Clinicopathological and biological analysis of *PIK3CA* mutation and amplification in cervical carcinomas

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Received December 19, 2017; Accepted July 19, 2018

DOI: 10.3892/etm.2019.7771

Abstract. The aim of the present study was to evaluate the mutation and amplification status of the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA)* gene, as well as the association with clinicopathological characteristics and prognosis, in Japanese patients with cervical cancer. Fluorescence *in situ* hybridization and polymerase chain reaction were performed to assess *PIK3CA* gene amplification and mutation. The inhibitors temsirolimus and NVP-BEZ235 were used to inactivate the phosphatidylinositide 3-kinase (PI3K)/AKT serine/threonine kinase (AKT)/mechanistic target of rapamycin kinase (mTOR) pathway to clarify the roles of PI3K/AKT activation in cervical carcinoma cells harboring associated mutations. Four somatic point mutations (4/71, 5.6%) were found in exon 20 in cervical squamous cell carcinoma samples, whereas three (3/53, 5.7%) were found in exon 9 in cervical adeno/adenosquamous cell carcinoma samples. Amplification of *PIK3CA* was also observed in this study and amplification was more commonly found in adeno/adenosquamous carcinomas than in cervical squamous cell carcinomas (20.7 vs. 1.4%, respectively, P=0.0003). No significant correlation was obesrved between *PIK3CA* amplification and progression free survival (P=0.7576) or overall survival (P=0.8859). Moreover, no association between *PIK3CA* mutation and sensitivity to PI3K/AKT/mTOR inhibitors was observed in cervical carcinoma cells. These results suggest that in Japanese patients with cervical cancer, *PIK3CA* mutation and amplification cannot act as biomarkers for individualized molecular targeted therapy.

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Key words: cervical cancer, *PIK3CA*, gene amplification, gene mutation, squamous cell carcinoma, adeno/adenosquamous cell carcinoma

Introduction

Cervical cancer was previously one of the leading causes of cancer-related death in Japanese women and the second most common malignancy in women worldwide (1). This disease comprises several histologic types, of which squamous cell carcinoma is predominant and accounts for approximately 85-90% of cases. In contrast, adenocarcinomas/adenosquamous carcinomas are less common and represent 10-25% of cases (2-3). The incidence of invasive cervical adenocarcinoma and its variants has risen dramatically among younger women over the past few decades (4). The cause of this is not clear but is of concern as several studies have indicated that adenocarcinoma is associated with a worse prognosis compared to that of squamous cell carcinoma. Compared to those with squamous cell carcinoma, a higher proportion of lymph node involvement and distant metastases, as well as a decline in survival across stages, are typically found with adenocarcinoma (5).

Recently, the phosphatidylinositide 3-kinase (PI3K)/AKT signaling pathway has been found to be a major survival signal for cancer cells. Cell proliferation, growth, apoptosis, autophagy, invasion, and migration are regulated by the phosphatidylinositide 3-kinase (PI3K)/AKT serine/threonine kinase (AKT)/mechanistic target of rapamycin kinase (mTOR) pathways, which are putatively activated by key signals in different tumor types (6,7). Activation is commonly conferred by mutations in the p110α subunit of PI3K, *PIK3CA*, with most mutations (>80%) occurring either in the helical domain exon 9 or the kinase domain exon 20. Until recently, molecular genetic studies on cervical tumors have been limited. Cervical carcinomas in U.S. populations are frequently associated with mutations in *PIK3CA*, with a mutation frequency of 31% (8). Lou *et al* (9), analyzed 675 Latin American patients with cervical tumors and found that 31% of squamous carcinomas and 24% of adeno and adenosquamous carcinomas harbored mutations in

the helical domain, and specifically at the E542 and E545 residues, which were thought to result in activation of this subunit and the PI3K/AKT pathway. In Chinese patients with cervical cancer, it was revealed that *PIK3CA* mutations are more common in squamous cell carcinomas (15.3%) than in non-squamous cell carcinomas (7.3%) (10). However, the prevalence of mutations in *PI3KCA* and how they are associated with clinicopathological characteristics and prognosis in Japanese patients with cervical cancer have not been studied until now. Therefore, the objective of this work was to analyze the relationship between amplification and somatic mutations in *PIK3CA* and various clinicopathologic variables including prognosis, in cervical cancer cell carcinomas from Japanese patients. Furthermore, we analyzed whether mutations in *PIK3CA* can predict response to PI3K/AKT/mTOR inhibition in cervical cancer.

Materials and methods

Tissue samples. A total of 71 paraffin-embedded tumor tissue samples were obtained from the Department of Obstetrics and Gynecology at Shimane University Hospital; all samples were cervical squamous cell carcinomas. In addition, 53 adenocarcinomas/adenosquamous carcinomas were obtained from the Department of Obstetrics and Gynecology at Seirei Hamamatsu General Hospital. Patients had received appropriate therapy at either Shimane University Hospital or Seirei Hamamatsu General Hospital between January 1994 and December 2013. All specimens from cervical cancer patients were obtained after operation and prior to any treatment. Tumor staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification (Shepherd, 2014). The invasive squamous cell carcinomas consisted of 28 cases of stage I disease, 11 of stage II disease, 24 of stage III disease, and eight of stage IV disease. All tumors were classified histologically according to the World Health Organization criteria. The median patient age was 55 years (range 32-84 years). The invasive adenocarcinomas/adenosquamous cell carcinomas consisted of 39 cases of stage I disease, eight of stage II disease, five of stage III disease, and one of stage IV disease. World Health Organization criteria were used to classify all tumors histologically. The median patient age was 46 years (range 27-82 years). Stage I and II patients were treated with class II or class III radical hysterectomies with pelvic lymph node dissection. Stage I patients were treated with positive lymph node metastasis or positive lymphovascular space invasion and all stage II patients received concurrent chemoradiotherapy or radiotherapy as adjuvant therapy. Stage III and IV patients were treated with concurrent chemoradiotherapy or radiotherapy alone.

Patients with an incomplete response to radiotherapy and patients with recurrent tumors were treated with a variety of salvage chemotherapy agents including cisplatin, carboplatin, and paclitaxel. The follow-up period ranged from 5 to 142 months, with a median of 65 months. Acquisition of tissue specimens and clinical information was approved by an institutional review board (Shimane University and Seirei Hamamatsu General Hospital), and written informed consent was obtained from all patients. Only patients with follow-up data were included. The paraffin tissue blocks were organized into tissue microarrays, each produced by removing 3-mm diameter cores of tumors from the block. Selection of the area for the core was made by a gynecologic oncologist (KN) and pathology technician (KI), and was based on review of the H&E slides.

DNA isolation. Unstained paraffin-embedded tissues were serially sectioned at a thickness of 10 μm and one adjacent hematoxylin and eosin-stained section was taken for identification and selection of tumor tissue. The carcinoma, comprising at least 85% of the total area was marked, and using a sterile needle, gross macroscopic dissection was performed. The dissected tissues were placed in microcentrifuge tubes and DNA isolation was performed as described previously (11).

Cell culture and cell lines. Hela and Hela P35 (adenocarcinoma), as well as ME180, TCS, and CaSki (squamous cell carcinoma) human cervical cell lines were obtained from Tohoku University (Sendai, Japan), whereas SKGIIIa, SKGIIIb, HCS2, and BOKU (also squamous cell carcinoma) cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). All human cervical cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in an atmosphere of 5% CO_2.

Fluorescence in situ hybridization (FISH). FISH was performed on 5-μm paraffin sections contained on a tissue microarray consisting of both carcinoma and normal tissue cores. Zytolight® SPEC *PIK3CA*/CEN 3 dual color probes (Zytovision, Bremerhaven, Germany) were used according to the manufacturer's protocol. Slides were denatured for 10 min at 75˚C and hybridized at 37˚C overnight with the probe mix. Cell nuclei were stained with 4',6-diamidino-2-phenylindole. Signals were evaluated by two independent researchers using an Olympus Bx41 fluorescence microscope (Olympus Corporation, Tokyo, Japan). Separate narrow band-pass filters were used to detect the ZygGreen™, ZyRed™, and DAPI signals. Approximately 100 tumor cells were examined for each specimen at magnification x60; a signal ratio of experimental probe/reference probe greater than three was considered amplification.

Mutational analysis of PIK3CA, KRAS, and BRAF. The mutational status of *KRAS* and *BRAF* was determined for all paraffin-embedded cervical cancer tissue samples, whereas *PIK3CA* mutation status was determined for all paraffin-embedded tissue samples as well as cell lines. Polymerase chain reaction (PCR) was performed, followed by nucleotide sequencing using the iCycler (Bio-Rad, Hercules, CA, USA). Exons 9 and 20 of *PIK3CA*, exon 2 of *KRAS*, and exon 15 of *BRAF* were sequenced because together, mutational hot spots in these regions harbor nearly all published mutations (12-15). The primers for PCR and sequencing were manufactured by GeneLink, Inc., (Hawthorne, NY, USA), and their sequences were described in a previous report (16). The sequences were analyzed using the Lasergene program, DNASTAR, Inc., (Madison, WI, USA).

Table I. Frequency of *PIK3CA*, *KRAS*, *BRAF* and *PIK3CA* amplification in cervical carcinoma.

Histological subtype	*PIK3CA* (%)	*KRAS* (%)	*BRAF* (%)	*PIK3CA* amplification (%)
SCC	4/17 (5.6)	0/71 (0.0)	0/71 (0.0)	1/71 (1.4)
AD/ASC	3/53 (5.7)	3/53 (5.7)	1/51 (2.0)	11/53 (20.7)

PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; SCC, cervical squamous cell carcinoma; AD/ASC, cervical adeno/adenosquamous cell carcinoma.

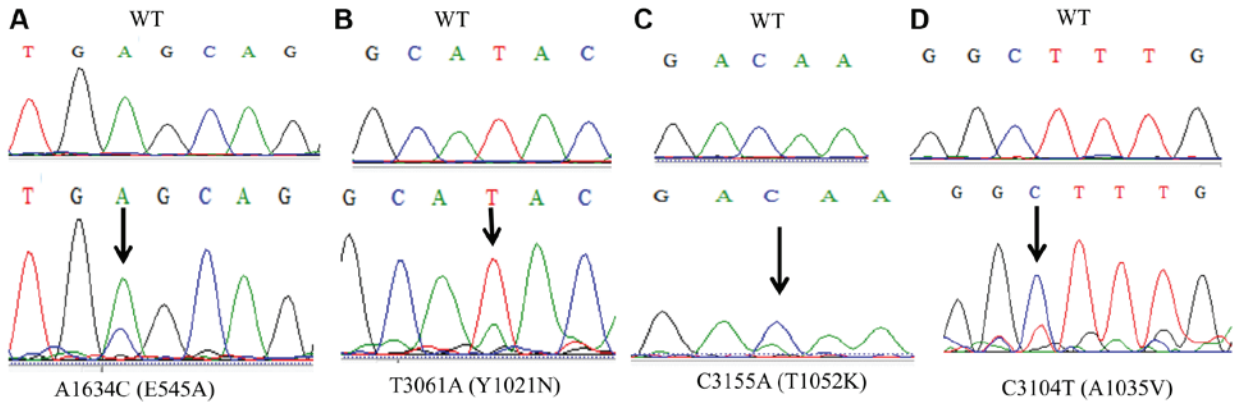

Figure 1. *PIK3CA* mutations in cervical cancer. (A) Upper chromatogram sequence represent wild type and lower mutant sequence E545A (1634 A >C) in exon 9. (B) Upper-wild type *PIK3CA* gene and lower mutant sequence Y1021N (3061 T>A) in exon 20. (C) Upper-wild type *PIK3CA* gene in exon 20 and lower mutant sequence T1052K (3155 C>A). (D) Upper-wild type *PIK3CA* gene in exon 20 and lower mutant sequence A1035V (3104 C>T). The bottom arrow indicates the position of missense mutations in each case.

Cell growth assays. Cells were plated in 96-well plates at a density of 3,000 cells per well and treated with or without a potent PI3K or mTOR inhibitor. An MTT growth assay was performed to determine the number of cells (17). Potent inhibitors such as temsirolimus (Selleck Chemicals, Houston, TX, USA) and NVP-BEZ235 (Selleck Chemicals) were used to treat each of the cell lines at doses of 50, 500, 1,000, and 3,000 nM to inhibit PI3K/mTOR function, and cell viability was measured 98 h later. DMSO was used at an equal amount as a control. The data were expressed as percentage relative to the DMSO control. The mean and SD were obtained from three experiments.

Statistical analysis. Progression-free survival was calculated as the time between diagnosis and recurrence of disease, whereas overall survival was calculated as the time between diagnosis of disease and death. Kaplan-Meier curves were used to plot the survival data and log-rank tests were performed to determine the statistical significance of survival differences. Data were censored when patients were lost to follow-up. The χ^2 test was used for comparisons of categorical data. Student's t-test (for comparison of two groups) or one-way analysis of variance followed by Tukey's post hoc test (for comparison of more than two groups) was used to evaluate numerical data.

Results

Identification of PIK3CA, KRAS, and BRAF mutations. Somatic mutations in *PIK3CA* were found in four (5.6%) of 71 cervical squamous cell carcinoma samples and three (5.7%) of 53 cervical adeno/adenosquamous cell carcinoma samples (Table I). Interestingly, mutations in adeno/adenosquamous carcinomas only occurred within exon 9 (E545A) (Table II; Fig. 1A) and no mutations were identified in the catalytic domain encoded by exon 20. In contrast, mutations in squamous cell carcinoma only occurred within exon 20 (Y1021N, A1035V, and T1052K) and no mutations were identified in the helical domain encoded by exon 9 (Table II; Fig. 1B-D). Somatic mutations in KRAS were identified in three (5.7%) of 53 cases and *BRAF* mutations were identified in one (2%) of 53 cases of cervical adeno/adenosquamous cell carcinoma (Table I). *KRAS* and *BRAF* mutations were not present in the same samples harboring *PIK3CA* mutations. No squamous cell carcinomas had detectable oncogenic mutations in *KRAS* (0%, of 71) or *BRAF* (0%, of 71).

Frequency of PIK3CA gene amplification is higher in adeno/adenosquamous cell carcinomas than in squamous cell carcinomas. *PIK3CA* amplification was identified in one (1.4%) of 71 cervical squamous cell carcinomas. In contrast, *PIK3CA* amplification was identified in 11 (20.7%) of 53 adeno/adenosquamous cell carcinomas. Amplification of *PIK3CA* was more frequently found of adeno/adenosquamous carcinomas than in squamous cell carcinomas ($P<0.0003$, χ^2-test; Table II; Fig. 2). Interestingly, amplification of *PIK3CA* was not found in the same patients harboring *PIK3CA* mutations.

Prognostic effect of PIK3CA amplification. Next, we examined the prognostic ability of *PIK3CA* amplification. Kaplan-Meier curves were used to plot progression free survival and overall

Table II. Association between *PIK3CA* mutation, amplification and cervical carcinoma histological subtype.

		PIK3CA mutation (exon 9)			*PIK3CA* mutation (exon 20)			*PIK3CA* amplification		
Histological subtype	Patients	-	+	P-value	-	+	P-value	-	+	P-value
SCC	71	71	0	0.0424	67	4	0.848	70	1	0.0003
AD/ASC	53	50	3		53	0		42	11	

PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; SCC, cervical squamous cell carcinoma; AD/ASC, cervical adeno/adenosquamous cell carcinoma; -, negative; +, positive.

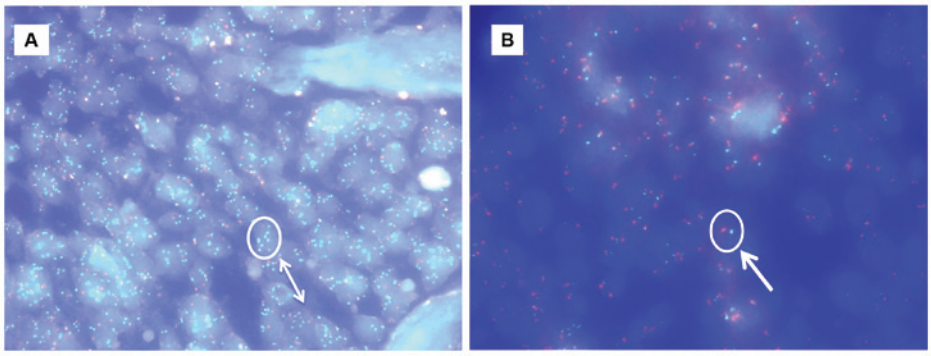

Figure 2. Dual-color FISH was used to detect amplification of the *PIK3CA* gene amplification cervical carcinomas. (A) FISH analysis revealed a homogeneously stained region in the cervical carcinoma case with *PIK3CA* gene amplification (↔) as indicated by multiple green signals in each nucleus. (B) In contrast, another case contained signals for both *PIK3CA* (green) and reference probes (red) at a ratio of approximately 1:1 (→). FISH, fluorescence in situ hybridization; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α.

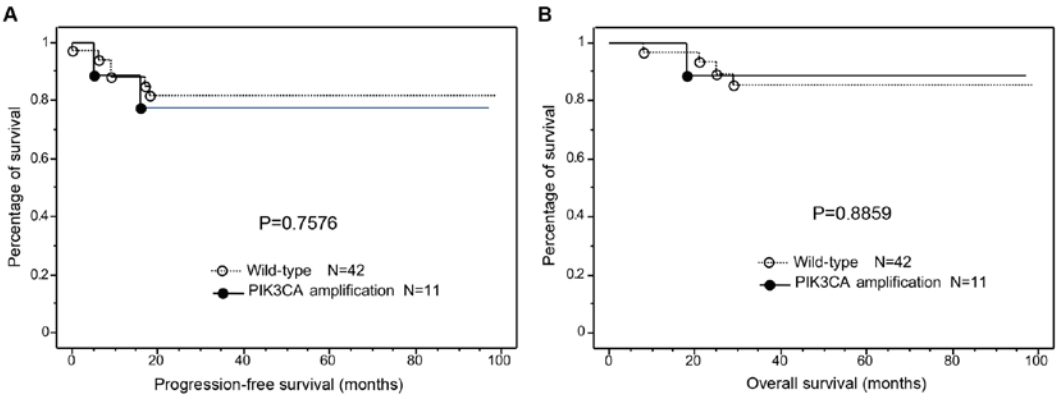

Figure 3. Kaplan-Meier survival analysis in 53 patients with cervical carcinoma according to *PIK3CA* amplification. *PIK3CA* amplification did not correlate with (A) progression-free survival and (B) overall survival. *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α.

survival (Fig. 3), and we observed no significant relationship between *PIK3CA* amplification and progression free/overall survival (P=0.7576 and P=0.8859, respectively).

Association between PIK3CA mutational status and growth inhibition. Squamous cell carcinoma and adeno/adenosquamous cell lines were first analyzed for *PIK3CA* mutational status. Of nine cervical cancer cell lines, *PIK3CA* mutations were observed in four (ME180, TCS, HCS-2, and CASKI), but only in Exon 9. Next, we used the potent inhibitor temsirolimus (formerly known as CCI-799) and NVP-BEZ235 to examine the relationship between mutational status and growth inhibition. Growth of the *PIK3CA* mutation-containing cell lines was not inhibited by any of the inhibitors (Figs. 4 and 5). Treatment with PI3K/AKT/mTOR inhibitors failed to inhibit proliferation in all four cell lines harboring *PIK3CA* mutations.

Discussion

PIK3CA mutations were found in only four of 71 (5.6%) cervical squamous cell carcinoma samples, and were identified in exon 20 (P=0.848). Similar mutations were present in three of 53 (5.7%) cervical adeno/adenosquamous cell carcinoma samples, and these were identified in exon 9 (P=0.042). No tumors harbored mutations in both the helical and kinase domain. These results indicated that mutations are more common in

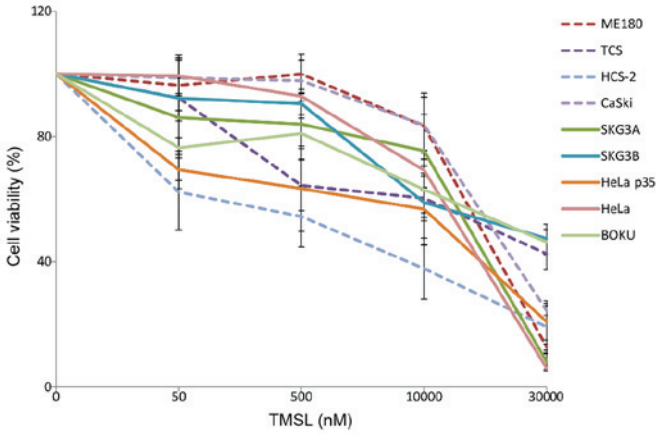

Figure 4. Effect of temsirolimus on cell proliferation in cervical carcinoma cell lines. Cells were counted after 72 h of temsirolimus and DMSO (control) treatment. The presence of a mutation in *PIK3CA* was not related to sensitivity to growth inhibition by temsirolimus. *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; M: *PIK3CA* mutation containing cell line; WT: wild-type cell line.

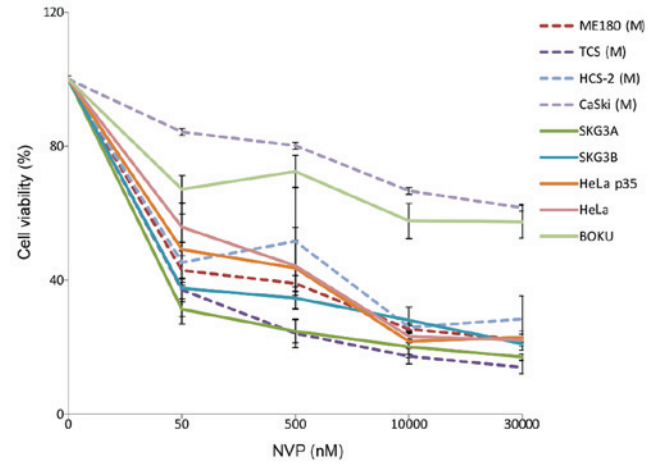

Figure 5. Effect of NVP-BEZ235 on cell proliferation in cervical carcinoma cell lines. Cells were counted after 72 h of temsirolimus and DMSO (control) treatment. The presence of a mutation in *PIK3CA* was not related to sensitivity to growth inhibition by NVP-BEZ235. *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; M: *PIK3CA* mutation containing cell line; WT: wild-type cell line.

exon 9 for adeno/adenosquamous cell carcinoma and in exon 20 for cervical squamous carcinomas in Japanese cervical cancer patients. Our findings suggest that adeno/adenosquamous carcinomas could be distinguished from squamous cell carcinomas based on genetic alterations. Lou *et al* (9), found that *PIK3CA* mutations in the helical domain are significantly more prevalent in squamous cell carcinomas than in adenocarcinomas (P=0.017) in Latin American patients with cervical cancer, which is not consistent with the results of our study. Xiang *et al* (10), identified 105 cases (13.6%) with *PIK3CA* mutations from a cohort of Chinese patients with cervical carcinoma. The most common mutations were found in exon 9, at residues 545 and 542, in 59 and 32 samples, respectively. The H1047R mutation has been reported in four cases, and several rare non synonymous base substitutions were also identified in Chinese patients, some of which have been reported in the Catalog of Somatic Mutation in Cancer (COSMIC) database. Two mutations were identified in Chinese patients that were not previously reported. In addition, mutations in *PIK3CA* were found in 93 (15.3%) of the 606 patients with squamous cell carcinomas, nine (8.9%) of the 101 patients with adenocarcinomas, and one (2.3%) of the 44 patients with adenosquamous carcinoma. Therefore, *PIK3CA* mutations also occurred more frequently in squamous cell carcinomas than in non-squamous cell carcinomas in Chinese patients; moreover, *PIK3CA* mutations were mostly activating helical domain mutations, specifically E542K and E545K (10). The cancer Genome Atlas Research Network described the genomic and molecular characterization of cervical cancer and observed that most *PIK3CA* mutations occurred at the helical domain of exon 9 (E542K and E545K) and that *PIK3CA* (P=0.01) were differentially expressed between keratin-low and keratin-high squamous cluster gene family members (18). Comparing the results of the current study with those of previous studies, we hypothesized that the difference in prevalence is due to a difference in genetic background between the Japanese population and other ethnic groups.

PIK3CA gene amplification was also detected in this study and our results suggested that 11 of 53 (20.7%) samples were associated with *PIK3CA* gene amplification in adeno/adenosquamous cell carcinoma. In contrast, one of 71 (1.4%) cervical squamous cell carcinomas exhibited *PIK3CA* gene amplification. This event occurred at a significantly higher prevalence (P=0.0003) in adeno/adenosquamous cell carcinoma than in cervical squamous cell carcinomas. Our data suggest that cervical squamous cell carcinoma and adenocarcinoma have distinct gene expression profiles that might arise from different pathways involved in carcinogenesis. We previously found that Notch3 is significantly overexpressed in cervical squamous cell carcinomas compared to expression in cervical cancer adenocarcinomas (19). Wright *et al* (5), identified *KRAS* mutations only in adenocarcinomas (17.5% (AC) vs. 0% (SCC); P=0.01), which is similar to our results; in addition, a novel *EGFR* mutation was previously detected only in squamous cell carcinomas (0% (AC) vs. 7.5% (SCC); P=0.24). Taken together, our results and those of previous studies suggest that cervical squamous cell carcinoma and adenocarcinoma have distinct molecular profiles.

Activating mutations and amplification of *PIK3CA*, the gene that encodes the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), have also been reported in 23-36% of cervical cancer specimens (20-22). Based on observational studies, activation of the PI3K pathway has been associated with higher rates of local recurrence after radiotherapy and decreased survival (22,23). Next, we examined the relationship between *PIK3CA* amplification and prognosis and found that *PIK3CA* amplification did not correlate with progression free survival and overall survival. Possible reasons for the different results in our study compared to those of others, including *PIK3CA* mutation rate in cervical cancer and the prognostic effect of *PIK3CA* amplification, might be different patient cohorts or sample sizes.

Mutations in *PIK3CA* are becoming a promising target for newly discovered anticancer drugs. Janku *et al* (24), observed that in patients with advanced breast, ovarian, endometrial, and cervical cancers, an association between *PIK3CA* mutations

and positive response to PI3K/AKT/mTOR inhibitors was apparent. McIntyre *et al* (22), recently identified that among cervical cancer patients with *PIK3CA* mutations treated with radical chemoradiotherapy, there was a strong association with overall survival in FIGO stage IB/II but not stage III/IVA. In the present study, PI3K/AKT/mTOR inhibitors such as temsirolimus and NVP-BEZ235 were tested in cervical cancer cell lines; it was found that mutational status does not correlate with growth inhibition with any of the tested inhibitors. These observations suggest that *PIK3CA* inhibition might not represent a potential therapeutic option for the treatment of cervical cancers with associated mutations.

In conclusion, our data demonstrate that a proportion of Japanese cervical cancer patients harbor mutations in *PIK3CA*. Helical domain-encoding *PIK3CA* mutations are more frequent in adeno/adenosquamous cell carcinomas, whereas kinase domain-encoding *PIK3CA* mutations are more frequent in cervical squamous cell carcinomas. Our study also suggests that *PIK3CA* amplification occurs at a significantly higher rate (P=0.0003) in adeno/adenosquamous cell carcinoma than in cervical squamous cell carcinomas, and that *PIK3CA* amplification does not correlate with progression free survival or overall survival. The mutation status of *PIK3CA* also did not predict sensitivity to PI3K/AKT/mTOR inhibitors in cervical carcinoma cells *in vitro*. This result suggests that *PIK3CA* mutations might not be an important parameter for predicting treatment response in Japanese cervical cancer patients.

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan (grant no. 15K10717).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on responsible request.

Authors' contributions

SR and KeN drafted the manuscript. KoN, TI, MI, and TM performed data collection, analysis and interpretation of data. SR and KI conducted the experimental trials. YO, SN and NI performed pathological diagnosis. KeN participated in the design of the study. SK conceived the study, participated in its design and coordination, and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Shimane Medical University. All patients provided written informed consent for the procedure and study participation.

Patient consent for publication

All patients approved their data for publication.

Competing interests

The authors declare that they have no competing interests.

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