

## Original Article

# Upregulation of *URI/RMP* gene expression in cervical cancer by high-throughput tissue microarray analysis

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**Abstract:** URI, or RMP, is a RNA polymerase II subunit RPB5-associated protein known to play essential roles in ubiquitination and transcription. Recently, we and others have shown that URI/RMP is also important for progression of hepatocellular carcinoma, ovarian, and prostate cancers. To identify the mechanistic basis of URI/RMP during multiple cellular processes, we investigated URI/RMP expression in a tissue microarray (TMA) containing multiple normal human tissues. The results showed that URI/RMP is ubiquitously but differentially expressed in these human tissues which partially explains its multiple cellular functions. To elucidate the role of URI/RMP during oncogenesis of multiple malignancies, especially the tumors of reproductive system, we analyzed URI/RMP expression in a TMA containing multiple reproductive system tumors. We did not observe significant difference of URI/RMP expression between cancerous and adjacent tissues of the prostate, breast, ovarian, and endometrial cancers. However, increased URI/RMP expression was observed in two of the three cases of cervical SCC (squamous cell carcinoma) cells compared to their adjacent epithelial cells. Moreover, we detected significantly upregulated URI/RMP expression not only in cervical cancers but also in pre-cancerous CINs (cervical intra-epithelial neoplasias) in a TMA that covers the whole spectrum of normal cervix, CINs, and cervical cancers. No difference of URI/RMP expression was observed between CINs and cervical cancers. Given the high risk of CINs (especially CIN3) turning into cervical cancer if left untreated, the increased URI/RMP expression in CINs as well as in cervical cancers suggest a clinical relevance of URI/RMP upon cervical cancer tumorigenesis and worth further investigation.

**Keywords:** URI/RMP, tissue microarray, immunohistochemistry, cervical cancer

## Introduction

URI (unconventional prefoldin RPB5 interactor) or RMP (RPB5-mediating protein), is a member of the prefoldin family of molecular chaperones. It has been demonstrated that URI/RMP functions as a scaffolding protein and plays critical roles in ubiquitination and transcription. This may be partially attributed to URI/RMP interaction with the RNA polymerase II subunit RPB5, as well as through formation of protein complexes with many other protein peptides [1, 2]. Previous studies have also shown that URI/RMP participates in the TOR (Target of Rapamycin) signaling pathway, suggesting its potential involvement in cancer development

[3]. Indeed, we have recently demonstrated that URI/RMP is important for the progression of hepatocellular carcinoma [4]. This oncogenic property was further confirmed by a recent study which indicated that *URI* is amplified in ovarian cancer cells [5]. URI/RMP is known to contain both nuclear and cytoplasm localization signals making *URI/RMP* a possible ubiquitous gene [3]. However, currently, the exact expression profile of *URI/RMP* gene in many human tissues is largely unknown. Meanwhile, besides hepatocellular carcinoma and ovarian cancer, how URI/RMP functions during development of other malignancies, especially the tumors of reproductive system, have not been elucidated. Here, we report analysis of *URI/*

## URI/RMP expression in cervical cancer

**Table 1.** URI/RMP expression in cytoplasm of major cell type in normal human tissues

Tissues	Cell Components	Score	Tissues	Cell Components	Score	
Tonsil	Lymphocytes	(+)	Brain	Granulose cells, Pyramidal cells	(+)	
	Epithelial cells	(++)		Liver	Liver cells	(++)
Lung	Alveolar cells	(++)	Bone marrow		Granulocytes, Erythroid cells	(++)
	Macrophages	(+++)			Lymphocytes	(+)
Testicle	Spermatogonia, Spermato- cytes, Sperm cells	(++)	Thyroid Gland	Follicular cells, Parafollicular cells	(++)	
	Sperm	(++)		Spleen	Lymphocytes	(+)
Parathyroid gland	Chief cells	(++)	Breast gland		Plasma cells	(++)
	Oxyphil cells	(+++)		Epithelial cells	(++)	
Colon	Columnar cells, Goblet cells	(++)	Adrenal gland	Cortical cells, Medullary cells	(+++)	
	Acidophil cells, Basophil cells	(+++)	Salivary gland	Acinar cells	(++)	
Prostate gland	Epithelial cells	(++)	Small Intestine	Epithelial cells	(++)	
Kidney	Epithelial cells	(+++)	Myocardia	Myocardial cells	(++)	
Esophagus	Mucosal epithelial cells	(+++)		Pancreas	Islet cells	(+++)
Stomach	Gastric parietal cells	(++)	Acinar cells		(+)	
	Principal cells	(++)				
Cerebellum	Granule cells	(++)				
Thymus	Lymphocytes	(+)				

“+” means weak, “++” means moderate, and “+++” means strong expression.

RMP gene expression in multiple normal human tissues as well as in tumors of the reproductive system by focusing on tissues from precancerous cervical intraepithelial neoplasia (CIN) and invasive cervical cancer.

### Materials and methods

#### *Tissue microarrays (TMAs)*

Three tissue microarrays (TMAs) were used for this study. The multiple normal human tissues TMA (FDA808-1) was purchased from the Ailina Biotechnology Co., Ltd. (Xi'an, China). This TMA contains 24 different kinds of human tissues that are partially listed in **Table 1**. Each tissue was dotted three times which is from three different individuals. The TMA from tumors of reproductive system (OD-CT-Rp03-002) was purchased from Shanghai Outdo Biotech Co., Ltd (Shanghai, China). This TMA was prepared from 31 cases of tumors that include prostate (10 cases), breast (7 cases), ovarian (7 cases), endometrial (4 cases) and cervical (3 cases) cancers. The TMA of cervical cancer and cervical intraepithelial neoplasias (CINs) was a product of Pantomics, Inc. (CIN481, Richmond, CA USA.). This tissue array contains tissues from 48 cases that cover the whole spectrum of nor-

mal cervix (4 cases), CINs (CIN 1-3, 32 cases) and invasive cervical cancers (12 cases).

#### *Immunohistochemistry (IHC)*

Immunohistochemical staining was performed on arrayed tissue samples using RMP antibody (K-17, sc-103869, Santa Cruz, CA USA). Briefly, after deparaffin and rehydration, the TMA slides were pretreated with 10mM sodium citrate buffer (pH 6.0) for 10 minutes in a microwave for antigen retrieval. The endogenous peroxidase was quenched by adding the hydrogen peroxide (3% H<sub>2</sub>O<sub>2</sub> in 100% ethanol) at room temperature for 15 minutes. After washing, the slides were blocked by rabbit serum for 1 hour. The blocking buffer was removed and the slides were then incubated overnight with primary RMP antibody (K-17) at 1:100 dilutions at 4°C. Slides were washed with the 1xTBST (Tris Buffered Saline with 0.1% Tween-20) solution and further incubated with poly-HRP anti-Goat IgG (PV-9003, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 15 minutes. Detection was using the 3, 3'-diaminobenzidine as instructed (DAB kit, ZLI-9032, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). Slides were counterstained with hematoxylin before microscopic analysis.

### *Microscopic analysis of URI/RMP in normal TMA*

The URI/RMP expression in TMA of normal human tissues was subjected to microscopic analysis. Briefly, after IHC staining, a cell was recorded as positive if it was stained from light yellow to brown either in the nucleus or cytoplasm of that cell. The cell type and subcellular (nucleus and cytoplasm) localization of URI/RMP expression in different tissues were determined by an experienced histologist. Cells from five randomly selected visions under high magnification (400x) were counted for statistical analysis. The expression levels of URI/RMP were quantified using a modified scoring method as previously described [6]. Specifically, if the percentage of positively stained cells over total number of cells is  $\leq 5\%$ , the score is "0"; 6-25%, scores "1"; 26-50%, scores "2"; 51-75%, scores "3", and more than 75%, scores "4". The intensity of the staining signal which represents relative URI/RMP expression level was also scored: "0" means no expression and "1-3" represents weak, moderate, and strong expression respectively. We also calculated the weight score of each specimen by multiplying percentage of positive cells and the score of the signal intensity. Weight score "0" means negative staining or no expression, while scores "1-4", "5-8", and "9-12" represents weak, moderate, and strong positive staining respectively.

### *Image analysis of URI/RMP in tumor TMA*

The URI/RMP expression in tumor TMAs were measured by analyzing the staining signal intensity using the Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Inc. Silver Spring, MD USA). The mean densitometry of the digital image ( $\times 400$ ) is designated as representative URI/RMP staining intensity. For the TMA of reproductive system tumors, the areas from both cancer and its adjacent normal tissue were selected for analysis of the staining intensity of URI/RMP. For the cervical cancer-related TMA, the cervical epithelium of normal cervix and the cervical lesions in CINs and invasive cervical cancers were analyzed.

### *Statistical analysis*

One-way ANOVA (SPSS16.0) was used to assess the significance of URI/RMP staining intensity across sample groups of cervical can-

cer and CIN TMA. Values (scores or signal intensities) are presented as means  $\pm$  SEM (standard error of mean). If there is a statistical significance, SNK-q test will be used for pairwise comparison among the multiple groups.  $p < 0.05$  was considered statistically significant.

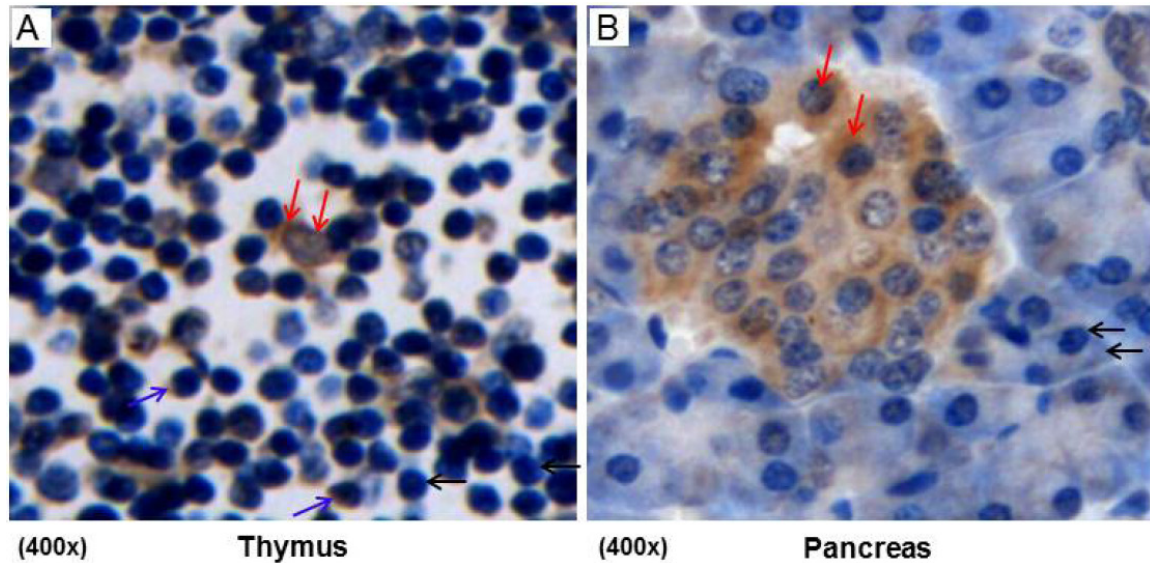
## Results

### *URI/RMP expression in normal human tissue*

IHC analysis of the TMA containing multiple normal human tissues (FDA808-1) indicates that, URI/RMP is ubiquitously expressed in all the human tissues examined, although more URI/RMP in cytoplasm was detected than in nuclei. We analyzed URI/RMP expression by focusing on the major cell type of each tissue. As illustrated in **Table 1**, URI/RMP were moderately expressed in cytoplasm of most of the major cell types examined, while weak or strong URI/RMP expression can be occasionally detected in some of the major cell types of the corresponding tissues. URI/RMP may be differentially expressed in same cell type of a tissue. Take lymphocytes of the thymus as an example, no URI/RMP expression was appreciated in the nuclei. However, weak URI/RMP expression was observed in cytoplasm of most of the lymphocytes, whereas there are lymphocytes that show either strong or negative URI/RMP expression in the cytoplasm (**Figure 1A**). URI/RMP expression can be significantly different among different cell types of a tissue. For instance, URI/RMP is strongly expressed in islet cells of the pancreas, while URI/RMP is barely detectable in the adjacent acinar cells (**Figure 1B**). URI/RMP is weakly expressed in nuclei of most of the tissues examined. We do not include data from ovarian tissue, as no follicular and luteal structure was observed except some connective tissue (**Table 1**).

### *URI/RMP expression in tumors of reproductive system*

To investigate the correlation of URI/RMP expression with tumors of the reproductive system, we performed IHC analysis using RMP antibody (K-17, sc-103869) and the TMA containing tissues from prostate, breast, ovarian, and endometrial cancers, as well as tissues from cervical squamous cell carcinoma (SCC) and their adjacent tissues (OD-CT-Rp03-002). We did not observe significant difference of



**Figure 1.** URI/RMP expression in tissues of thymus and pancreas. A. Thymus: Lymphocytes without URI/RMP expression in nuclei and cytoplasm were indicated by black arrows. Lymphocytes without URI/RMP expression in nuclei but weakly expressed in cytoplasm were indicated by blue arrows. Strong URI/RMP expression was observed both in nuclei and in cytoplasm of the epithelial reticular cells (red arrows) compared to the negative or weak signals in the nuclei and cytoplasm of the lymphocytes. B. Pancreas: URI/RMP is highly expressed both in nuclei and in cytoplasm of islet cells (red arrows). URI/RMP is barely detectable in nuclei and very weakly expressed in cytoplasm of the acinar cells (black arrows).

URI/RMP expression between cancerous and the adjacent tissues in prostate, breast, ovarian, and the endometrial cancers. However, enhanced immunostaining indicating upregulated URI/RMP expression was observed in two of the three cervical SCC cells (**Figure 2A, 2C**) compared to their adjacent epithelial cells (**Figure 2B, 2D**).

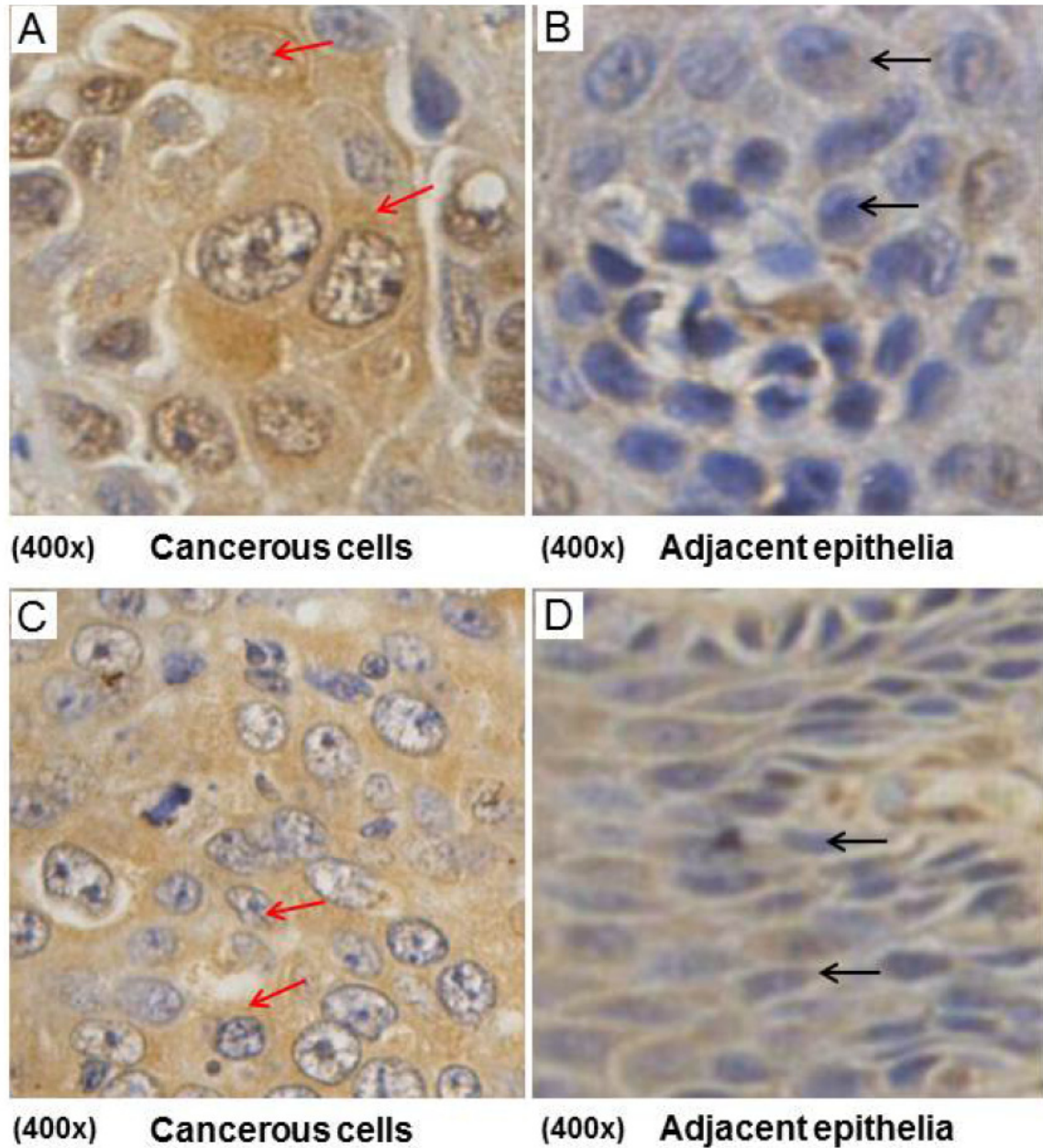
*URI/RMP expression in tissues of CINs and cervical cancers*

To attest above finding of upregulated URI/RMP expression in cervical SCC cells, we specifically performed URI/RMP immunostaining using a CIN and cervical cancer related TMA from Pantomics (Cat. No: CIN481, Set 1, California). This TMA covers the whole spectrum of tissues from normal cervix (4 cases), CIN 1 (12 cases), CIN 2 (11 cases), CIN3 (9 cases), and invasive cervical cancers (including 10 cases of cervical SCC, 1 case of mucinous adenocarcinoma, and 1 case of adenocarcinoma). As shown in **Figure 3A**, no or only weak URI/RMP expression was detected in the normal epithelial cells of the cervix, whereas high-level URI/RMP expression was shown in the nuclei and cytoplasm of the pre-cancerous cells of the CIN1, CIN2, and CIN3, as well as in cells from invasive cervical

cancers (**Figure 3B-F**). We also quantified URI/RMP expression in tissues of normal cervix, CINs and invasive cancers by analyzing their immunostaining signal intensity (densitometry). The results showed that URI/RMP expression in tissues of CINs and cervical SCC is significantly upregulated compared to its expression in tissues of normal cervix (**Table 2** and **Figure 4**,  $p < 0.05$ ). There is no difference of the signal intensity between CINs and cervical SCC (**Table 2** and **Figure 4**,  $p > 0.05$ ).

**Discussion**

According to the NCBI (National Center for Biotechnology Information) database, *URI/RMP* is a conserved gene among species (<http://www.ncbi.nlm.nih.gov/gene/8725>). The subcellular (nucleus and cytoplasm) localization of URI/RMP suggests that URI/RMP might be ubiquitously expressed and play essential functions during multiple cellular processes [3]. Indeed, URI/RMP has been shown to play important roles in DNA damage response, transcription elongation, and translation initiation [7-9]. These nuclear related functions are critical for cellular growth, apoptosis, and cell death [10-12]. URI has also been shown to participate in the cytoplasmic assembly of RNA polymerase

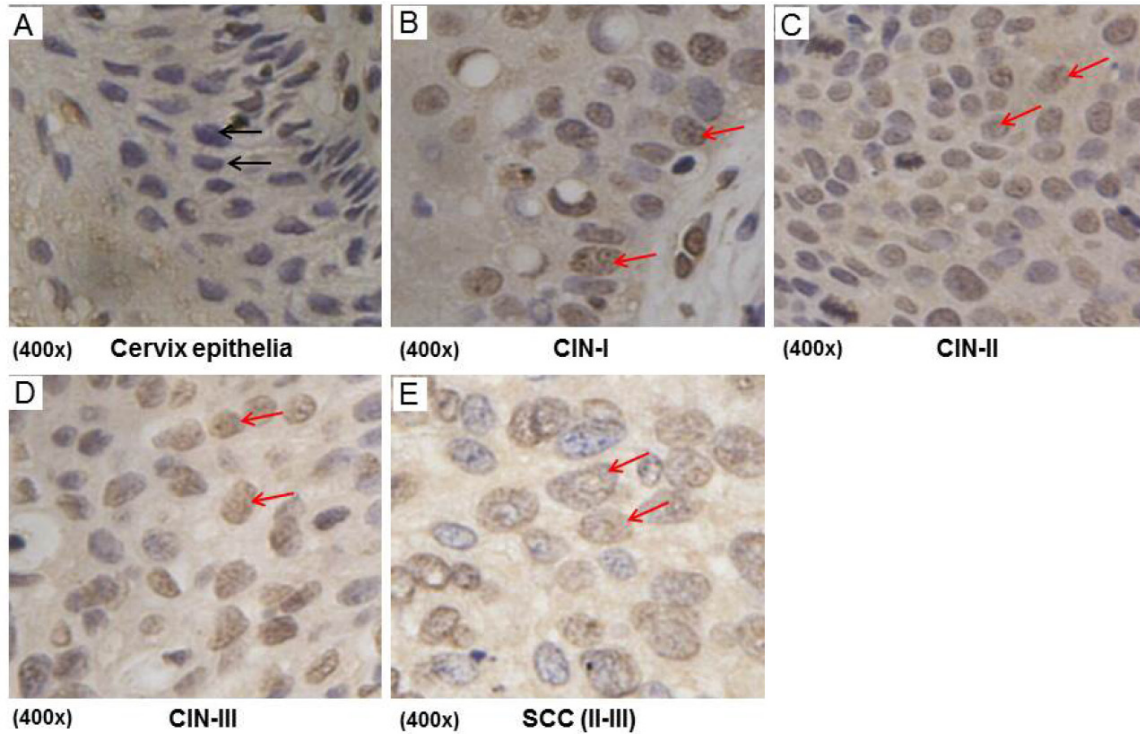


**Figure 2.** URI/RMP expression in cervical cancer and adjacent tissues. URI/RMP is highly expressed both in nuclei and in cytoplasm of the cervical SCC (squamous cell carcinoma) cells (A, C, red arrows). The tumor cells are enlarged and show irregular morphology and multiple nuclei. URI/RMP is barely detectable or weakly expressed in nuclei and cytoplasm of the adjacent epithelial cells (B, D, black arrows).

II by formation of a prefoldin-like complex [13]. To identify the potential mechanistic basis of URI/RMP and its correlation with multiple cellular functions, we investigated URI/RMP expression in a TMA containing multiple normal human tissues (FDA808-1). We observed ubiquitous URI/RMP expression in all the human tissues examined with more URI/RMP in cytoplasm than in nuclei (Table 1). Notably, URI/

RMP was found differentially expressed not only among different cell types, but also in same cell type of a tissue. In thymus, URI/RMP is non-detectable in nuclei of lymphocytes. Interestingly, we clearly detected two kinds of lymphocytes that show either positive or negative URI/RMP expression in the cytoplasm (Figure 1A, blue and black arrows). Although the biological significance of this expression

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**Figure 3.** URI/RMP expression in normal cervix, CINs, and cervical cancer. URI/RMP is barely detectable in nuclei and cytoplasm of the epithelial cells within the normal cervix (A, black arrows). URI/RMP is highly expressed both in nuclei and in cytoplasm of the epithelial cells at the CIN1 (B), CIN2 (C), and CIN3 (D) stages (red arrows). The epithelial cells of the CINs show nuclear atypia. URI/RMP is also highly expressed in nuclei and cytoplasm of the cervical SCC cells (E, red arrows). The cancer cells are enlarged and show irregular morphology and multiple nuclei. Illustrated are representative images from each of the normal cervix, CINs and cervical SCC groups.

**Table 2.** Densitometry calculation of URI/RMP in different groups

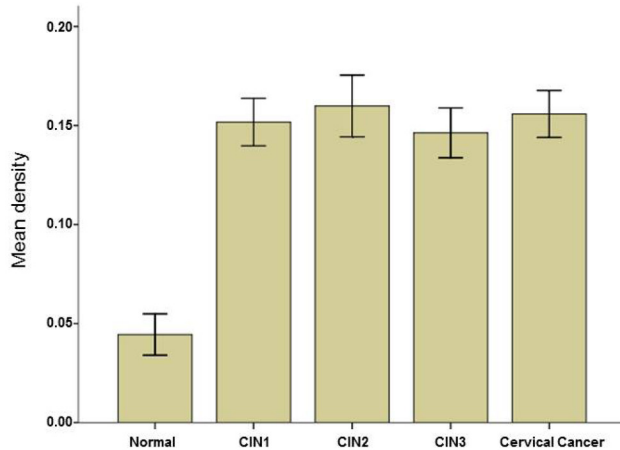
Groups	Cases	Mean	Std Error
Normal	4	0.044433	0.0104337
CIN1	12	0.151727	0.0119604
CIN2	11	0.159850	0.0155759
CIN3	9	0.146300	0.0125643
Cervical SCC	10	0.155833	0.0118321

The densitometry of CINs/cervical SCC is more intense than that of normal cervix ( $p < 0.05$ ). There is no significant densitometry difference between CINs and cervical SCC ( $p > 0.05$ ).

pattern is not clear, it may partially explain the multiple cellular functions of URI/RMP. Meanwhile, the differential or specific expression of URI/RMP in different cell types of a tissue provides a biological basis for human diseases. Here, our finding of high-level URI/RMP expression in islet (beta) cells, a cell population known to synthesize large amount of insulin [14], suggests a putative function of URI/RMP upon DM (Diabetes Mellitus) pathogenesis.

While it is not clear whether URI/RMP is important for DM pathophysiology, there is growing evidence which suggests an essential role of URI/RMP upon carcinogenesis of multiple malignancies. We have previously shown that URI/RMP regulates the growth, apoptosis, and death of hepatocellular carcinoma cells (HCC) both in vitro and in vivo [4]. URI was also identified as an oncogene that is amplified and over-expressed in ovarian cancer cell lines and human ovarian carcinomas. In addition, URI/RMP has been shown to play important function in meiotic cell cycle regulation and participate in AR (androgen receptor) signaling, a pathway critical for prostate cancer progression [8, 15-17]. These findings suggest a role of URI/RMP in development and cancer formation that majorly involves the reproductive system. However, expression analysis of TMA (OD-CT-Rp03-002) containing multiple reproductive system tumors did not show difference between cancerous and adjacent tissues in prostate, breast, ovarian, and endometrial cancers. This

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**Figure 4.** Quantitation of URI/RMP expression in normal cervix, CINs, and cancer cells. Densitometry of the immunostaining signal intensity of URI/RMP within normal cervix, CINs and cancers was calculated and subjected to statistical analysis. The mean signal intensity indicating URI/RMP expression in tissues of CINs and invasive cancers is significantly higher than that of normal cervix ( $p < 0.05$ ). There is no difference of the signal intensity between CINs and cervical cancers ( $p > 0.05$ ).

is possibly due to the limited number of specimens we analyzed and that contamination between cancerous and adjacent tissues may not be excluded. We did observe significant difference of URI/RMP expression between cancerous and adjacent tissues in two of the three cases of cervical SCC (Figure 3). Moreover, our further studies using TMA (CIN481) containing tissues from CINs and more cervical SCC cases demonstrated that URI/RMP is indeed significantly upregulated not only in cancerous cervical tissues but also in pre-cancerous CINs compared to the normal epithelium of the cervix (Figures 3, 4).

The finding that CINs show upregulation of URI/RMP suggests a potential clinical relevance of URI/RMP upon oncogenesis of cervical SCC. CIN, or cervical dysplasia, is the abnormal growth of epithelial cells on the cervix surface. CIN can be divided into LSIL (low-grade squamous intraepithelial lesions, or CIN1) and HSIL (high-grade squamous intraepithelial lesions, which subdivides into CIN2 and CIN3). The HSILs, especially cells at stage CIN3, are often referred to as cervical carcinoma in situ, given their high risk of turning into cervical cancer (usually cervical SCC) without proper treatment [18, 19]. We observed upregulated URI/RMP expression in cervical tissues from as early stage as in CIN1 to invasive cancers. Although,

there is no detectable difference of URI/RMP expression among CINs, or between CINs and cervical cancers, the increased URI/RMP expression in CINs compared to normal epithelial cells of the cervix suggests its involvement in cervical SCC initiation. We notice that cervical cancer has been largely attributed to HPV (human papillomavirus) infection, while CINs, which may take years to turn into invasive cervical cancers, are also associated with HPV infection. HPV oncoproteins E6 and E7 are the primary viral factors that play important roles in cervical cancer tumorigenesis, possibly through inactivation of *pRB* and *P53*, two famous tumor suppressor genes [20-23]. Correspondingly, reduced p53 and Rb expression was observed in cervical cancer [24]. More recently, mTOR inhibitors have been suggested as molecular targets for the treatment of HPV-associated malignancies including cervical cancer [25]. However, whether URI1/RMP works through the canonical HPV-P53-RB or mTOR signaling pathway and plays a role in cervical cancer oncogenesis remains to be determined and worth further investigation.

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### Declaration

All authors have no conflict of interest.

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