EJGO European Journal of Gynaecological Oncology

RAPH1 in cervical cancer proliferation, migration, and invasion: in vitro studies with SiHa and HeLa cells

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Summary

Background: Ras association and pleckstrin homology domains 1 (RAPH1) may play a critical role in cervical cancer development, prompting us to investigate its potential role(s) in proliferation, migration, and invasion by cervical cancer cells. *Materials and Methods:* RAPH1 was knocked down in SiHa and HeLa cervical cancer cells using short interfering RNA. Effects of RAPH1 knock-down were assessed on cell proliferation using the CCK8 assay, and on cell migration and invasion using *in vitro* transwell assays. *Results:* Efficient RAPH1 knock-down significant reduced the proliferation, migration, and invasion abilities of SiHa and HeLa cells ($p < 0.05$). *Conclusions:* RAPH1 may promote tumor cell proliferation, migration, and invasion during cervical cancer development.

Key words: RAPH1; Cervical cancer; Cell migration

Introduction

Cervical cancer is one of the most common gynecological malignancies, causing approximately 527,600 new cases and 265,000 deaths every year around the world [1]. Most cervical cancer cases, and nearly 90% of related deaths, occur in less developed countries [2]. Although the principal cause of cervical cancer appears to be infection with HPV, only a fraction of HPV-infected individuals develop such cancer [3]. This suggests that factors beyond HPV infection alone are needed for cervical cancer to develop. Elucidating the molecular mechanisms of cervical cancer development may help identify novel treatment targets and guide prevention efforts.

One candidate gene in cervical cancer development is Ras association and pleckstrin homology domains 1 (RAPH1), also known as lamellipodin, and encoded at genomic locus 2q33. RAPH1 is a ubiquitously expressed member of the MRL family of Ras effector proteins. Since it interacts with actin regulatory protein and controls actin movement [4], the present authors suspected that it might play important roles in adhesion, growth, and migration of cervical cancer cells. Unpublished work from our laboratory suggested that RAPH1 transcript and protein levels were significantly higher in cervical cancer tissue than normal cervical tissue. This prompted us to examine the effects of RAPH1 knock-down on the ability of two cervical cancer cell lines to proliferate, migrate, and invade *in vitro*.

Materials and Methods

Short interfering RNAs (siRNAs) targeting RAPH1 expression were designed based on the human RAPH1 gene sequence in Genbank (accession no. 65059). The following target sequences were synthesized: si-h-RAPH1-001 (catalog no. SiG15080-6084304), GAAGTACACTCTATTAGTA; si-h-RAPH1-002 (catalog no. SiG150806084323), GGCTGATCTTTGCTCTATA; and si-h-RAPH1-003 (catalog no. SiG150806084337), and GAGTCAGCCTATGATTGGA. As a negative control, garbled RNA (Nontrol_05815, catalog no. SiN05815122147) was used.

Human cervical cancer cell lines SiHa and HeLa were obtained from the Laboratory of Molecular and Translational Medicine of West China Second University Hospital of Sichuan University. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells in the logarithmic growth phase were seeded into six-well plates at densities of 10×10^4 cells/well (SiHa) or 8×10^4 cells/well (HeLa). At 24 hours after seeding, all wells were transfected with anti-RAPH1 siRNAs or garbled siRNA (100 nmol/well in all cases) using Polyplus transfection reagent according to the manufacturer's instructions.

At 48 hours after transfection, total RNA was extracted from cultures using Trizol, and 500 ng was used as template for reverse transcription (RT) in the PrimeScript RT reagent kit according to the manufacturer's instructions. The resulting cDNA was used as template in PCR amplification reactions (10 µl) containing ROX qPCR Master Mix (5 µl), primers (0.4 µl), cDNA (0.5 µl). Reactions also contained 1× SYBR Green to allow measurement of fluorescence intensity during amplification. Reactions were carried out at 95 °C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. All samples were measured in triplicate, and levels of RAPH1 mRNA were determined relative to the levels of GAPDH mRNA using the $2^{-\Delta\Delta}$CT method [5].

Cell proliferation was measured using a cell-counting kit.

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Revised manuscript accepted for publication November 20, 2017

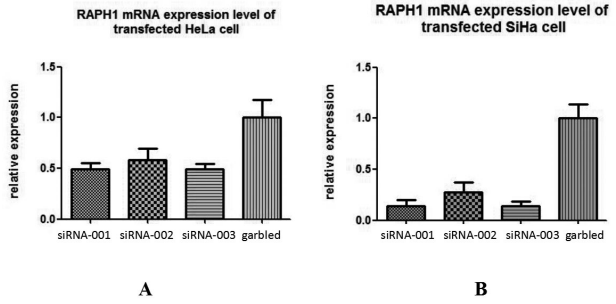

Figure 1. — RAPH1 expression after siRNA-mediated knock-down. Cultures transfected with different siRNAs against RAPH1 show significantly lower levels of RAPH1 transcription than cultures transfected with negative-control garbled siRNA ($p < 0.01$ in all cases). (A) Relative RAPH1 transcript levels in HeLa cultures: si-h-RAPH1-001, 0.49 ± 0.06; si-h-RAPH1-002, 0.58 ± 0.11; si-h-RAPH1-003, 0.49 ± 0.05; garbled siRNA, 1.00 ± 0.18. (B) Relative RAPH1 transcription levels in SiHa cultures: si-h-RAPH1-001, 0.14 ± 0.06; si-h-RAPH1-002, 0.28 ± 0.10; si-h-RAPH1-003, 0.14 ± 0.05; garbled siRNA, 1.00 ± 0.14.

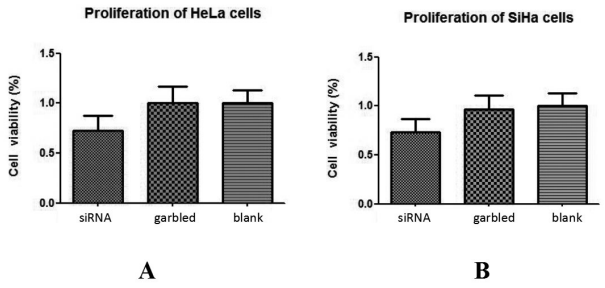

Figure 2. — Effects of RAPH1 knock-down on cell proliferation. Cell proliferation was measured using the CCK8 kit. Cell viability after transfection with si-h-RAPH1-001 or negative-control garbled siRNA is expressed as a percentage of viability in the blank control. (A) Results with HeLa cultures. (B) Results with SiHa cells.

Briefly, SiHa or HeLa cells (approximately 5×10^3) transfected with si-h-RAPH1-001 or garbled siRNA or left untreated (blank control) were seeded into 96-well plates at 48 hours after transfection and cultured in normal medium for 24 hours at 37°C. CCK8 reagent (20 µl) was added to each well, and plates were incubated for one hour under normal conditions. Absorbance at 450 nm was measured using a micro-plate reader. Viable cell counts in the siRNA cultures were expressed as a percentage of the counts in blank control cultures.

Cell migration and invasion abilities were measured using transwell migration assays. Briefly, cells transfected with siRNA or garbled RNA or left untreated (blank control) were suspended in serum-free medium to a density of 100 cells/µL and 200 µL of the suspension was seeded into the upper chamber of transwell plates. A porous membrane was positioned between the upper and lower chambers, and this membrane was coated with Matrigel in invasion assays but left uncoated in migration assays. To attract

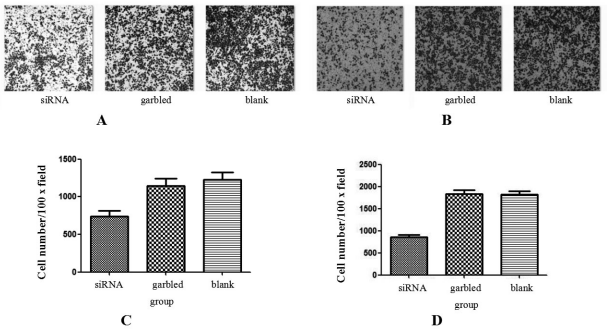

Figure 3. — Effects of RAPH1 knock-down on cell migration. Cell migration was measured using a transwell migration assay. Numbers of migrated cells in each group were determined from five random fields at ×10 magnification. (A, C) Results with HeLa cultures. (B, D) Results with SiHa cultures. siRNA, si-h-RAPH1-001.

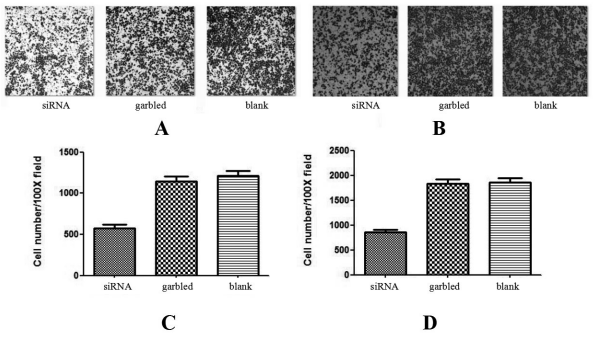

Figure 4. — Effects of RAPH1 knock-down on cell invasion. Cell invasion was measured in a transwell assay. Numbers of invading cells in each group were determined from five random fields at ×10 magnification. (A, C) Results with HeLa cultures. (B, D) Results with SiHa cultures. siRNA, si-h-RAPH1-001.

cells, 500 µL of RPMI medium containing 20% FBS was added to the lower chamber. After allowing cells to migrate for 24 hours or invade for 48 hours, cells that had penetrated the filter were fixed in dried methanol and stained in crystal violet. Numbers of migrating or invading cells were determined in five randomly selected fields of view at ×10 magnification and averaged.

Experimental data were analyzed using SPSS 21.0. Data in figures were reported as mean ± SD. Comparisons among three or more groups were conducted using one-way ANOVA. $P < 0.05$ was considered significant.

Results

Based on qRT-PCR analysis, all three siRNAs targeting the RAPH1 gene significantly decreased the level of RAPH1 mRNA compared to the control (Figure 1, all $p < 0.01$). For simplicity, si-h-RAPH1-001 was used in subsequent experiments.

Relative cell viability was significantly lower in siRNA-

treated HeLa cells (0.68 ± 0.14) than in control cells (1.00 ± 0.16) or blank cells (1.00 ± 0.13) ($p < 0.05$, Figure 2A). Similar results were obtained for SiHa (0.73 ± 0.14 *vs.* 0.97 ± 0.14 *vs.* 1.0 ± 0.13; $p < 0.05$, Figure 2B). These results suggest that RAPH1 knock-down may inhibit cell proliferation in cervical cancer cells *in vitro*.

Significantly fewer cells migrated through the membrane pores in the case of siRNA-treated SiHa cells (741 ± 73) than in the case of control cells (1143 ± 98) or blank cells (1231 ± 97) ($p < 0.01$, Figures 3A and 3C). Similar results were observed for HeLa cells (887 ± 109 *vs.* 1799 ± 150 *vs.* 1824 ± 148; $p < 0.01$, Figures 3B and 3D).

Significantly fewer cells invaded through the membrane in the case of siRNA-treated SiHa cells (575 ± 47) than in the case of control cells (1143 ± 61) or blank cells (1,212 ± 64) ($p < 0.01$, Figures 4A and 4C). Similar results were observed for HeLa cells (854 ± 58 *vs.* 1832 ± 94 *vs.* 1857 ± 90; $p < 0.01$, Figures 4B and 4D). These results suggest that RAPH1 may help promote cervical cancer migration and invasion *in vitro*.

Discussion

RAPH1 (lamellipodin) interacts with Ena/VASP proteins in the lamellipodial protrusion, where it modulates actin cytoskeleton dynamics to enhance cell mobility and contribute to cell migration [6, 7]. De-regulation of RAPH1 expression appears to be involved in some human carcinomas including osteosarcoma, breast cancer and ovarian cancer [8-10]. The present authors' earlier unpublished work suggested that RAPH1 over-expression correlates with progression and aggressiveness of cervical carcinoma in *vivo*. The present *in vitro* study extends this previous work by providing direct evidence that RAPH1 promotes proliferation and metastasis of cervical cancer: RAPH1 knock-down significantly reduced proliferation, migration, and invasion of two cervical cancer cell lines *in vitro*. These findings, together with previous work, suggest a tumor-promoting role of RAPH1 in cervical cancer development and progression.

The precise mechanisms by which RAPH1 promotes cell proliferation or migration remain unclear. RAPH1 may trigger various signaling pathways related to cellular development [11]. For example, RAPH1 may trigger changes in actin dynamics leading to signaling involving epidermal growth factor receptor and serum response factor, ultimately promoting cell growth and proliferation [12]. RAPH1 may also act via Ras GTPase signaling to regulate actin polymerization and thereby facilitate cell migration [13]. Further studies should examine in detail how RAPH1 may promote cervical cancer development.

Regardless of the mechanism, the present results establish an *in vitro* correlation between RAPH1 expression and cervical cancer cell proliferation, migration, and invasion. This justifies further work in cell culture and animal models to explore potential preventive and therapeutic approaches against this malignancy.

Acknowledgments

The authors gratefully acknowledge technical assistance from Professors Xia Wang and Xuelian Zheng of the Western Women's and Children's Research Institute at West China Second Hospital of Sichuan University.

This work received a grant from the National Natural Science Foundation of China (81001158).

References

[1] Torre L.A., Bray F., Siegel R.L., Ferlay J., Lortet-Tieulent J., Jemal A.: "Global cancer statistics, 2012". *CA Cancer J. Clin.*, 2015, *65*, 87.
[2] Chen W., Zheng R., Baade P.D., Zhang S., Zeng H., Bray F., *et al.*: "Cancer statistics in China, 2015". *CA Cancer J. Clin.,* 2016, *66*, 115.
[3] Li J., Mei J., Wang X., Hu L., Lin Y., Yang P.: "Human papillomavirus type-specific prevalence in women with cervical intraepithelial neoplasm in western China". *J. Clin. Microbiol.*, 2012, *50*, 1079.
[4] Krause M., Leslie J.D., Stewart M., Lafuente E.M., Valderrama F., Jagannathan R., *et al.*: "Lamellipodin, an Ena/VASP ligand, is implicated in the regulation of lamellipodial dynamics". *Dev Cell.*, 2004, *7*, 571.
[5] Livak K.J., Schmittgen T.D.: "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method". *Methods*, 2001, *25*, 402.
[6] Hansen S.D., Mullins R.D.: "Lamellipodin promotes actin assembly by clustering Ena/VASP proteins and tethering them to actin filaments". Elife, 2015, *4*. doi: 10.7554/eLife.06585.
[7] Wang J., King J.E., Goldrick M., Lowe M., Gertler F.B., Roberts I.S.: "Lamellipodin Is Important for Cell-to-Cell Spread and Actin-Based Motility in Listeria monocytogenes". *Infect. Immun.*, 2015, *83*, 3740.
[8] Eppert K., Wunder J.S., Aneliunas V., Tsui L.C., Scherer S.W., Andrulis I.L.: "Altered expression and deletion of RMO1 in osteosarcoma". *Int. J. Cancer*, 2005, *114*, 738.
[9] Dahl E., Sadr-Nabavi A., Klopocki E., Betz B., Grube S., Kreutzfeld R., et al.: "Systematic identification and molecular characterization of genes differentially expressed in breast and ovarian cancer". *J. Pathol.*, 2005, *205*, 21.
[10] Batistela M.S., Boberg D.R., Andrade F.A., Pecharki M., de S F Ribeiro E.M., Cavalli I.J., *et al.*: "Amplification and deletion of the RAPH1 gene in breast cancer patients". *Mol. Biol. Rep.*, 2013, *40*, 6613.
[11] Gorai S., Paul D., Haloi N., Borah R., Santra M.K., Manna D.: "Mechanistic insights into the phosphatidylinositol binding properties of the pleckstrin homology domain of lamellipodin". *Mol. Biosyst.*, 2016, *12*, 747.
[12] Lyulcheva E., Taylor E., Michael M., Vehlow A., Tan S., Fletcher A., *et al.*: "Drosophila pico and its mammalian ortholog lamellipodin activate serum response factor and promote cell proliferation". *Dev. Cell*, 2008, *15*, 680.
[13] Chang Y.C., Zhang H., Brennan M.L., Wu J.: "Crystal structure of Lamellipodin implicates diverse functions in actin polymerization and Ras signaling". Protein Cell., 2013, *4*, 211.

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