ORIGINAL RESEARCH

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Expression and significance of FOX M1 in human cervical cancer: A tissue micro-array study

Abstract

Objective: This study was designed to investigate the expression and significance of the Forkhead Box M1 (FOX M1) transcription factor in human cervical cancer.

Methods: The expression of FOX M1 protein was assessed in tissue microarrays containing 102 cervical cancer tissues by the Streptavidin–Peroxidase (SP) immunohistochemistry technique. The relationship between FOX M1 protein and clinico-pathological features (pathological stages, pathological types, TNM stage) was analyzed.

Results: FOX M1 protein was located in the cytoplasm and/or nucleus. The overall expression of FOX M1 in the cytoplasm and nucleus was not associated with T stages ($P=0.217$) or lymph node status ($P=0.313$). The nuclear expression of FOX M1 protein was not associated with T stage ($P=0.508$) or lymph node status ($P=0.345$). Elevated translocation and activity of FOX M1 were discovered with a secondary analysis that showed that the differences of the nuclear expression of FOX M1, among different pathological stages, were statistically significant ($P<0.05$). The nuclear expression of FOX M1 in low differential cervical cancer tissues was significantly higher than in high differential cervical cancer tissues ($P<0.05$).

Conclusion: The overexpression of FOX M1 protein in cervical cancer maybe associated with the progression of cervical cancer, and could be a potentially novel tumor marker useful for diagnosis and therapy of cervical cancer.

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Although the 5-year relative survival rate of cervical cancer has improved, as a potentially preventable disease, cervical cancer is still the seventh in frequency overall and the second most common cancer among women worldwide [1, 2]. It has been evidenced that human papillomavirus (HPV) is one of the most important risk factors and maybe the most essential etiological agent in cervical cancer [3]. Recent studies have demonstrated that HPV alone is insufficient in cervical cancer development. The average of women with invasive cervical cancer is approximately 50 years of age, whereas the mean age of women with detectable HPV is much earlier than 28 years, which suggests in most cases a long precancerous state exist that allows the accumulation of secondary genetic changes [4, 5]. Therefore, there must be other factors involving in the development of cellular genetic changes or in the activation of signal transduction pathways in the progress of carcinogenesis and tumor progression.

Cancer is often defined as a disease of cell proliferation, and the majority of cancer studies have focused on examining functional consequences of activating and/or inactivating mutations in critical genes and signaling pathways regulating cell proliferation and/or cell death [6]. Forkhead box (FOX) is such a critical gene and FOX protein family is a growing group of transcription factors that plays important roles in cellular proliferation, differentiation and organ morphogenesis [7]. FOXM1, an essential member of FOX family, is broadly expressed in actively dividing cells and is crucial for cell cycle-dependent gene expression in the process of the cell cycle. As more and more studies have uncovered the mechanism by which FOXM1 controls the transcriptional network of genes that are essential for cell division and exit from mitosis, it has been proposed that FOXM1 may also mediate cellular transformation into cancer cells [8, 9]; however, little is known about FOXM1 expression in human cervical cancer and its clinical implications. In the present study, the expression and significance of FOXM1 protein were examined by immunohistochemistry in a tissue micro-array composed of human cervical cancer tissues.

Methods

Patients and Tissue Micro-array

Tissue micro-array analysis of 102 cervical specimens, obtained from patients who underwent surgery, was prepared by Fanpu Biotech, Inc (Guilin, China). All specimens were fixed with formalin postoperative and embedded with paraffin before constructed as a slide of tissue micro-array (1.5 mm each core, 13×8). The TNM stage of tumors was determined according

to the TNM classification system of UICC staging criteria version 6 with 83 T1 stage cases and 14 T2-4 stages cases. No distant metastasis was found in any cervical cancer patients.

Immunohistochemistry

Immunolocalization staining of FOXM1 protein was performed using the streptavidin-biotin peroxidase complex method (SP) [10]. Tissue micro-arrays and two control slides were first deparaffinized in xylene, ethanol, and water, and then endogenous peroxidase was blocked by immersing the sections in 3% H₂O₂ in methanol for 10 min to prevent any nonspecific binding. For staining, the slides were pretreated in 10 mM citrate buffer (pH 6.0) and then heated in a microwave oven (98°C) for 10 min. After blocked with bovine serum albumin (BSA), the slides were incubated with the primary antibodies for FOXM1 protein (rabbit polyclonal, Proteintech Group, Inc, China, dilution 1:300) for 90 min at 37°C, and then incubated with the secondary antibody (biotin-labeled anti-rabbit IgG goat antibody, (Santa Cruz Biotechnology, USA; dilution 1:250) for 15 min at 37°C, and finally incubated with peroxidase-labeled streptavidin (Maixin Biotechnology, Inc., China,) for 15 min. The reaction products were visualized with diaminobenzidine (DAKO, Denmark). The slides were counterstained with haematoxylin. As a negative control, the primary antibody was replaced with Tris-buffered saline on sections that were proven to be positive for FOXM1 in preliminary experiments.

Evaluation of Immunohistochemical Variables

The slides were examined under Olympus BX51 fluorescence microscope equipped with Olympus DP72 camera (Olympus Optical Co., Ltd., Tokyo, Japan). Positive cells were stained brownish granules either in cytoplasm or in nucleus. The percentage of immunoreactive positive cells and intensity for FOXM1 protein in cervical tumors and normal epithelia were assessed semiquantitatively. Three high power fields in each core were selected randomly and observed double blind by two pathologists. The staining score of each core was calculated by staining intensity and percentage of positive cancer cells. For the quantification of staining intensity, the intensity of staining was scored as 1 (very weak), 2 (weak), 3 (moderate), 4 (intense) and 5 (very intense). The overall expression level of FOXM1 protein in each positive cell was scored as percentage of positive cells multiplied by intensity of staining ranging from 0 to 5. Furthermore, nuclear FOXM1 positive rate was obtained by counting the cells with FOXM1 localized in nucleus, thus nuclear FOXM1 expression was defined as nuclear positive rate

FIGURE 1. Immunohistochemical expression of FOXM1 protein in cervical cancer tissues using a tissue micro-array. (A, B) Squamous cell carcinoma is detected with intense and no FOXM1 expression. (C, D) FOXM1 protein is located in the nucleus both in squamous cell carcinoma and adenocarcinoma (black arrows). (E, F) The characteristic distribution pattern of FOXM1 in stage III cervical squamous cell carcinoma is diffused expression, invasion progression was revealed (red arrows).

Magnifications: A, B: 100 $\times$; C, D, F: 400 $\times$, E: 200 $\times$.

multiplied by overall expression scores (Nuclear expression = % nuclear positive cells $\times$ intensity of nuclear staining). All those scores ranging from 0 to 5 are documented as overall and nuclear FOXM1 protein level.

Statistical Analysis

All the experiment data were integrated into a comprehensive data set. Numerical data were recorded directly and measurement data were described as median and range. A secondary analysis was performed to assess the relationship between clinopathological characteristics and FOXM1 expression. For the comparison of individual variables, *t* tests, LSD and Welch tests were carried out as appropriate. Statistical analysis was performed on SPSS software version 13.0 (SPSS Inc. Chicago, IL),

TABLE 1. Major clinico-pathologic characteristics of 97 cancer cases

Items	Value (%)
Pathological types	
Adenocarcinoma (%)	17 (17.5)
squamous cell carcinomas (%)	74 (76.3)
Adeno-squamous cell carcinoma (%)	5 (5.2)
muroid adenocarcinoma (%)	1 (1.0)
Pathological stages	
Highly differentiated (%)	12 (13.2)
Moderately differentiated (%)	24 (26.4)
Moderately –poorly differentiated (%)	17 (18.7)
Poorly differentiated (%)	38 (41.8)
Invasive extent	
Confined to uterus (%)	83 (85.6)
Beyond the uterus (%)	14 (14.4)
Lymph node status	
N0 (%)	73 (75.3)
N1 (%)	24 (24.7)

and two-sided $P < 0.05$ was considered indicative of statistically significant.

Results

Overall Data

Among the 102 cases, 97 specimens were obtained from cervical cancer patients and 5 healthy controls. The patients ranged in age from 24 to 68 years (mean age $M \pm SD$, 44.5 ± 9.3 years). The major clinico-pathologic characteristics are summarized in Table 1.

Immunohistochemical Characteristics

There was a clear background, with no nonspecific staining, in control slides and FOXM1 protein was stained brownish granules in positive cells, mainly located in the cytoplasm and nucleus (Fig. 1. A, B). A significant percentage of nuclear FOXM1 protein expression was observed both in squamous cell carcinomas and adenocarcinoma tissues (Fig. 1. C, D). Additionally, the progress of invasion was showed as the invasive cancer cells were stained brownish with no staining in circumjacent normal cells (Fig. 1. E, F). The characteristic distribution pattern of FOXM1 was scattered expression in tumor tissue, although small areas of diffused expression were also observed (Fig. 1).

FIGURE 2. The expression of FOXM1 protein in cervical cancer with different pathological stages. A: The expression of FOXM1 protein in well differentiated carcinomas. B: The expression of FOXM1 protein in moderately differentiated carcinomas FOXM1. C: The expression of FOXM1 protein in moderately differentiated carcinomas FOXM1. Significant difference of nuclear expression of FOXM1 was found in different pathological stages. D: Decreasing expression of FOXM1 protein was found from basal layer to cutinized layer (blue arrow). E: Most FOXM1 proteins were located in neoplasm though the overall expression was intensive (red arrow). F: The FOXM1 proteins were mainly located in nuclear (black arrow). Magnification: A, B, C: 100 $\times$; D, E, F: 200 $\times$.

The overall expression of FOXM1 in cervical cancer tissues

The overall expression of FOXM1 in 5 normal tissues and 97 cancer tissues were scored as 0.140 ± 0.147 and 1.397 ± 1.122 (mean $\pm$ SD), respectively. To address the relationship between the overall expression of FOXM1 and cervical cancer patients' clinicopathological data in terms of pathological types and stages, adeno-squamous cell carcinoma (5 cases) and mucoid adenocarcinoma (1 case) were excluded because of lack of complete pathological stages and types information. The overall FOXM1 expression was 1.386 ± 1.137 in 91 cervical cancer cases. The difference between the overall expression of

TABLE 2. The relationship between the overall expression of FOXM1 and clinico-pathological characteristics

Variables	N (%)	Overall Expression*	P value
Pathological types			
Squamous cell carcinoma	74(81.3)	1.326±1.104	P=0.297
Adenocarcinoma	17(18.7)	1.647±1.272	
Pathological stages			
Well differentiated	12(13.2)	1.675±1.070	P=0.770
Moderately differentiated	24(26.4)	1.281±1.152	
Moderately - Poorly differentiated	17(18.7)	1.277±1.271	
Poorly differentiated	38(41.7)	1.411±1.114	
Invasive extent			
Confined to uterus (T1)	83(85.6)	1.455±1.119	P=0.217
Beyond the uterus(T2~T4)	14(14.4)	1.054±1.113	
Lymph node status			
N0	73(75.3)	1.464±1.130	P=0.313
N1	24(24.7)	1.196±1.095	

*Overall Expression = percentage of positive cells multiplied by intensity of staining

FOXM1 in adenocarcinoma and squamous cell carcinomas was not statistically significant ($P=0.297$). To evaluate the relationship between overall FOXM1 expression and invasive extent, FOXM1 expression was assessed in two groups; determined by T stage as cervical carcinoma confined to uterus (T1) and tumors invade beyond the uterus (T2, T3, T4). The overall FOXM1 expression in the cytoplasm and nucleus was not associated with pathological stages, pathological types, invasive extent or lymph node status.

The nuclear expression of FOXM1 in cervical cancer tissues

The nuclear expression of FOXM1 protein in 5 normal tissues and 97 cancer tissues were scored as 0.042 ± 0.044 and 0.900 ± 0.784 (M $\pm$ SD), respectively. The nuclear expression of FOXM1 protein was 0.862 ± 0.764 in 91 cervical cancers cases with complete pathological information. The nuclear expression of FOXM1 was not related to pathological types ($P=0.101$). Significant difference of nuclear expression of FOXM1 was found in different pathological stages ($P=0.027$) followed by a secondary analysis. The difference between well differentiated carcinoma and poorly differentiated group was statistically significant ($P=0.030$). The difference between moderately differentiated carcinoma and poorly differentiated group was statistically significant too ($P=0.039$). The nuclear expression of FOXM1 was higher in stage III compared to

stage I and II ($P < 0.05$). All major information was summarized in Table 2 and Fig. 2.

Discussion

FOXM1 protein is a typical proliferation-associated transcription factor that stimulates proliferation by promoting S-phase entry as well as M-phase entry and is essential for proper execution of mitosis [11]. Furthermore, in accordance with the observed role of FOXM1 protein in cell cycle progression and cell proliferation, FOXM1 is indicated in malignant transformation and elevated expression of FOXM1 has been reported in several tumors; including pancreatic carcinoma, hepatocellular carcinoma, glioblastoma, lung cancer, prostate cancer and leukemia [12-16]. Although few studies exploring the functional significance of FOXM1 in human cervical cancer have been reported, oncoprotein E7 of transforming human papillomavirus 16 (HPV), one of the major oncogene contributes to cervical carcinogenesis, can directly bind to FOXM1 and enhance the transactivation of human c-myc P1 and P2 promoter by FOXM1 [14, 17]. In our study, morphology analysis showed that the expression of FOXM1 protein was decreasing from basal layer to cutinized layer (Fig. 2), which indicated that FOXM1 protein may play an important role in tumorigenesis. Though our study showed that the overall expression of FOXM1 was higher in cervical cancer tissues (1.397 ± 1.122) than in normal cervical tissues (0.140 ± 0.147), the sample size for normal patients was too limited to allow a statistical analysis.

An increase in expression of FOXM1 protein may promote tumorigenesis through driving proliferation and cell cycle by regulating critical events such as DNA replication, DNA repair, G2/M progression, centrosome duplication, chromosome segregation and oxidative stress [18, 19]. Recent research has validated the critical role of FOXM1 protein in tumorigenesis. Wang *et al.* [20] showed that FOXM1 is associated with poor prognosis of non-small cell lung cancer (NSCLC). Furthermore, a striking reduction in the number and size of lung tumors was found following the deletion of FOXM1 from lung epithelial cells prior to tumor initiation in transgenic mice, which resulted primarily from a significant decrease in TOPO-2 α , a direct target of FOXM1. FOXM1B, the predominant FOXM1 isoform, could directly promote gastric tumorigenesis by regulating the expression of genes key to cancer biology overall [21]. Disruption of the FOXM1-cyclin B1 regulatory loop is one of the mechanisms of the negative regulation of hepatocarcinogenesis [22].

The role of FOXM1 protein in cancer extends beyond malignant transformation, and, in certain instances, deregula-

TABLE 3. The relationship between the nuclear expression of FOXM1 and clinico-pathological characteristics

Variables	N (%)	Nuclear Expression*	P value
Pathological types			
Squamous cell carcinoma	74(81.3)	0.908±0.813	P=0.101
Adenocarcinoma	17(18.7)	0.662±0.463	
Pathological stages			
Well differentiated	12(13.2)	0.620±0.378	P=0.027
Moderately differentiated	24(26.4)	0.643±0.538	
Moderately-Poorly differentiated	17(18.7)	0.641±0.501	
Poorly differentiated	38(41.8)	1.177±0.956	
Invasive extent			
Confined to uterus (T1)	83(85.6)	0.922±0.773	P=0.508
Beyond the uterus (T2~T4)	14(14.4)	0.771±0.866	
Lymph node status			
N0	73(75.3)	0.943±0.828	P=0.345
N1	24(24.7)	0.768±0.630	

*Nuclear Expression = Percentage nuclear positive cells multiplied by intensity of nuclear staining

tion of FOXM1 proteins could result in disease progression. Thus, FOXM1 may be used as a prognostic marker. FOXM1 protein expression was confirmed by immunohistochemistry on a separate panel of human oral tissues showing significant increase in FOXM1 protein expression in moderate and severely dysplastic lesions, primary HNSCC [23]. In contrast to this finding, no significant relationship was found between overall FOXM1 expression and cervical cancer stage in our study; however, nuclear positive rates were calculated and a secondary analysis revealed that increased positive rate of nuclear FOXM1 expression was associated with upgraded pathological stage, and FOXM1 expression in poorly differentiated tumors were much higher than in others. This is reasonable given that FOXM1 protein is known to be a transcriptional factor, exercising its biological effect primarily in the nucleus. FOXM1 protein is localized mainly in the cytoplasm in cells at late-G1 and S phase, nuclear translocation occurs just before entry into the G2/M phase and FOXM1 is activated by phosphorylation [24]. The transcriptional activity of FOXM1 increases as cells progress from S to G(2)/M phases and is controlled in a cell cycle-dependent fashion by temporally regulated phosphorylation and dephosphorylation of Ser-251, a critical target for the activation of FOXM1 within the forkhead domain [25]. For this reason, we propose that FOXM1 promotes cancer progression by increasing the nuclear translocation and activity of FOXM1.

What is even more exciting is the accumulating evidence that FOXM1 protein plays an important role in tumor invasion, tumor angiogenesis and metastasis. Acquisition invasive phenotype is a turning point during cancer progression, through which cells evolve progressively from normalcy through a series of premalignant states into invasive cancers. Several pathways have been proposed and recent studies have pointed to a considerable role for FOXM1 in invasion and metastasis [8, 26]. It FOXM1 has been shown to regulate transcription of c-Jun N-terminal kinase (JNK1) gene to promote the G1/S transition and tumor cell invasiveness, partly by up-regulating the expression of the matrix metalloproteases (MMPs) 2 and MMP9, both of which are crucial in the processes of tumor cell invasion and metastasis for MMPs can directly degrade the basement membrane collagen at tumor-invasive front and enable cancer cells to overcome the extracellular matrix (ECM) barrier leading to metastasis [27, 28]. In lung cancer cells, combining radiation therapy with MMP2 down regulation is more effective than radiation alone, and such a strategy involves abrogation of FOXM1 activity [29]. In addition to MMPs, VEGF (another important factor in tumor cell invasion, angiogenesis, and metastasis) has been shown to be a direct transcriptional target of FOXM1 [15, 30]. Down regulation of FOXM1 inhibited the activity of VEGF and led to a significant reduction in the invasive potential of malignant breast cancer cell lines [31]. Although the phenomenon of invasion was revealed in our study (Fig. 1 a significant correlation could not be detected between FOXM1 expression and T stage or lymph node status; consistent with the results of a study by Bektas *et al.* [32]. This lack of correlation might be related to the fact that previous studies were mainly based on *in vitro* experiments using only cancer cell lines, whereas the studies analyzed primary human cancer tissues take into account the role of tumor microenvironment. The major limitation of our study was insufficient patient numbers, and may be another reason for this negative result. Large scale studies are needed for more detailed analyses.

In summary, we found a positive correlation between FOXM1 expression and pathological stage. Tissue micro-array is a high throughput way to study the expression of FOXM1 protein in cervical cancer tissues. In addition, FOXM1 may represent a novel cervical tumor marker with clinical significance that could be included into multi-marker panels for cervical cancer detection and treatment.

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