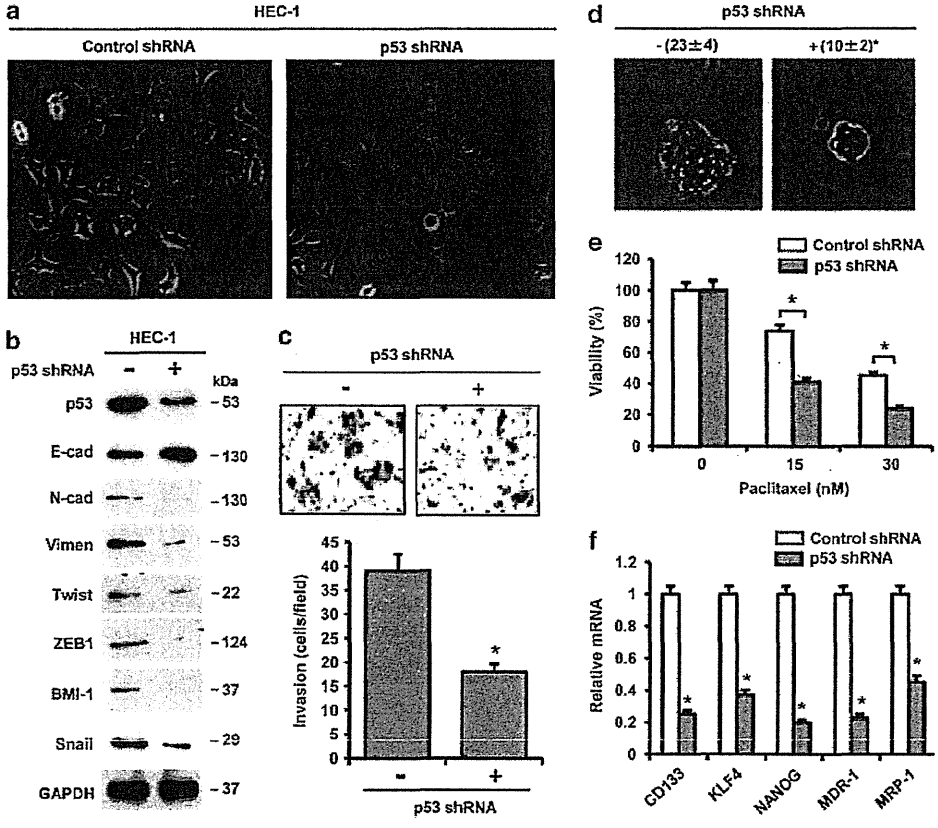

Figure 2. Knockdown of mutant p53 in EC cells causes a reversal of EMT and inhibition of cell invasion ability. (**a**) Morphology of endometrial cancer HEC-1 cells transfected with control shRNA vector or p53 shRNA vector (scale bar = 100 μm). (**b**) Protein levels of p53 and EMT markers as analyzed by western blot. (**c**) Invasion of HEC-1 cells after p53 shRNA transfection (mean ± s.d.; $n = 3$; $^*P < 0.01$). Representative images of invaded cells are shown. (**d**) Images show mammosphere formation in HEC-1 cells after p53 silencing by shRNA. Number of spheres obtained from 1000 cells at 12 days after plating (scale bar = 50 μm; mean ± s.d.; $n = 3$; $^*P < 0.01$). (**e**) Control- or p53 shRNA-transfected HEC-1 cells were treated with paclitaxel (0, 15, and 30 nmol/l) for 48 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (mean ± s.d.; $n = 3$; $^*P < 0.01$). (**f**) Relative mRNA expression of stemness markers (normalized to GAPDH) in HEC-1 cells after p53 silencing, determined by qRT–PCR (mean ± s.d.; $n = 4$; $^*P < 0.01$).

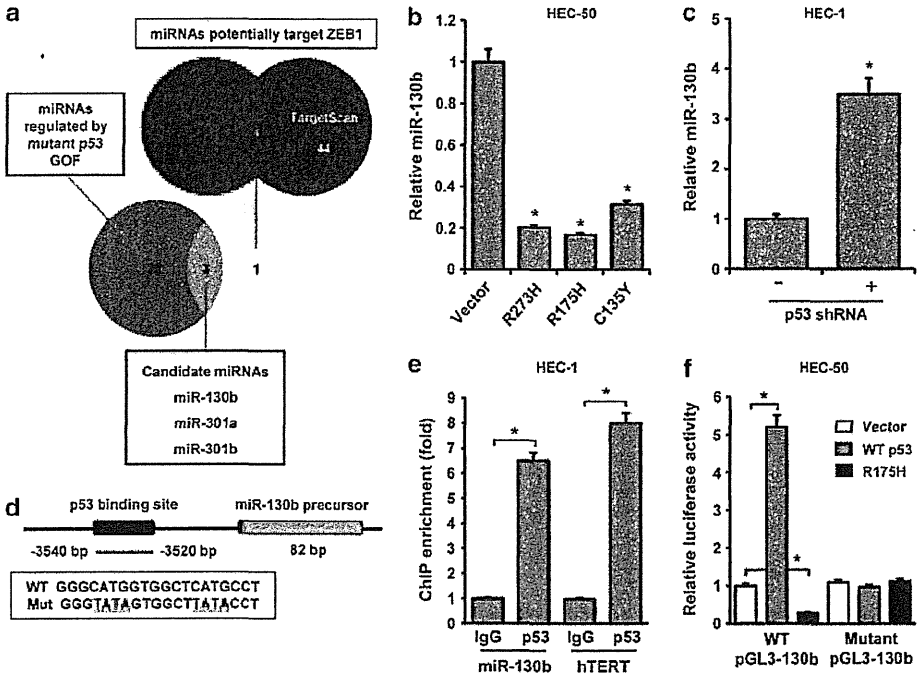

Figure 3. Mutant p53 binds to and transrepresses the promoter of miR-130b. (**a**) Schematic of algorithm used to select candidate microRNAs that potentially target ZEB1, and are negatively regulated by mutant p53s. (**b**, **c**) Relative miR-130b expression levels in HEC-50 cells transfected with mutant p53 vector (**b**), or in HEC-1 cells after p53 silencing by shRNA (**c**), were determined by qRT–PCR (mean ± s.d.; $n=4$; $^*P<0.01$). (**d**) Location and sequence of predicted p53-binding sites in the promoter of miR-130b gene. Mutated residues (red) are indicated at the bottom. (**e**) ChIP–qPCR analysis of mutant p53 (DO-7 antibody) binding to the miR-130b promoter region in HEC-50 cells. Human telomerase (hTERT) was used as a positive control. The fold enrichment over the IgG control is represented (mean ± s.d.; $n=3$; $^*P<0.01$). (**f**) HEC-50 cells were transfected with luciferase reporter plasmid pGL3-130b or empty pGL3-basic vector, along with control vector, wild-type p53 or mutant p53 R175H vector, and relative luciferase activity were assayed (mean ± s.d.; $n=3$; $^*P<0.01$). All qRT–PCR or luciferase values were normalized to GAPDH or Renilla activity, respectively.

146[19] were significantly reduced in p53-mutant-expressing cells (Supplementary Figure S4B). Thus, these results suggest that global repression of miRNA expression is likely to be a critical mechanism for p53 GOF mutant-enhanced EC tumorigenesis.

Mutant p53 binds to and transrepresses the promoter of miR-130b

Considering the important roles of ZEB1 in regulating EMT, we next sought to determine whether any of the 23 miRNAs may target ZEB1, and if repression of these miRNAs by mutant p53 may contribute to increased ZEB1 expression in EC cells. We first searched for all predicted miRNA-*ZEB1* interactions by using two target-prediction algorithms: TargetScan (http://www.targetscan.org) and microRNA.org (http://www.microrna.org), and detected four miRNAs that potentially bind to the 3′ untranslated regions (3′-UTR) of *ZEB1* mRNA. We then cross-referenced these four miRNAs with the 23 miRNAs identified by miRNA microarray. We found three miRNAs (miR-130b, miR-301a and miR-301b) that were downregulated in p53-mutant-expressing cells and predicted to bind to *ZEB1* 3′-UTR (Figure 3a). We selected *miR-130b* to investigate its effects on EMT and EC cell invasion because transfection of miR-301a and miR-301b did not substantially alter the protein expression of ZEB1 in EC cells (data not shown).

To evaluate whether the p53 mutants (R273H, R175H and C135Y) control the expression of miR-130b, we examined the effects of overexpression of mutant p53 on the expression of miR-130b in HEC-50 cells. The qRT–PCR analysis confirmed a significant decrease in mature miR-130b levels following transfection with the p53 mutants (Figure 3b). Similarly, the p53 mutation R248Q, but not the empty vector, inhibited the expression of *miR-130b* when expressed transiently (Supplementary Figure S2B). On the other hand, HEC-1 cells transfected with *p53* shRNA exhibited a marked elevation in the level of miR-130b (Figure 3c). These results suggest that the endogenous expression of miR-130b is negatively regulated by p53 mutants.

Recent evidence has established an association between p53 and several miRNAs, such as miR-34,[20] miR-192[21] and miR-200c.[5] Transcription of these miRNAs is directly regulated by p53. In particular, several studies have suggested that miR-200c is downregulated in EC tissues,[22] and restoration of miR-200c expression in HEC-50 cells decreases cell invasion.[23] Using qRT–PCRs to compare miRNA levels in HEC-50 cells, we found that overexpression of mutant p53 R175H and C135Y decrease the expression of miR-200c by 30–20%, whereas mutant R273H has no effects on its expression (Supplementary Figure S5), indicating that downregulation of miR-200c is involved in mutant p53 GOF-induced EC cell invasion.

However, it remains unknown whether p53 mutants function as a transcription regulator of miR-130b. Therefore, we searched for p53-binding sites in the miR-130b promoter using a bioinformatics approach.[24] Importantly, we found a conserved p53-binding site (5′-GGGCATGGTGGCTCATGCCT-3′) with a ranking score of 83 (Figure 3d). To determine whether an endogenous p53 mutant can bind this site, chromatin immunoprecipitation (ChIP)–qPCR analysis was performed on HEC-1 cells. The human telomerase (*hTERT*) promoter served as a positive control, as it has been previously shown that p53 mutants can bind this promoter.[25] Both miR-130b (sixfold) and *hTERT* (eightfold) promoter sequences were specifically enriched by anti-p53 antibodies, but not by

non-specific antibodies (Figure 3e). These data suggest that miR-130b is a direct target of mutant p53 in EC cells.

To assess if the downregulation of *miR-130b* expression is mediated by transrepression of the p53 mutants, we cloned the p53-binding sequence of the miR-130b promoter upstream of firefly luciferase to yield a WT plasmid pGL3-130b, and further generated mutant pGL3-130b luciferase vectors containing mutations in the candidate p53-binding site. The WT pGL3-130b or mutant pGL3-130b vector was transfected into HEC-50 cells with either a control vector, mutant p53 R175H or WT p53. Interestingly, the luciferase activity of WT pGL3-130b was significantly repressed by R175H, but was transactivated by WT p53. However, expression of mutant p53 or WT p53 did not affect the luciferase activity of mutant pGL3-130b (Figure 3f). Therefore, our observations by qRT–PCR, ChIP–qPCR and the luciferase assay collectively demonstrate that a GOF p53 mutant binds to and transrepresses the miR-130b promoter.

Our results showing a fivefold increase in the ability of WT p53 to transactivate the promoter of miR-130b (Figure 3f, lane 2) raised an interesting possibility that WT p53 controls metastasis through modulation of miR-130b. Therefore, we transiently transfected the WT p53 expression vector into HEC-50 cells (Figure 4a). A qRT–PCR analysis revealed that expression of WT p53 protein significantly induced the levels of miR-130b and also slightly increased the expression of miR-200c (Figure 4b), which is a known target of WT p53.[6] In WT p53-expressing HHUA cells, activation of p53 in response to the Mdm2 antagonist Nutlin-3 (Figure 4c) enhanced the level of miR-130b, but this was abolished by the shRNA-mediated knockdown of p53 (Figure 4d). Consistent with an earlier report,[6] the knockdown of WT p53 in HHUA cells induced changes associated with EMT, such as a mesenchymal morphology (Figure 4e), low expression of *E-cadherin*, upregulation of *ZEB1* and *BMI-1* (Figure 4f), and increased cell invasion (Figure 4g). We also observed an enrichment of WT p53 binding to both the miR-130b and p21 promoters using a ChIP–PCR analysis (Figure 4h). In addition, WT p53 was able to transactivate a pGL-3-miR-130b luciferase reporter gene (Figure 4i). Silencing of p53 by shRNA abrogated Nutlin-3-stimulated luciferase activities of the miR-130b promoter (Figure 4i, compare lane 5 to lanes 7 and 8). These data indicated that mutant and WT p53 exert opposite effects on miR-130b expression, which supports the hypothesis that a p53 GOF mutant contributes to EC carcinogenesis by altering the expression of miR-130b.

Despite direct transcriptional regulation by mutant p53, some GOF effects of the p53 mutants may depend on their ability to inactivate p53 family members p63 or p73.[26] Furthermore, p63 has been shown to inhibit metastasis through transactivation of miR-130b.[27] Therefore, we determined whether p63 inhibition by the p53 mutants is involved in the p53 GOF mutant-induced suppression of *miR-130b* in HEC-50 cells. We found that downregulation of p63 protein expression by *p63* siRNA (Supplementary Figure S6A) resulted in a dose-dependent decrease in *p21* luciferase activity (Supplementary Figure S6C). As expected, transient transfection of the WT p53 expression vector markedly transactivated the *p21* promoter (Supplementary Figure S6C). However, the mRNA expression of *miR-130b* did not substantially change after *p63* knockdown (Supplementary Figure S6B). Thus, p63 inhibition is not likely to be responsible for p53 GOF mutant-induced suppression of miR-130b in EC cells.

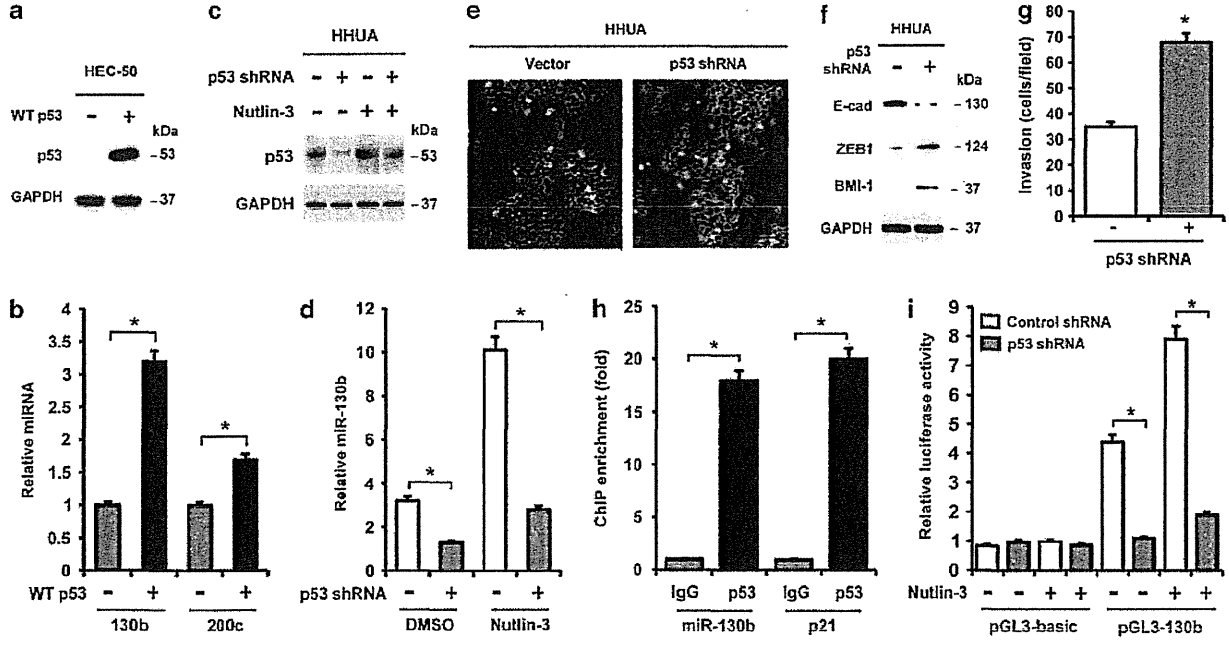

Figure 4. WT p53 transactivates the promoter of miR-130b. (**a**) WT p53 protein level in HEC-50 cells transfected with WT p53 expression vector or control vector. (**b**) qRT–PCR for miR-130b and miR-200c in HEC-50 cells transfected with WT p53 expression vector or control vector (mean ± s.d.; $n=4$; $^*P<0.01$). (**c**, **d**) HHUA cells transfected with p53 shRNA vector or control vector were treated with 5 μmol/l of Nutlin-3 or dimethyl sulfoxide (DMSO) for 12 h. WT p53 protein (**c**) and miR-130b expression (**d**) were detected by western blot analysis and qRT–PCR (mean ± s.d.; $n=4$; $^*P<0.01$), respectively. (**e**) Morphology of HHUA cells after p53 silencing. Scale bars represent 200 μm. (**f**) Western blot analysis for EMT markers in HHUA cells after p53 silencing. (**g**) Invasion assay of HHUA cells after transfection with p53 shRNA (mean ± s.d.; $n=3$; $^*P<0.01$). (**h**) ChIP–qPCR analysis of WT p53 (DO-7 antibody) binding to the miR-130b promoter region in HHUA cells. *p21* was used as a positive control. The fold enrichment over the IgG control is represented (mean ± s.d.; $n=3$; $^*P<0.01$). (**i**) Indicated HHUA cells were transfected with luciferase reporter plasmid pGL3-130b or empty pGL3-basic vector, and treated with 5 μmol/l of Nutlin-3 or DMSO for 12 h. Relative luciferase activity was determined (mean ± s.d.; $n=3$; $^*P<0.01$). All qPCR or luciferase values were normalized to GAPDH or Renilla activity, respectively.