Immunoreactivities of human nonmetastatic clone 23 and p53 products are disassociated and not good predictors of lymph node metastases in early-stage cervical cancer patients

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Summary

Objective: To assess the relation between expressions of *human nonmetastatic clone 23 (nm23-H1)* and p53 in cervical cancer, their relationships with lymph node metastasis, and further to examine their predictive of lymph node metastases.

Materials and Methods: nm23-H1 and p53 expression profiles were visualized by immunohistochemistry in early-stage cervical cancer specimens.

Results: Immunoreactivities of nm23-H1 and p53 were disassociated. The independent variables related with lymph node metastases were grade of cancer cell differentiation ($p < 0.029$) and stromal invasion ($p < 0.039$). Sensitivity, specificity, positive and negative predictive values, and accuracy for lymph node metastasis were calculated to be 91.7%, 13.5%, 25.6%, 83.3%, and 32.7% for nm23-H1 and 66.7%, 51.4%, 30.8%, 82.6%, and 55.1% for p53.

Conclusion: Nm23-H1 and p53 are disassociated and not good predictors of lymph node metastases in early-stage cervical cancer patients. However, stromal invasion and cell differentiation can predict lymph node metastasis.

Key words: Early-stage cervical cancer; Human nonmetastatic clone 23; p53; Lymph node metastasis; Grade of cell differentiation; Stromal invasion.

Introduction

Cervical cancer is the second most common type of cancer among women, accounting for 15% of all female cancers worldwide with approximately 500,000 new cases diagnosed annually [1] . The probability of survival for cervical cancer patients is highly dependent on lymph node involvement [2, 3]. Indeed, Denschlag and colleagues reported that lymph node metastasis is the only significant independent predictor of survival [2]. Cervical cancer patients with negative nodes had a 85% to 95% five-year survival rate [2, 3]. However, once metastasis to the lymph nodes was documented, five-year survival rates declined significantly, ranging from as low as 20% to 70% [2, 3]. Introduction of molecular markers as predictors of lymph node metastasis in cervical cancer may help us to identify patients with a higher mortality risk while planning treatment.

The involvement of nm23 gene family members in tumor progression and metastasis has been shown in numerous studies [4-8]. The first *nonmetastatic clone 23* gene (*nm23* gene) was isolated by Steeg *et al.* in 1989 and identified as a gene associated with metastatic potential [4]. These investigators analyzed the relative RNA levels of 24 cDNA clones selected by differential hybridization from seven K-1735 melanoma cell lines and identified clone 23, which exhibited high RNA levels and was associated with low metastatic potential [4]. The gene was designated as *"nonmetastatic clone 23"* and encodes proteins that possess nucleoside diphosphate (NDP) kinase catalyzing the phosphorylation of nucleoside diphosphates into nucleoside triphosphates [4, 5]. In 1989, the first *human nonmetastatic clone 23* gene was isolated by screening a human fibroblast cDNA library [9]. Rosengard *et al.* identified the *nm23* gene, for which RNA levels were reduced in tumor cells of high metastatic potential. This gene was referred to as *nm23-H1*. To date, at least eight human isotypes of the *nm23*/NDP kinase family have been discovered [5]. Among these gene isotypes, *nm23*-H1 has been regarded as the most representative [4, 5].

p53, a tumor suppressor gene which can modify the ability of tumor cells to metastasize, is the most frequently mutated gene in human cancer. Although p53 and nm23-H1 proteins (p53 and nm23-H1) play an important part in regulating tumor progression, the relationship between these proteins is currently unknown. However, Chen *et al.* studied the relationship between p53 and nm23-H1 expresions and found that wild-type p53 upregulated the expressions of nm23-H1 mRNA and protein in breast and colorectal cancer cell lines [10]. To the best of our knowledge, there are no studies correlating the expression of nm23-H1 with that of p53 in cervical cancer. The purposes of this study were to assess the immunoreactivities of nm23-H1 and p53 in early-stage cervical cancer tissues, to estimate their possible association or disassociation and their relationships with lymph node metastasis, and then to further examine whether the results of immunohistochemical analysis of these parameters can be applied to predict lymph node metastases.

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Materials and Methods

Forty-nine paraffin-embedded pathological samples of early-stage cervical cancer were collected from patients enrolled in this study who were admitted to the Department of Gynecology and Obstetrics of Chung Shan Medical University Hospital. These cervical cancer patients were staged Ia, Ib or IIa according to the International Federation of Gynecology and Obstetrics (FIGO) classification. They underwent abdominal radical hysterectomy (RH) and pelvic lymph node dissection between January 1997 and January 2000. Of the specimens, 42 were squamous cell carcinomas (SCCs), six were adenocarcinomas and one was a clear cell variant. The clinicopathological characteristics of all cervical cancer patients are presented in Table 1. The mean age and parity of the patients were 51 ± 11.2 years (range 29-73) and 4 (range 1-7), respectively. Of these cases, five (10%) were Stage 1a, 31 (63%) were Stage 1b, and 13 (27%) were Stage IIa based on clinical staging proposed by FIGO. The Chung Shan Institutional Review Board approved the study protocol and informed consent was obtained from all patients.

Table 1. — *Characteristics of cervical cancer patients*.*

Characteristic	Patients (no. = 49)
Age (years)	51.1 ± 11.2 (29-73)
Parity	4 (1-7)
Stage	
Ia	5
Ib	31
IIa	13
Histology	
Squamous cell carcinoma	42
Adenocarcinoma	6
Clear cell carcinoma	1

* Abdominal radical hysterectomy and bilateral pelvic lymphadenectomy were performed for all cervical cancer patients.

Immunohistochemistry

Immunohistochemical methods were used to evaluate nm23-H1 and p53 immunoreactivities. Immediately after the tissues were excised they were fixed with 10% buffered formalin for 8-16 hours. Then, the tissues were dehydrated with increasing concentrations of ethanol and infiltrated with paraffin using a tissue processor (Medite Medizintechnik, TPC 15). When immunohistochemistry was performed, paraffin sections (5 μm) of tissue samples were collected on slides, dewaxed in xylene and rehydrated in a series of ethanol solutions of decreasing concentration. Endogenous peroxidase activity was quenched by incubation in a 3% hydrogen peroxide solution for 20 minutes at room temperature. Next, the sections were then treated twice for 5 min each in citrate buffer (2.1g/1000ml; pH 6.0) with microwave heating at 60°C for antigen retrieval. Tissues sections were then incubated overnight at 4°C with mouse monoclonal antibodies against the nm23 protein (Novocastra; NCL-nm23-H1, 1:200 dilution) or the p53 protein (DAKO, Glostrup, Denmark; DO7, 1:175 dilution). Following primary antibody incubation, the sections were stained using the streptavidin-biotin-peroxidase method. The chromagen (brown color) was visualized following incubation in 0.04% 3',3'-diaminobenzidine tetrahydrochloride. Finally, sections were counterstained with Gill's Hematoxylin before mounting. Phosphate-buffered saline was substituted for primary antibody for the sections used as negative controls. Nm23-H1 and p53 immunohistochemical expressions in all samples were determined by the same pathologist. The nm23-H1 immunoreactivity was evaluated according to the intensity and proportion of the stained cells [11]. p53 immunoreactivity was quantified based on the percentage of stained nuclei. Expression of p53 was scored as being high if at least 30% [12] of cervical carcinomal cell nuclei exhibited a greater staining intensity than that of the adjacent acellular stroma. Expression of p53 was scored as being low when the percentage of stained nuclei was less than 30%.

Statistical analysis

McNemar's chi-square test was used to analyze the association or difference between expressions of nm23-H1 and p53. Chi-square or Fisher's exact tests were used to assess the clinicopathological variables and the expressions of nm23-H1 and p53 in relation to lymph nodes metastases. These clinicopathological variables include age (≥ 50 or < years), grade of cell differentiation (well and moderate or poor differentiation), stromal invasion (≥ half depth of stromal invasion or < half depth of stromal invasion), FIGO stage (Ia, Ib or IIa) and histologic types (SCC, adenocarcinoma or clear cell carcinoma). When nm23-H1 and p53 expressions were provided for the prediction of lymph node metastases in cervical cancer patients, we calculated the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy. A p value less than 0.05 was considered statistically significant.

Results

Nm23-H1 immunoreactivity was cytoplasmic (Figure 1A), however, p53 was nuclear (Figure 1B). The expression profiles of nm23-H1 and p53 are summarized in Table 2. Immunoreactivities of nm23-H1 and p53 products are different or disassociated.

Subgroup analyses revealed that the independent variables predictive of lymph node metastases include grade of tumor cell differentiation ($p < 0.05$) and stromal invasion ($p < 0.05$). Age, FIGO stage, and histopathology were not predictive of lymph node metastasis (Table 3). No patients with Stage Ia cancer exhibited lymph node metastasis and a higher percentage of Stage IIa patients exhibited lymph node metastasis compared to Stage Ib patients (46.2% versus 19.4%). Although no significant differences could be reached between Stage Ia and Ib as well as Stage Ia and IIa, the proportions of lymph node metastases showed an increasing tendency in Stage Ia, Ib and IIa cases (0%, 19.4% and 46.2%, Figure 2). Furthermore, lymph node metastasis was found in the only patient with clear cell carcinoma. Finally, neither nm23-H1 nor p53 expression was associated with lymph node metastasis in our patients.

Table 2. — *Disassociation between nm23-H1 and p53 immunohistochemical expressions in cervical cancer patients.*

Expression of nm23-H1	Expression of p53		Disassociation*
	High	Low	
High	26	17	$p < 0.05$
Low	0	6	

* McNemar's chi-square test shows the difference or disassociation between expressions of p53 and nm23-H1. The significant difference is defined as $p < 0.05$. Nm23-H1: human nonmetastatic clone 23.

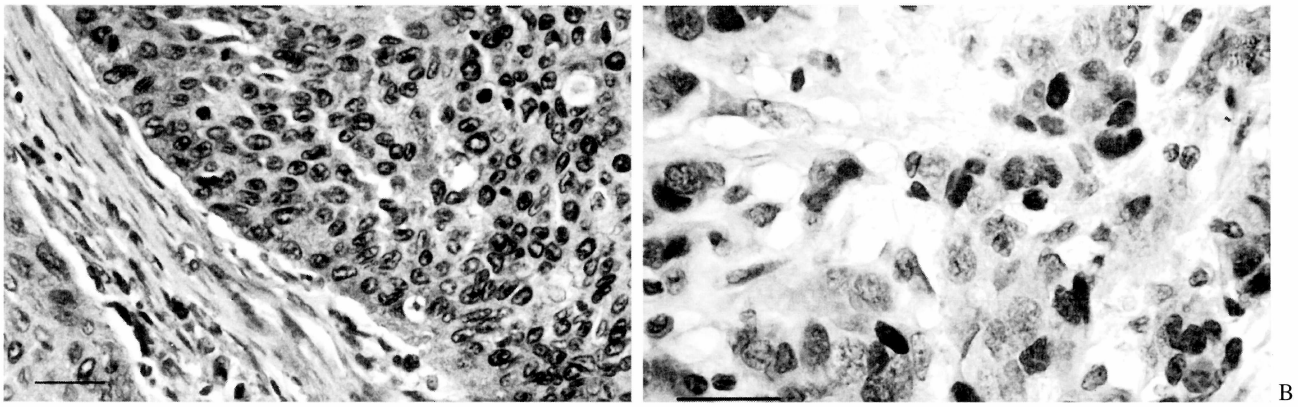

Figure 1. — (A) A moderately differentiated squamous cell carcinoma sample of the uterine cervix revealing high nm23-H1 expression with strong brown-stained cytoplasm. (nm23-H1: human nonmetastatic clone 23; immunohistochemistry, streptavidin-biotin peroxidase immunostaining; scale marker: 100 μm). (B) A well differentiated squamous cell carcinoma sample of the uterine cervix revealing high p53 expression with strong brown-stained nucleus (immunohistochemistry, streptavidin-biotin peroxidase immunostaining; scale marker: 100 μm).

Table 3. — *Clinicopathological characteristics and expressions of nm23-H1 and p53 in relation to lymph node metastases.*

Characteristics	Case numbers Percentage (%)	Lymph node metastases Yes	No	p value
Age				NS
≥ 50	25	7	18	
< 50	24	5	19	
Grade of cell differentiation[a]				0.029
Well and moderate	34	5	29	
Poor	15	7	8	
Stromal invasion[b]				0.039
≥ Half depth of invasion	19	8	11	
< Half depth of invasion	30	4	26	
FIGO staging				0.068
Ia	5	0	5	
Ib	31	6	25	
IIa	13	6	7	
Histology				NS
SCC	42	10	32	
Adenocarcinoma	6	1	5	
Clear cell carcinoma	1	1	0	
Expression of p53				NS
High	26 (53)	8	18	
Low	23 (47)	4	19	
Expression of nm23				NS
High	43 (88)	11	32	
Low	6 (12)	1	5	
Nm23/p53expression				NS
High/Low	17	3	14	
High/High	26	8	18	
Low/High	0	0	0	
Low/Low	6	1	5	

Statistical analysis: Chi-square or Fisher's exact tests. [a,b] A significant difference between cancer cell grading or stromal invasion and lymph nodes metastases, respectively. NS: not significant. Nm23-H1: human nonmetastatic clone 23.

Table 4. — *Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of nm23-H1 and p53 expression for lymph node metastases in cervical cancer patients.*

	Sensitivity	Specificity	PPV	NPV	Accuracy
Nm23-H1	11/12 (91.7%)	5/37 (13.5%)	11/43 (25.6%)	5/6 (83.3%)	16/49 (32.7%)
p53	8/12 (66.7%)	19/37 (51.4%)	8/26 (30.8%)	19/23 (82.6%)	27/49 (55.1%)

Nm23-H1: human nonmetastatic clone 23.

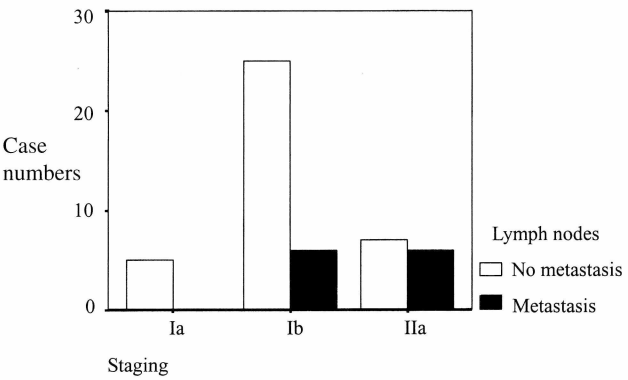

Figure 2. — An increasing tendency for lymph node metastases in ascending order of Stages Ia, Ib and IIa in early cervical cancers (from 0%, 19.4% to 46.2%).

The sensitivity, specificity, PPV, NPV, and accuracy of nm23-H1 and p53 for the prediction of lymph node metastases in cervical cancer were 91.7%, 13.5%, 25.6%, 83.3%, 32.7% and 66.7%, 51.4%, 30.8%, 82.6%, and 55.1%, respectively (Table 4).

Discussion

Our results revealed that the expression of nm23-H1 is not associated with that of p53 in early cervical cancers patients (Table 2). In contrast, Chen *et al.* found that wild-type p53 can upregulate the expression of nm23-H1 at the protein level in breast and colorectal cancer cell lines [10]. The lack of correlation between p53 and nm23-H1 in our study seems to document that p53 cannot regulate nm23-H1 in cervical cancer. Moreover, Chen *et al.* demonstrated that this capacity of wild-type p53 to regulate nm23-H1 expression could not be reproduced with mutant p53 [10]. Which is the same as our finding of the dissociation of p53 and nm23-H1, since the expression of p53 protein in our study likely reflects the accumulation of mutated protein in the nucleus of cancer

cells. Mutations in the *p53* gene usually result in stabilization and accumulation of p53 proteins in the nucleus and prevent entry to the cytoplasm, where the protein would be metabolized [13, 14]. Our results found 53.1% of SCC cases had high p53 immunoreactivity (26/49, Table 3), which is in agreement with the results of Mittal *et al.* [13]. Furthermore, Sirotkovic-Skerlev *et al.* demonstrated a tendency of a negative correlation between the expression of nm23-H1 and mutated p53 in breast cancer [15]. However, this negative relationship also cannot be found in cervical cancer (Table 2).

It was reported that the incidence of pelvic lymph node metastasis ranged from 10-30% [16]. Most studies have evaluated the prognostic significance of histopathologic factors of survival rate and progression-free survival in cervical cancer [17, 18]. However, few of them concerned the risk factors for predicting lymph node metastasis. A score of the invasive front grading system has been proposed to be an associated risk factor for lymph node metastasis [19]. In our study, we correlated the clinicopathological variables and the expressions of nm23-H1 and p53 in relation to lymph node metastases, and found only stromal invasion and grade of cell differentiation to be associated with lymph node metastases in early-stage cervical cancer. Although the proportions of lymph node metastases increased in ascending order among Stage Ia, Ib and IIa cases, no significant difference was noted between FIGO staging and lymph node metastasis (Table 3; Figure 2). In addition, most lymph node metastasis cases had high nm23-H1 immunoreactivities (11/12, 91.7%; Table 3), however a significant correlation could not be reached between nm23-H1 expression and lymph node metastasis. Our findings are consistent with the findings of Krestensen *et al.*, who found 75.8% of cases with lymph node metastasis exhibited intense nm23-H1 immunoreactivity [20]. In contrast, Marone *et al.* demonstrated that cervical cancer patients with lymph node involvement were shown to have significantly lower protein levels of nm23-H1 [21]. Concerning p53 expression, the ratios of high p53 immunoreactivity in SCC tissues are similar between our findings and those of Mittal *et al.* and Akasofu *et al.* (53.1% vs 59% and 43.5%), which may indicate p53 nuclear accumulation [13, 14]. However, p53 also did not show a significant correlation with lymph node metastasis in early-stage cervical cancer patients (Table 3). This finding is further supported by that of Graflund *et al.* [12].

The current study is the first to examine the combined biologic behavior of nm23-H1 and p53 in cervical cancer. Although high levels of nm23-H1 and p53 expression were found in patients with lymph node metastasis, they did not reach statistical significance (Table 3). In grade 3 transitional cell carcinoma of the bladder, combined analysis of p53 and nm23-H1 expressions and lymph nodal status was also not significant (Table 3) [22].

Pelvic lymphadenectomy is an invasive procedure used to evaluate the status of lymph node metastasis in cervical cancer patients. To avoid having to perform such an invasive procedure, it would be beneficial to use biological markers or imaging techniques which can be reliably used as predictors of lymph node metastasis. The analysis of biological parameters from cervical samples using biopsies for cervical cancer lesions is not only safer but also less complicated. Because the likelihood of false-negative results associated with magnetic resonance imaging or computed tomography is high and positron emission tomography is too expensive in Taiwan [23, 24], nm23-H1 and p53, which are involved in tumor progression, were introduced. Therefore, we calculated the sensitivity, specificity, PPV, NPV and accuracy of nm23-H1 and p53 expressions for the prediction of lymph node metastasis in early-stage cervical cancer patients. However, the specificity and PPV for nm23-H1 expression were only 13.5% and 25.6%, respectively. The PPV for p53 expression was only 30.8%. This implies that these parameters cannot substitute for pelvic lymphadenectomy in the detection of lymph node metastasis in early-stage cervical cancer patients.

In conclusion, nm23-H1 and p53 do not show significant associations with lymph node metastases in early-stage cervical cancers. The only variables that showed a significant association with lymph node metastasis were stromal invasion and grade of cell differentiation in this study. Nm23-H1 and p53 cannot be used as good predictors of lymph node metastasis in early-stage cervical cancer patients.

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References

[1] Saraiya U.B.: "Preventable but not prevented: the reality of cervical cancer". *J. Obstet. Gynaecol. Res.,* 2003, *29*, 351.

[2] Denschlag D., Gabriel B., Mueller-Lantzsch C., Tempfer C., Henne K., Gitsch G. *et al.*: "Evaluation of patients after extraperitoneal lymph node dissection for cervical cancer". *Gynecol. Oncol.*, 2005, *96,* 658.

[3] Cheng X., Cai S., Li Z., Tang M., Xue M., Zang R.: "The prognosis of women with Stage IB1-IIB node-positive cervical carcinoma after radical surgery". *World J. Surg. Oncol.,* 2004, *2,* 47.

[4] Steeg P.S., Bevilacqua G., Kopper L., Thorgeirsson U., P Talmadge., J E.Liotta. L.A. *et al.*: "Evidence for a novel gene associated with low tumor metastatic potential". *J. Natl. Cancer Inst.*, 1988, *80*, 200.

[5] Lacombe M.L., Milon L., Munier A., Mehus J.G., Lambeth D.O.: "The human Nm23/nucleoside diphosphate kinases". *J. Bioenerg. Biomembr.,* 2000, *32,* 247.

[6] Ouatas T., Salerno M., Palmieri D., Steeg P.S.: "Basic and translational advances in cancer metastasis: Nm23". *J. Bioenerg. Biomembr.*, 2003, *35*, 73.

[7] Wang P.H., Ko J.L., Chang H., Lin L.Y.: "Clinical significance of high nm23-H1 expression in intraepithelial neoplasia and early-stage squamous cell carcinoma of the uterine cervix". *Gynecol. Obstet. Invest.,* 2003, *55*, 14.

[8] Ravazoula P., Aletra C., Kourounis G., Ladopoulos I., Tzigounis V.: "Immunohistochemical analysis of nm23-H1 expression in human cervical lesions". *Eur. J. Gynaecol. Oncol.,* 2000, *21,* 510.

[9] Rosengard A.M., Krutzsch H.C., Shearn A. Biggs J. R., Barker E., Margulies I. M. *et al.*:"Reduced Nm23/Awd protein in tumour metastasis and aberrant Drosophila development". *Nature*, 1989, *342,* 177.

[10] Chen S.L., Wu Y.S., Shieh H.Y., Yen C.C., Shen J.J., Lin K.H.: "p53 is a regulator of the metastasis suppressor gene Nm23-H1". *Mol. Carcinog.*, 2003, *36,* 204.

[11] Wang P.H., Chang H., Ko J.L., Lin L.Y.: "Nm23-H1 immunohistochemical expression in multisteps of cervical carcinogenesis". *Int. J. Gynecol. Cancer,* 2003, *13,* 325.
[12] Graflund M., Sorbe B., Karlsson M.: "Immunohistochmical expression of p53, bcl-2, and p21 (WAF1/CIP1) in early cervical carcinoma: correlation with clinical outcome". *Int. J. Gynecol. Cancer,* 2002, *12,* 290.
[13] Mittal K.R., Lin O., Chan W., Goswami S., Demopoulos R.I.: "Cervical squamous dysplasias and carcinomas with immunodetectable p53 frequently contain HPV". *Gynecol. Oncol.,* 1995, *58,* 289.
[14] Akasofu M., Oda Y.: "Immunohistochemical detection of p53 in cervical epithelial lesions with or without infection of uman papillomavirus type 16 and 18". *Virchows Arch.,* 1995, *425,* 593.
[15] Sirotkovic-Skerlev M., Krizanac S., Kapitanovic S., Husnjak K., Unusic J., Pavelic K.: "Expression of c-myc, erbB-2, p53 and nm23-H1 gene product in benign and malignant breast lesions: coexpression and correlation with clinicopathologic parameters". *Exp. Mol. Pathol.,* 2005, *79,* 42.
[16] Lee Y.N., Wang K.L., Lin M.H., Liu C.H., Wang K G., Lan C.C. *et al.*: "Radical hysterectomy with pelvic lymph node dissection for treatment of cervical cancer: a clinical review of 954 cases". *Gynecol Oncol.,* 1989, *32* (2), 135.
[17] Takeda N., Sakuragi N., Takeda M., Okamoto K., Kuwabara M., Negishi H. *et al.*: "Multivariate analysis of histopathologic prognostic factors for invasive cervical cancer treated with radical hysterectomy and systematic retroperitoneal lymphadenectomy". *Acta Obstet. Gynecol. Scand.*, 2002, *81* (12), 1144.
[18] Zreik T.G., Chambers J.T., Chambers S.K.: "Parametrial involvement, regardless of nodal status: a poor prognostic factor for cervical cancer". *Obstet Gynecol.,* 1996, *87* (5 Pt 1), 741.
[19] Graflund M., Sorbe B., Bryne M., Karlsson M.: "The prognostic value of a histologic grading system, DNA profile, and MIB-1 expression in early stages of cervical squamous cell carcinomas". *Int. J. Gynecol. Cancer,* 2002, *12,* 149.
[20] Kristensen G.B., Holm R., Abeler V.M., Trope C.G. :"Evaluation of the prognostic significance of nm23/NDP kinase protein expression in cervical carcinoma: an immunohistochemical study". *Gynecol. Oncol.,* 1996, *61,* 378.
[21] Marone M., Scambia G., Ferrandina G., Giannitelli C., Benedetti-Panici P., Iacovella S. *et al.*: "Nm23 expression in endometrial and cervical cancer: inverse correlation with lymph node involvement and myometrial invasion". *Br. J. Cancer,* 1996, *74,* 1063.
[22] Park D.S., Lee Y.T., Lee J.M.: "Prediction of lymph node metastasis based on p53 and nm23-H1 expression in muscle invasive grade III transitional cell carcinoma of bladder". *Adv. Exp. Med. Biol.*, 2003, *539,* 67.
[23] Camilien L., Gordon D., Fruchter R.G., Maiman M., Boyce J.G.: "Predictive value of computerized tomography in the presurgical evaluation of primary carcinoma of the cervix". *Gynecol. Oncol.,* 1988, *30,* 209.
[24] Choi H.J., Roh J.W., Seo S.S.: "Comparison of the accuracy of magnetic resonance imaging and positron emission tomography/computed tomography in the presurgical detection of lymph node metastases in patients with uterine cervical carcinoma: a prospective study". *Cancer,* 2006, *106,* 914.

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