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Association of RAPH1 expression with cervical cancer progression and aggressiveness

Guangqiong Yang[1,2], Huafang Chen[1], Yong Lin[2], Pei Yang[1], Jinke Li[1,2]

[1]Department of Gynecology and Obstetrics, West China Second Hospital, Sichuan University, Chengdu, Sichuan
[2]Key Laboratory of Birth Defects and Related Disease of Women and Children (Sichuan University), ministry of Education, Chengdu, Sichuan (China)

Summary

Objective: The gene known as Ras association (RalGDS/AF-6) and pleckstrin homology domains 1 (RAPH1) play a role in cellular motility and lamelipodial protrusion, raising the possibility that it may contribute to development of cervical carcinoma and aggressive invasion by tumor cells. *Materials and Methods:* RAPH1 expression was analyzed in 153 cervical tissue samples at the mRNA level using quantitative RT-PCR and at the protein level using immunohistochemistry. Correlations were explored between expression and clinicopathological features of tumors. *Results:* Levels of RAPH1 mRNA were significantly higher in carcinoma tissues than in precancerous squamous intraepithelial lesions (SIL) or normal tissue (both $p < 0.05$). The percentage of RAPH1-positive samples increased progressively from normal tissue (10.0%) and low-grade SIL (31.3%) to high-grade SIL (45.8%) and carcinoma tissue (72.3%; $\chi^2_{CMH} = 30.0$, $p < 0.001$). RAHP1 expression positively correlated with grade of tumor differentiation ($p = 0.04$), vascular invasion ($p < 0.001$), interstitial invasive depth ($p < 0.01$), and tumor size ($p < 0.01$). *Conclusions:* RAPH1 expression is associated with cervical cancer development and aggressive behavior.

Key words: RAPH1; Cervical cancer; Carcinogenesis; Tumor development.

Introduction

Cervical cancer is the second most common malignant tumor affecting women worldwide, particularly in developing countries: 530,000 new cases and 275,000 deaths occur every year [1]. In China alone, nearly 100,000 women were newly diagnosed with invasive cervical cancer in 2015, and 30,800 women died from the malignancy [2].

Human papillomavirus (HPV) is the most important risk factor for cervical cancer development. More than 100 different HPV genotypes have been detected in the female reproductive tract, with at least 15 showing strong oncogenic potential [3]. Other events appear to be necessary for cervical carcinogenesis, since most HPV infections vanish spontaneously, and only 1% of infections appear to progress to carcinoma. This has made it difficult to understand the oncogenesis of cervical cancer in detail; it is thought that normal cervical epithelial cells are transformed into carcinoma by progressing through precancerous stages known as low-grade squamous intraepithial lesions (SIL) followed by high-grade SIL. Understanding the events that trigger this progression may help in understanding and treating cervical cancer.

The gene named Ras association (RalGDS/AF-6) and pleckstrin homology domains 1 (RAPH1) have been implicated in several process that can affect cancer onset and progression, including lymphocyte chemotaxis, axon guidance, angiogenesis, and tumor cell metastasis [4-6]. Indeed, RAPH1 is down-regulated in breast and ovarian cancers as well as in metastatic osteosarcomas, suggesting that it plays a role in cancer development [7, 8]. RAPH1 encodes the lamellipodin protein (Lpd), which binds to Ena and VASPs to assist in actin cytoskeleton assembly [4]; in this way, the gene plays an important role in cellular motility and lamelipodial protrusion. Whether RAPH1 is involved in cervical carcinoma development is unclear.

Work in the present laboratory based on cDNA microarrays has identified RAPH1 as one of the genes significantly up-regulated during *in vitro* transformation of immortalized cervical epithelial cells (data not shown). The present study verifies and extends that previous work by showing for the first time that RAPH1 mRNA and protein are up-regulated in cervical carcinoma tissues. The authors also found that greater RAPH1 expression correlates with tumor progression, poor differentiation, and aggressive behavior, suggesting a tumor-promoting role for RAPH1 during cervical cancer development.

Material and Methods

This study protocol was approved by the Ethics Committee of West China Second Hospital, Sichuan University, and informed consent was obtained from all individual participants included in the study.

Between 2012 and 2014, a total of 153 cervical tissue samples (28 fresh tissues and 125 paraffin-embedded tissue tissues) were

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collected at West China Second Hospital, Sichuan University for RAPH1 mRNA detection and immunohistochemistry (IHC). The fresh tissues including eight cases of squamous carcinoma, six cases of HSIL, six cases of LSIL, and eight normal tissues were collected for mRNA detection. The paraffin-embedded tissue samples (n=125) include 20 normal tissues, 16 LSILs, 24 HSILs, and 65 cervical squamous cancer tissues which were used for IHC study. Normal samples were taken from patients undergoing hysterectomy because of benign uterine disease. Tumors and SIL tissues were collected when patients were receiving operation or cervical biopsy. Tissue samples were either processed for mRNA analysis or paraffin-embedded for later immunohistochemistry.

All patients were treated at West China Second Hospital. All patients with invasive tumors underwent radical hysterectomy and lymphectomy. Pathological diagnosis was made independently by two pathologists.

Data on the following clinicopathological characteristics were analyzed: tumor histology grade, vascular invasion, interstitial invasive depth, lymph node metastasis, and tumor size. Tumor histology was classified as squamous cell carcinoma that was well-differentiated (WDSCC), moderately differentiated (MDSCC) or poorly differentiated (PDSCC). Vascular invasion was defined as microscopic detection of a carcinoma embolus in a blood capillary or lymphatic capillary. Lymph node metastasis was defined as microscopic detection of carcinoma in at least one lymph node. Interstitial invasive depth was classified as less than one-half of the entire cervix layer, more than one-half of the layer without reaching the cervical fascia, and exceeding the entire layer. Tumor size was measured in terms of the greatest dimension (cm).

Fresh tissue was homogenized in liquid nitrogen and suspended in TRIzol reagent. Total RNA was isolated according to the manufacturer's instructions, and RNA quality was verified by 1% agarose gel electrophoresis. RNA samples were stored at -80 °C until further analysis, and used to synthesize cDNAs with the first strand cDNA synthesis kit according to the manufacturer's instructions. PCR was performed using the SYBR Green real-time PCR kit. PCR reactions (10 μL) contained: 2.5x RealMasterMix, 1x SYBR Green, 0.4 μM forward and reverse primers, and 0.8 μl cDNA. GAPDH was analyzed as an internal control. The following primers were used: RAHP1 forward, 5'- GGGATCTGGTGTGCTTTCTC-3', RAHP1 reverse, 5'-CTGGATTTGTGGATGCTTCA-3', GAPDH forward, 5'- AGCCACATCGCTCAGACAC-3'; and GAPDH reverse, 5'- GCCCAATACACCAAATCC-3'. PCR was performed on a FTC-2000 fluorescent quantitative PCR detection system with the following parameters: initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, and annealing at 60°C for 30 seconds, with a final extension at 72°C for 30 seconds. Ct values were determined from amplification curves, and fold-increases were determined relative to levels in normal tissue using the 2^{-Ct} method [9].

Immunohistochemistry was performed as described [10]. Briefly, sections were deparaffinized, rehydrated and incubated with 3% H_2O_2 for 10 minutes, followed by boiling in phosphate-buffered saline (PBS) for 20 minutes. After blocking with 10% bovine serum, sections were incubated overnight at 4°C with an anti-RAPH1 polyclonal rabbit antibody (1:50). Sections were washed with PBS and incubated first with a biotinylated goat anti-polyvalent, then with streptavidin peroxidase. RAPH1 protein was visualized by incubating sections with 0.05% DAB. Slides were counterstained with hematoxylin. A positive control was processed in parallel. Negative controls were processed by omitting the primary antibody or both primary and secondary antibodies.

RAPH1 expression was classified in terms of the percentage of positive cells as well as staining intensity, based on counts of at least 500 cells [10]. Points were assigned based on the percentage of positive cells as follows: 2 points, 11–50% positive cells;, 3 points, 51–80% positive cells, and 4 points, >80% positive cells. Points were assigned based on staining intensity as follows: 1 point, weak intensity, 2 points, moderate intensity, and 3 points, strong intensity. Points for intensity and percentage of positive cells were added together to give an overall score, which was used to assign each section to one of the following four expression groups: negative expression, <10% of positive cells, regardless of intensity, low expression, 2-3 points, moderate expression, 4-5 points, and high expression, 6-7 points. Overall scores were determined independently by two investigators. If these investigators assigned the same section to different expression groups, they reassessed their analyses until reaching consensus.

Differences between groups were assessed for significance using the chi-squared test (c^2 test), Cochran-Mantel-Hanszel chi-squared test (CMH c^2 test), and Cochran-Armitage trend test or analysis of variance (ANOVA) as appropriate. The threshold for significance was defined as $p < 0.05$.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Results

Relative RAPH1 mRNA levels detected by quantitative real-time PCR (Figure 1) were 1.0+1.4 for normal tissue (n = 8), 1.1+0.5 for low-grade SIL (n = 6), 1.4+2.0 for high-grade SIL (n = 6), and 6.5+7.2 for carcinoma (n = 8). Thus, levels increased progressively from normal tissue to SIL and finally to carcinoma, although ANOVA showed significant differences only between carcinoma tissue and either SIL tissue or normal tissue (both $p < 0.05$). Significant differences in RAPH1 mRNA levels were not observed among normal, low-grade or high-grade SIL tissues (all $p > 0.05$). These results probably reflect the small number of tissue samples examined.

Immunohistochemistry to detect RAPH1 in 125 paraffin-embedded tissues showed that, in agreement with previous studies, RAPH1 localized primarily to the cytoplasm (Figure 2). Only two of ten normal tissue samples were weakly RAPH1-positive (Table 1). Most carcinoma tissues (48/65, 73.8%) were RAPH1-positive and over half showed high expression. The percentage of RAHP1-positive samples gradually increased from normal tissue (10.0%) and low-grade SIL (31.3%) to high-grade SIL (45.8%), and carcinoma tissue (73.8%; $\chi^2_{CMH} = 30.0$, $p < 0.001$). Only one of 16 low-grade SIL samples (6.3%) showed moderate to high expression, compared to nine of 24 high-grade SIL samples (37.5%), and 34 of 65 carcinoma samples (52.3%). These findings, together with the PCR results, suggest that RAPH1 expression increases at early carcinoma stages and is associated with progression of cervical carcinoma.

RAPH1 expression varied inversely with grade of tumor

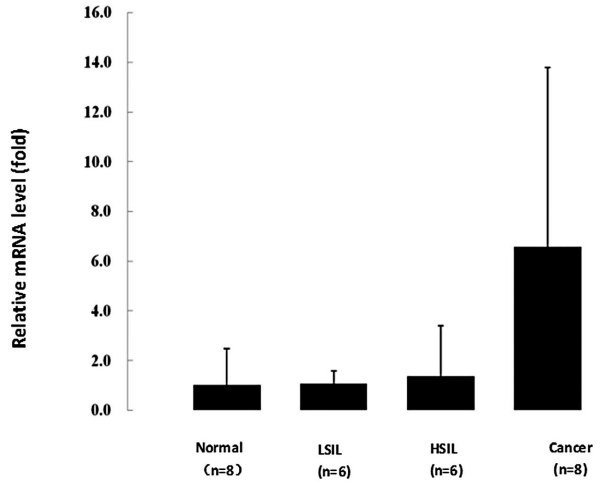

Figure 1. — RAPH1 mRNA expression in normal, low-grade SIL (LSIL), high-grade SIL (HSIL), and cancerous cervical tissues. GAPDH served as an internal control. Fold-increases are expressed relative to normal tissue.

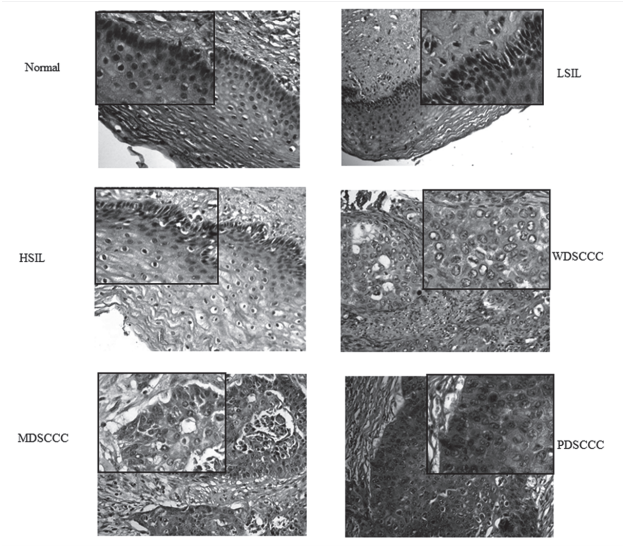

Figure 2. — RAPH1 protein expression based on immunohistochemistry of normal, low-grade SIL (LSIL), high-grade SIL (HSIL), and cancerous cervical tissues. RAPH1 staining (brown) localized mainly to the cytoplasm. Representative images are shown at ×200; inserts are at ×400. Squamous cell carcinoma tissue was classified as well-differentiated (WDSCC), moderately differentiated (MDSCC) or poorly differentiated (PDSCC).

differentiation (Table 2, Figure 2). Proportions of RAPH1-positive cells increased from WDSCC (57.1%) to MDSCC (75.0%) and finally to PDSCC (80.0%). Only four of 14 WDSCC samples (28.6%) showed moderate to high expression, compared to 10 of 16 MDSCC samples (62.5%) and 20 of 35 PDSCC samples (57.2%). In other words, higher RAPH1 protein expression was associated with less differentiated tumors ($c^2_{CMH} = 4.4$, $p = 0.04$). Since histological grade is regarded as an indicator of cancer progression, these results suggest that RAPH1 protein expression levels correlate directly with cervical cancer progression.

Possible correlations were explored between percent of RAPH1-positive samples and aggressiveness of cervical carcinoma in 65 samples for which clinicopathological data were available. This percentage of RAPH1-positive samples was significantly higher in those with vascular invasion (88.2%) than in samples without it (54.8%, $c^2 = 7.5$, $p < 0.001$). Similarly, RAPH1 positivity correlated with interstitial invasive depth and tumor size (both $p < 0.01$, Cochran-Armitage-trend test). In contrast, no significant

Table 1. — *RAPH1 expression in normal, precancerous, and carcinoma cervical tissues*.*

Tissue	No. cases	No. (%) RAPH1(+) cases	RAPH1 expression		
			low	moderate	high
Normal	20	2 (10.0)	2	0	0
LSIL	16	5 (31.3)	4	1	0
HSIL	24	11 (45.8)	2	8	1
Cancer	65	48 (73.8)	14	24	10
Total	125	66	22	33	11

*$c^2_{CMH} = 30.0$, $p < 0.001$. **Based on immunohistochemical staining intensity as described in Materials and Methods. LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesions.

Table 2. — *RAPH1 expression in cervical carcinoma tissues in different grades of differentiation*.*

Grade	No. cases	No. (%) RAPH1(+) cases	RAPH1 expression**		
			low	moderate	high
WDSCC	14	8 (57.1)	4	4	0
MDSCC	16	12 (75.0)	2	8	2
PDSCC	35	28 (80.0)	8	12	8
Total	65	48 (73.8)	13	25	10

* $c^2_{CMH} = 4.4$, $p = 0.04$ **Based on immunohistochemical staining intensity as described in Materials and Methods. Squamous cell carcinoma was classified as well-differentiated (WDSCC), moderately differentiated (MDSCC) or poorly differentiated (PDSCC).

Table 3. — *Correlation of RAPH1 expression with aggressiveness of cervical carcinoma.*

	No. cases	No. (%) RAPH1(+) cases	c^2	p
Lymph node metastasis	5	5 (100)	1.9	0.17
Vascular invasion	34	30 (88.2)	7.5	<0.001
Invasive depth			8.1	0.004
Less than 1/2	25	14 (56.0)		
More than 1/2	30	24 (80.0)		
Entire layer	10	10 (100)		
Tumor size, cm			7.4	0.006
$d \leq 2$	23	13 (56.5)		
$2 < d \leq 4$	24	18 (75.0)		
$d > 4$	18	17 (94.4)		

correlation was detected between RAPH1 expression and lymph node metastasis, probably reflecting the small sample. Nevertheless, all five samples showing lymph node metastasis also showed RAPH1 expression, compared to 43 of 60 samples without lymph node metastasis (71.6%, $p = 0.17$; Table 3). This trend should be investigated in larger samples. If verified, these results link RAPH1 expression to cervical cancer development and invasion.

Discussion

Building on the present authors' previous work (data not shown) in which RAPH1 was found to be one of the most strongly up-regulated genes following *in vitro* cervical transformation using nitrosopyrrolidine [11], the authors provide here evidence that RAPH1 expression correlates with progression and aggressiveness of cervical carcinoma. Levels of RAPH1 mRNA and protein were higher in cervical cancer tissues than in precancerous and normal tissue. RAPH1 protein expression correlated inversely with tumor differentiation, suggesting that RAPH1 promotes cervical cancer development. To the best of the present authors' knowledge, this is the first study focusing on the relationship between RAPH1 and cervical carcinogenesis.

The present finding that RAPH1 may act as a tumor promoter in cervical carcinoma contrasts with the observed associations of RAPH1 with other cancers, which are often contradictory. Studies suggest that RAPH1 is down-regulated in breast cancer and ovarian cancer [8], in primary osteosarcomas, and in metastatic tumors relative to primary tumors in the same patient [7]. These studies suggest that RAPH1 acts as a tumor suppressor gene. However, another study of RAPH1 in breast cancer has reported both deletion and amplification of the gene, leading those investigators to conclude that it does not affect mammary carcinoma tumor development [12]. The present results, combined with those in the literature, suggest that RAPH1 may act as a tumor suppressor, promoter, or neither depending on the tissue involved.

While the present results link RAPH1 expression with cervical carcinoma invasion and metastasis, further study is needed to uncover the detailed mechanisms involved. These studies may help identify molecular targets and signaling pathways useful for preventing or treating this malignancy.

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Corresponding Author:
JINKE LI, M.D., PHD
Department of Gynecology and Obstetrics
West China Second Hospital, Sichuan University
No 20, Sec 3, Renminnan Road,
Chengdu, Sichuan 610041 (China)
e-mail: leonljk@126.com