筑 波 大 学

博 士 (医 学) 学 位 論 文

Analysis of *PIK3CA* mutation in stage IIB to IVA cervical cancers treated by concurrent chemoradiotherapy with weekly cisplatin

(シスプラチン毎週投与併用同時化学放射線治療が行われた IIB～IVA 期子宮頸癌患者における *PIK3CA* 遺伝子変異の解析)

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List of Abbreviations:

CCRT	concurrent chemoradiotherapy
PI3-kinase	phosphatidylinositide 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
mTOR	mammalian target of rapamycin
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PDK1	phosphoinositide-dependent kinase 1
DNA	deoxyribonucleic acid
ATR	ataxia telangiectasia and Rad3 related
MAPK	mitogen-activated protein kinase
CDK	cyclin-dependent kinase
Mdm2	mouse double minute 2
TME	tumor micro-environment
HIF	hypoxia-iducible factor
VEGF	vascular endothelial growth factor
ATP	adenosine triphosphate
FFPE	formalin-fixed, paraffin-embedded
FIGO	International Federation of Gynecology and Obstetrics
PCR	polymerase chain reaction
SCC	squamous cell carcinoma
CR	complete response
PR	partial response
SD	stable disease
PD	progressive disease

ABSTRACT

[Aim] The standard treatment for locally advanced cervical cancer is cisplatin-based concurrent chemoradiotherapy (CCRT). Although the activated PI3-kinase/AKT pathway is known to be involved in both cisplatin-resistance and radioresistance, to date only a few studies have reported significant associations between *PIK3CA* gene mutational status and outcome by CCRT in the disease. The aim of this study was to clarify the prognostic significance of *PIK3CA* mutational status in cervical cancers treated by CCRT.

[Methods] We analyzed *PIK3CA* mutation in 59 patients diagnosed with stages IIB to IVA cervical carcinomas primarily treated by CCRT with weekly cisplatin at our institution. We used formalin-fixed paraffin-embedded biopsy specimens before treatment. Fifty-seven out of 59 patients (97%) were locally advanced cancers with stages IIIA to IVA. Clinicopathological data and patient survival were compared according to *PIK3CA* mutational status. We further performed cell viability assay examining the effect of a PI3-kinase inhibitor copanlisib (BAY80-6946) in combination with cisplatin on cell proliferation of CaSki cells harboring *PIK3CA* mutation.

[Results] *PIK3CA* mutation was present in 7 out of 59 patients (12%). No significant clinical differences were observed according to *PIK3CA* mutational status. Patients with wild-type *PIK3CA* showed significantly improved cancer-specific survival as compared with mutated patients ($p=0.044$). Subsequent survival analyses revealed

that *PIK3CA* mutation was a significant prognostic factor for poor overall survival (multivariate adjusted HR, 3.88; 95% CI, 1.28-11.78; p=0.017) and cancer-specific survival (multivariate adjusted HR, 3.60; 95% CI, 1.19-10.95; p=0.024). Our in-vitro study demonstrated that copanlisib in combination with cisplatin showed mild synergistic effect on proliferation of CaSki cells compared to single-drug treatments.

[Conclusion] The current findings suggest that molecular inhibitors targeting the PI3-kinase/AKT pathway may improve the outcome by CCRT in cervical cancers harboring *PIK3CA* mutation, providing significant implications for novel treatment strategy based on precision medicine in the disease.

1. Introduction

PHOSPHATIDYLINOSITIDE 3-KINASE (PI3-kinase), especially class I, has a well-established role in cancer development[1]. This protein is heterodimeric with a regulatory subunit and a catalytic subunit encoded by *PIK3CA* gene. *PIK3CA* gene is located on 3q26.3 (Figure 1) and has a major role in the PI3-kinase/AKT pathway (Figure 2). *PIK3CA* gene encodes p110α protein, which contains many domains, but the most effective are helical and kinase domains[2].

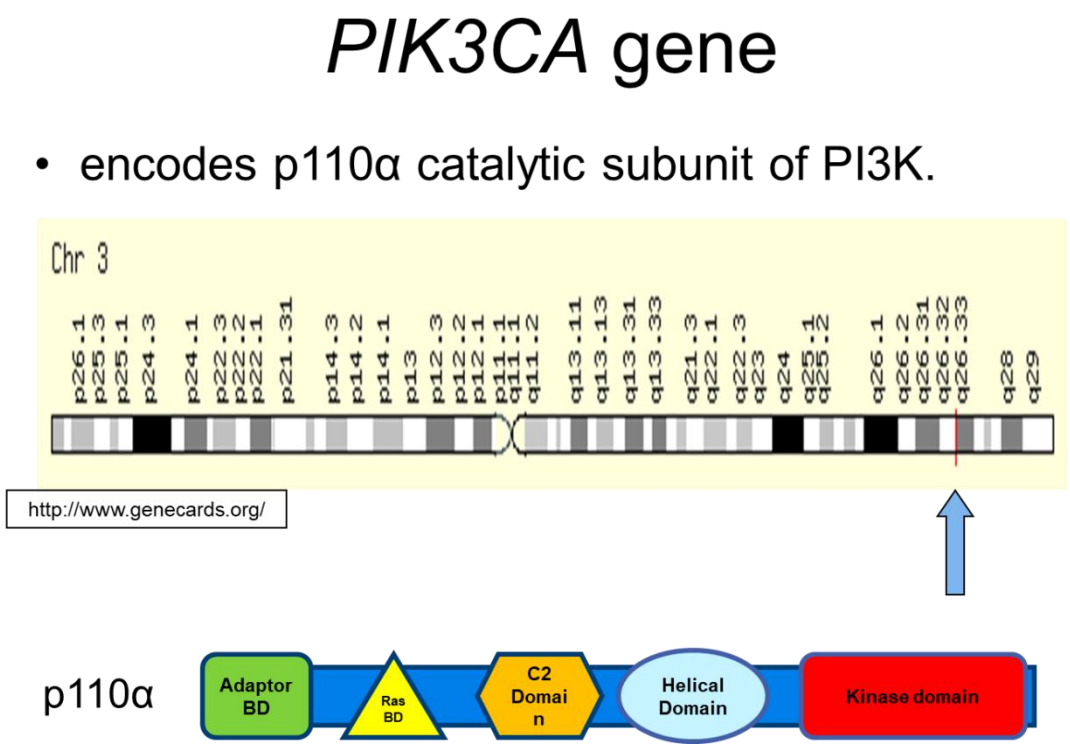

Figure 1: *PIK3CA* gene encodes the catalytic subunit of PI3-kinase, and is located at the long arm of chromosome 3. It contains many domains, but the most effective are helical and kinase domains.

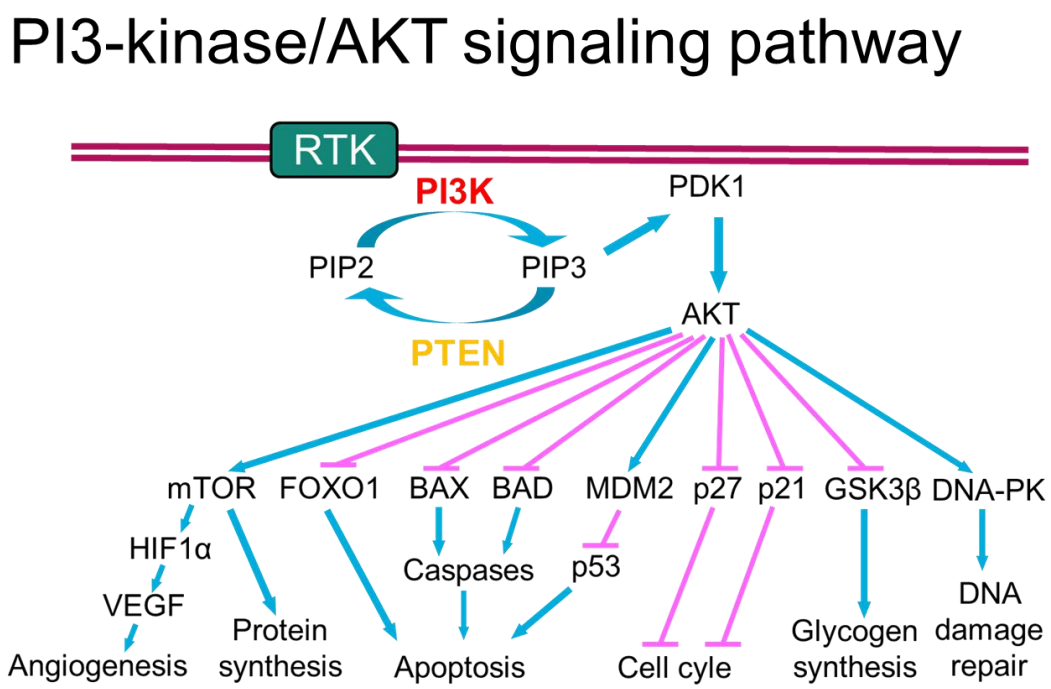

Figure 2: After binding of growth factors, the tyrosine kinase receptor on cell membrane is phosphorylated and activated. The active receptor will then turn on the PI3-kinase enzyme attached at its bottom. PI3-kinase phosphorylates PIP2 to PIP3. PIP3 activates PDK1 enzyme that will phosphorylate and activate AKT. The activated AKT will then regulate downstream proteins necessary for a variety of cell functions.

P110α protein phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) at the third position of the inositol ring, producing phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 is a second cellular messenger that is important for cell growth, proliferation, survival, motility, metabolism and angiogenesis[1,3]. In fact, *PIK3CA* mutation and genomic aberrations promote tumorigenesis through the upregulation of the PI3-kinase/AKT axis[4].

PIK3CA gene is closely related with cancer, making it an important and promising target for cancer treatment and drug development. Mutations of the *PIK3CA* gene are associated with poor prognosis in patients with different solid tumors of different organs, but they can lead to a favorable response to PI3-kinase/AKT inhibitors[5,6]. Moreover, the PI3-kinase/AKT/mTOR is the most commonly activated pathway in human cancer, present in 30-50% of all tumors[7]. *PIK3CA* mutations and modification

of p110α are reported to be the most frequent driver mutations in cancer[8]. *PIK3CA* gene is the most consistent genomic aberration in cervical cancer, and is implicated in the progression of dysplastic uterine cervical cells into invasive cancer[3,9]. More than 80% of mutations are located in helical or kinase domains, commonly referred to as exon 9 and exon 20[3]. Nowadays, cervical cancer (Table 1) is still among the leading cancer-related death world wide[10]. It was reported to represent 8% of all cancer mortality, with a very low 5-year survival[11]. Moreover, it is the 4th most common cancer among women and it represents 4% of all diagnosed cancer worldwide [12].

TNM clinical classification

TNM categories	FIGO stages	Definition
T – Primary Tumour		
TX		Primary tumour cannot be assessed
T0		No evidence of primary tumour
Tis		Carcinoma *in situ* (preinvasive carcinoma)
T1	I	Tumour confined to the cervix[a]
T1a[b,c]	IA	Invasive carcinoma diagnosed only by microscopy. Stromal invasion with a maximal depth of 5.0 mm measured from the base of the epithelium and a horizontal spread of 7.0 mm or less[d]
T1a1	IA1	Measured stromal invasion 3.0 mm or less in depth and 7.0 mm or less in horizontal spread
T1a2	IA2	Measured stromal invasion more than 3.0 mm and not more than 5.0 mm with a horizontal spread of 7.0 mm or less[d]
T1b	IB	Clinically visible lesion confined to the cervix or microscopic lesion greater than T1a/IA2
T1b1	IB1	Clinically visible lesion 4.0 cm or less in greatest dimension
T1b2	IB2	Clinically visible lesion more than 4.0 cm in greatest dimension
T2	II	Tumour invades beyond uterus but not to pelvic wall or to lower third of vagina
T2a	IIA	Tumour without parametrial invasion
T2a1	IIA1	Clinically visible lesion 4.0 cm or less in greatest dimension
T2a2	IIA2	Clinically visible lesion more than 4.0 cm in greatest dimension
T2b	IIB	Tumour with parametrial invasion
T3	III	Tumour involves lower third of vagina, or extends to pelvic wall, or causes hydronephrosis or non-functioning kidney
T3a	IIIA	Tumour involves lower third of vagina
T3b	IIIB	Tumour extends to pelvic wall, or causes hydronephrosis or non-functioning kidney
T4	IVA	Tumour invades mucosa of the bladder or rectum, or extends beyond true pelvis[e]
N – Regional Lymph Nodes[f]		
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Regional lymph node metastasis
M – Distant Metastasis[f]		
M0		No distant metastasis
M1		Distant metastasis (includes inguinal lymph nodes and intraperitoneal disease). It excludes metastasis to vagina, pelvic serosa, and adnexa

[a]Extension to corpus uteri should be disregarded.
[b]The depth of invasion should be taken from the base of the epithelium, either surface or glandular, from which it originates. The depth of invasion is defined as the measurement of the tumour from the epithelial–stromal junction of the adjacent most superficial papillae to the deepest point of invasion. Vascular space involvement, venous or lymphatic, does not affect classification.
[c]All macroscopically visible lesions even with superficial invasion are T1b/IB.
[d]Vascular space involvement, venous or lymphatic, does not affect classification.
[e]Bullous oedema is not sufficient to classify a tumour as T4.
[f]No FIGO equivalent.
TNM, tumour, node and metastasis.
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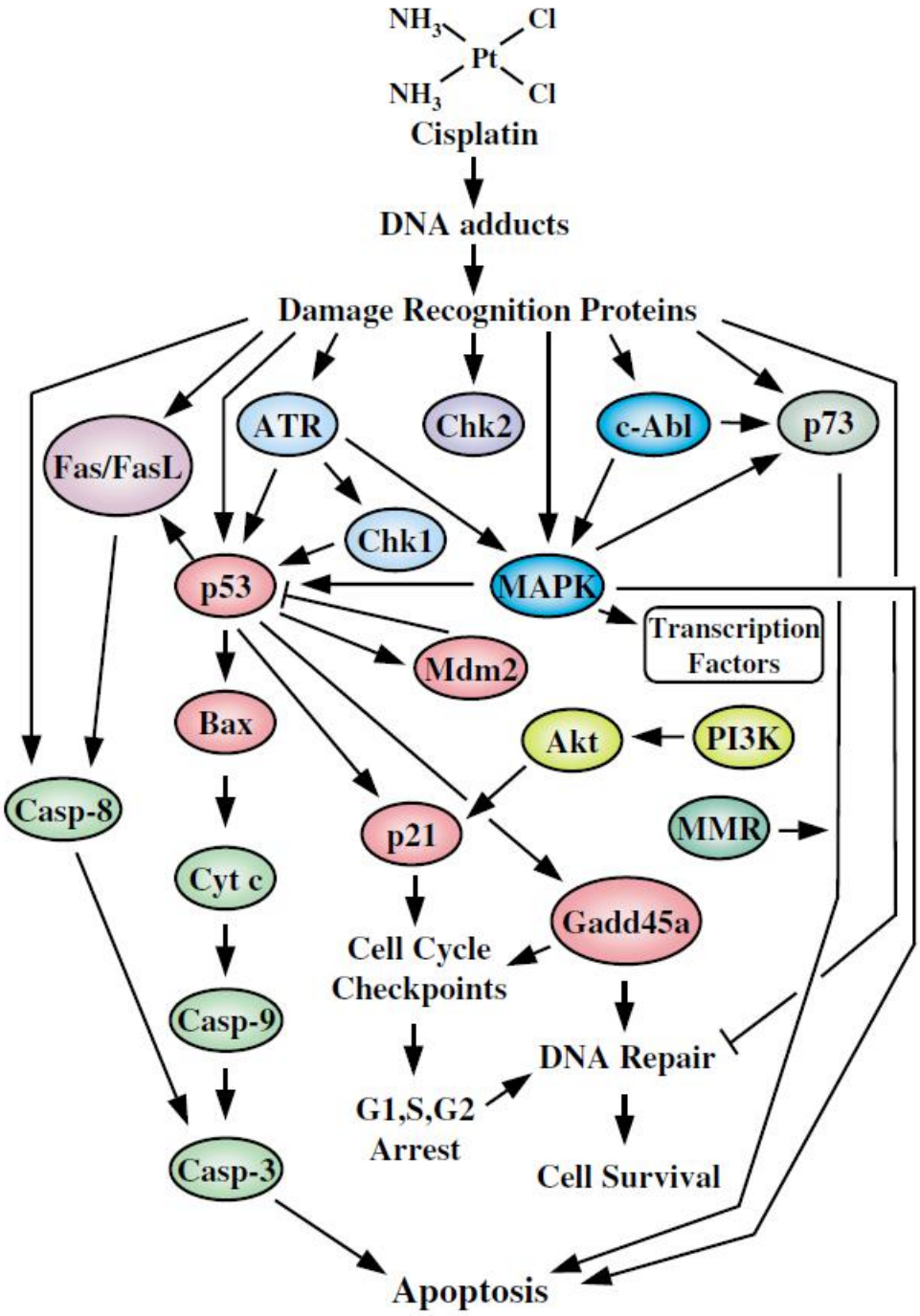
Marth C, et al. Ann Oncol. 2017;28(suppl_4):iv72-iv83

Table 1[13]: Staging of cervical tumors by the International Federation of Gynecology and Obstetrics (FIGO) and TNM classification.

CISPLATIN is one of the strongest chemotherapeutic agents. It has an anti-tumor activity against a large number of solid tumors, such as ovary, testis, head and neck. Cisplatin action consists of its interaction with DNA to form DNA adducts especially intrastrand crosslink adducts. Those adducts activate several signal transduction pathways, involving ATR, p53, and MAPK, all necessary for cellular apoptosis[14] (Figure 3). Unfortunately, even though many tumors have a good response to cisplatin at the beginning of treatment, several become cisplatin-resistant, like small cell lung cancer or ovarian cancer. This resistance limits the clinical use of cisplatin and increases its toxicity. In addition, tumors with cisplatin-resistance are fully cross-resistant to platinum-based chemotherapeutic agents and also usually resistant to other unrelated anti-tumor agents[14]. The administration of cisplatin results in inhibiting cell cycle on G_2/M phase and later the cells will undergo apoptosis, but before that an upstream of events have to occur, for instance, p53-dependent functions. First, cisplatin activates ATR kinase which phosphorylates p53. Some genes transactivated by p53 as a result of cisplatin exposure are associated with cell cycle arrest, DNA repair, and apoptosis, including CDK inhibitor p21. However, cisplatin resistance arises when cells develop the ability to replicate their DNA past the adducts. The p53 protein also transactivates Mdm2 that has a negative feedback regulation decreasing p53 activity. Additionally, other inhibitors of apoptosis may interfere with p53 activity. AKT protein, which is downstream of p110α, promotes the activation of Mdm2 oncoprotein and its translocation into the nucleus, where Mdm2 will downregulate p53 and instigate resistance. The cisplatin resistance may also be due to the inactivation of pro-apoptotic protein Bad after its phosphorylation by AKT. To increase the complexity of cisplatin resistance, the anti-apoptotic signal can be a result of AKT-mediated phosphorylation of procaspase 9 which leads to its inactivation, facilitating the inhibition of the caspase cascasde[14].



Siddik ZH. Oncogene. 2003;22(47):7265-79

Figure 3[14]: An outline of pathways implicated in mediating cisplatin-induced cellular effects. Cell death or survival will depend on the relative intensity of the signals engendered and the crosslink between the pathways involved.

RADIOTHERAPY is used to treat up to 50% of cancer patients and to manage up to 40% of cured patients. Radiotherapy affects mainly the tumor micro-environment

(TME). It was reported that radiotherapy has effects on hypoxia, fibrotic response and immune activation inside the TME[15]. Radiation causes endothelial cell dysfunction, by increasing their permeability, detachment from their basal membrane and their apoptosis, leading to post-irradiation inflammation and fibrosis. Within vessels, irradiation generates a pro-thrombotic state characterized by platelet aggregation, microthrombus formation and increased adhesion of inflammatory cells to endothelial cells with subsequent diapedesis into the perivascular space[4]. As a result of vessel fibrosis and microthrombus, hypo-vascularization is developed, followed by tissue necrosis. Additionally, endothelial cells within the TME are characterized by rapid proliferation rates, contributing to their radio sensitivity. However, many tumor cells become resistant to hypoxia. Vascular damage triggers immune response through increased production of cytokines, inducing immune cells recruitment. After that, tumor revascularization happens through hypoxia-inducible factor-1α (HIF-1α). Furthermore, markers of HIF-1 hypoxia-response pathways are strongly associated with radiotherapy failure and resistance in many cancers. To increase the tumors vascularization, abnormal vessels are used to decrease the impact of hypoxia. VEGF is the principal angiogenic molecule involved in the formation of abnormal vessels[4].

PI3-KINASE INHIBITORS, which bind competitively to the ATP-binding pocket of the catalytic domain, leaded to the development of pan-PI3-kinase and isoform-specific inhibitors. The pan-PI3-kinase may be most effective but the potential toxic side effects on glucose metabolism and immune response are not tolerated on the long term. On the other hand, the isoform-specific inhibitors may be well tolerated but are difficult to develop seeing the extremely conserved nature of ATP-binding pocket[4]. Copanlisib (BAY80-6946) is a novel, intravenous, potent, and highly selective pan-class I PI3-kinase inhibitor, but with preferential activity against the p110α and p110δ isoforms (IC_{50} values of 0.5, 3.7, 6.4, and 0.7 nM in cell-free assays for PI3-kinase α/β/γ/δ, respectively). Copanlisib (Figure 4) has demonstrated potent anti-tumor and pro-apoptotic activity in various tumor cell lines and xenograft models[16].

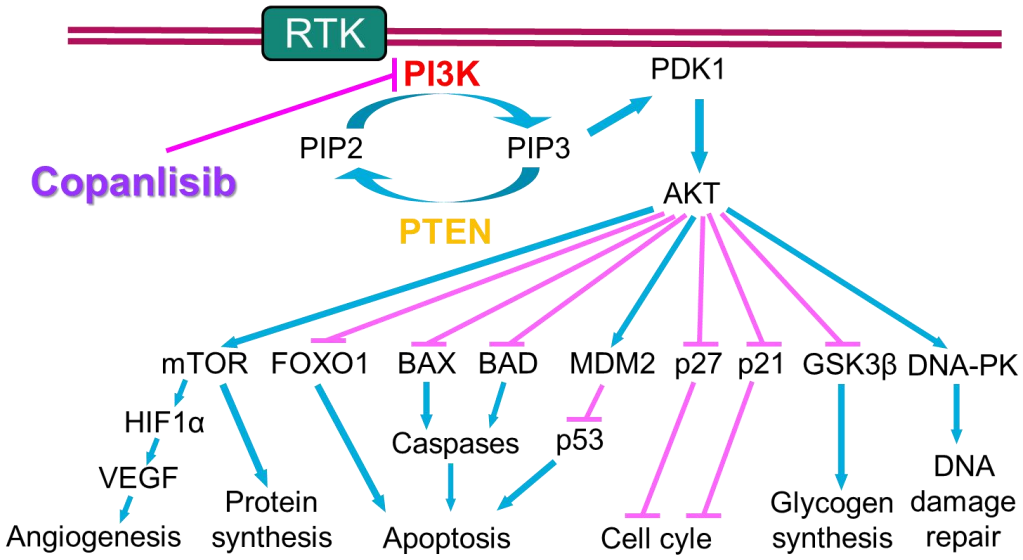
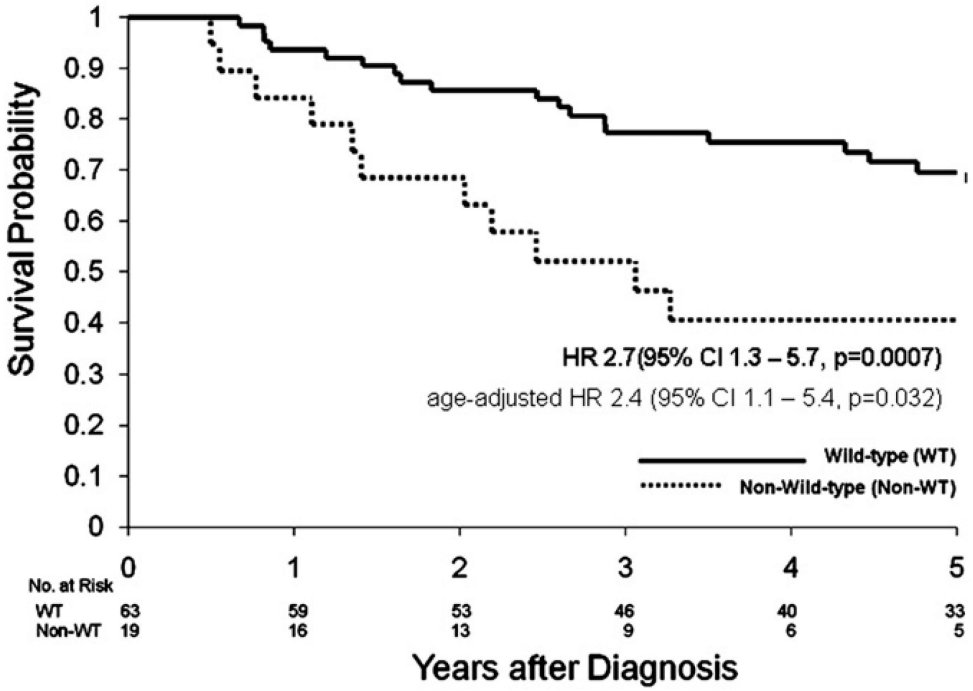


Figure 4: Molecular targeted agent against PI3-kinase/AKT signaling pathway.

Although the current standard treatment for locally advanced cervical cancer is cisplatin-based concurrent chemoradiotherapy (CCRT), the 5-year survival rate in advanced cervical cancer patients is still low (30-50%). Hence, the development of novel treatment strategy to improve outcome by CCRT is urgently required. To date only a few studies have reported the prognostic significance of *PIK3CA* mutation in cervical cancers treated by CCRT. McIntyre et al. reported that overall survival at 5 years after CCRT was significantly worse for *PIK3CA* mutant patients compared to *PIK3CA* wild type patients (40% vs. 70%; Figure 5)[6]. In another study conducted by Wang et al., patients with *PIK3CA* mutations had a significantly lower complete response rate to CCRT, 52% against 86% in patients with wild-type *PIK3CA* (Table 2)[12]. Further accumulation of evidence is warranted to clarify the prognostic significance of *PIK3CA* mutation for the outcome after CCRT. Hence, the aim of the current study was to clarify the prognostic significance of *PIK3CA* mutational status in cervical cancers treated by CCRT with weekly cisplatin[17]. Besides, we further examined the effect of a PI3-kinase inhibitor, copanlisib, in combination with cisplatin on the proliferation of human cervical cancer cells harboring *PIK3CA* mutation.



McIntyre JB et al. Gynecol Oncol. 2013;128(3):409-14

Figure 5: Overall survival at 5 years after CCRT was significantly worse for *PIK3CA* mutant patients compared to wild type patients (40% vs. 70%)[6].

	PIK3CA WT (n=37)	*PIK3CA* mutant (n=23)	*P*-value
Age			
≤55	46%	43%	0.852
>55	54%	57%	
Tumor size			
≤4 cm	13%	91%	0.107
>4 cm	87%	9%	
Histological grade			
Well and moderately differentiated	95%	87%	0.362
Poorly differentiated	5%	13%	
Node status			
N0	65%	48%	0.282
N1	35%	52%	
FIGO stage			
II	49%	52%	0.685
III	49%	48%	
IV	2%	0%	
Response			
CR	86%	52%	0.006
Non-CR	14%	48%	

Wang J, et al. Exp Mol Pathol. 2015;98(3):407-10

Table 2: Patients with *PIK3CA* mutations had a significantly lower complete response rate to CCRT than wild-type patients (52% vs. 86%)[12].

2- Methods

2.1 Patients and treatment :

Patients with cervical carcinomas who were primarily treated by concurrent chemoradiotherapy with weekly cisplatin at the University of Tsukuba Hospital between 2001 and 2015 were identified through our database, and their medical records were retrospectively reviewed. Staging at diagnosis was performed based on the International Federation of Gynecology and Obstetrics (FIGO) system. Patients mostly received whole pelvic irradiation of 50Gy and brachytherapy of 24Gy concomitant with 5 to 6 cycles of weekly administration of 40 mg/m^2 cisplatin. All samples were obtained with informed consent or opt-out procedure in accordance with protocols approved by the Ethics Committee University of Tsukuba Hospital. Median follow-up duration was 77 months. Follow-up data were retrieved until 2017-6-30. Overall survival, cancer-specific survival, and progression-free survival were defined as the time interval from initial diagnosis to death from any cause, to death due to cervical cancer, and to disease progression or death, respectively.

2.2 DNA extraction :

All the biopsy tissue specimens were collected before treatment. Archival FFPE biopsy tissues were used for DNA extraction. We prepared 8 to 10 slides of 5 μm FFPE tissues, and scratched the tissues from the slides into an autoclaved tube using sterile surgical blades. We used the blackPREP DNA Kit (GenoStaff, Tokyo, Japan) for DNA extraction according to the manufacturer's instructions. We measured the DNA quality and concentration using a Nanodrop.

2.3 DNA amplification:

We performed DNA amplification targeting exons 9 and 20 of the *PIK3CA* gene by PCR as described previously[18]. Four different sets of primers with different annealing

temperatures were used (Table 3, Figure 6).

Table 3: PCR primers		
Primer Pair	**Size of PCR product**	**Annealing Temperature**
(1) PIK3CA9f-PIK3CA9r2	322bp	53°C
(2) PIK3CA9f2-PIK3CA9r	224bp	59°C
(3) PIK3CA20f-PIK3CA20r3	261bp	55°C
(4) PIK3CA20f3-PIK3CA20r	318bp	55°C

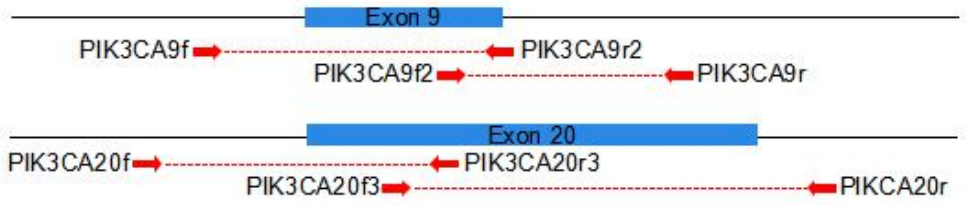


Figure 6: Location of the primers on the *PIK3CA* gene.

PCR was conducted in 40 μL reaction volumes containing 200 ng of genomic DNA, 200 μmol/L of each deoxynucleotide triphosphate, 1× PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 1.5 mmol/L $MgCl_2$, 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), and 1 μmol/L of each primer. PCR amplifications were performed with denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 53°C to 59°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. Electrophoresis was then performed to check the size of the target sequence.

2.4 DNA isolation:

Using the PCR samples, we performed an electrophoresis on a 1.5% agarose gel. Under UV light and after confirmation of the DNA band size, we cut and weigh the visible band of the gel, and then isolated the DNA using a Mini Elute Gel Extraction

Kit (Qiagen, Valencia, CA, USA). We measured the DNA quality and concentration using a Nanodrop.

2.5 DNA sequencing:

The isolated DNAs were submitted to Eurofins Genomics (Tokyo, Japan) for direct sequencing. We used BioLign software (version 4.0.6.2, http://en.bio-soft.net/) to look for the presence of mutations.

2.6 Cell viability assay:

Human cervical cancer cell line CaSki was purchased from American Type Culture Collection (Manassas, VA, USA). We used CaSki cells for cell viability assay (Figure 10), as these cells contain heterozygous *PIK3CA* mutation E545K. Cisplatin was purchased from Wako (Osaka, Japan). Copanlisib was purchased from LKT Laboratories (St. Paul, MN, USA). The drugs were dissolved in DMSO, aliquoted, and stored at -80℃ until use. CaSki cells were cultured in DMEM medium containing fetal bovine serum (10%), penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL) in a humidified atmosphere of 5% CO_2 at 37℃. Cells in the log growth phase were seeded on 96-well plates at 2,000 cells per well. Twenty-four hours later, cells were treated with increasing doses of cisplatin (0.01-100 μM), copanlisib (1-10,000 nM), or cisplatin plus copanlisib. Cellular proliferation was monitored after 72 hours of treatment using a WST-1 assay as follows. WST-1 (Dojindo, Kumamoto, Japan) and 1-methoxy PMS (Dojindo) were added to the wells and incubated for 4 hours, and then the absorbance at 450 nm was measured and normalized relative to the absorbance of cell cultures treated with DMSO alone. The mean and SD were obtained from independent 3 experiments.

To determine the dose effect of combination therapy, the Chou-Talalay method and CalcuSyn software (version 2.11, Biosoft, Cambridge, UK) were used. Cisplatin was combined with copanlisib at a constant ratio, and the interaction was quantified based on a combination index (CI) to assess synergism (CI < 0.9), additive effect (CI=0.9-

1.0), and antagonism (CI > 1.1).

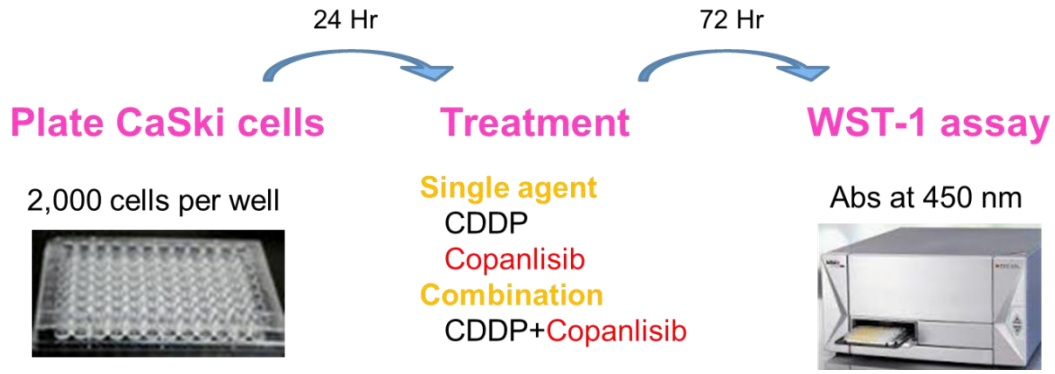


Figure 7: Cell viability assay.

2.7 Statistical analyses:

Differences in proportions were evaluated by the Fisher's exact test. Differences in continuous variables were evaluated by the t-test. Kaplan-Meier survival curves were calculated and compared using the log-rank test. The Cox proportional hazard model was used for univariate analysis and, after adjustment for baseline characteristics and prognostic factors (age, FIGO stage, histology, and pelvic node metastasis), multivariate analysis.

3. RESULTS

We analyzed DNA sequences on exons 9 and 20 of the *PIK3CA* gene in archival FFPE biopsy specimens before treatment by direct sequencing in 59 patients with stages IIB to IVA cervical carcinomas treated by CCRT with weekly cisplatin. Among those patients, 57 (97%) were locally advanced cancers with stages IIIA to IVA. Table 4 summarizes patient characteristics[17].

Table 4[17]. Patient characteristics.

Characteristic		**Number** (n=59)
Mean age (range)		51.3 (28-75)
FIGO stage (%)		
	IIB	2 (3)
	IIIA	2 (3)
	IIIB	52 (88)
	IVA	3 (5)
Histology (%)		
	SCC	53 (90)
	Poorly differentiated carcinoma	2 (3)
	Adenocarcinoma	2 (3)
	Mucinous adenocarcinoma	1 (2)
	Adenosquamous carcinoma	1 (2)
Pelvic node metastasis (%)		
	Present	39 (66)
	Absent	20 (34)
Paraaortic node metastasis (%)		
	Present	13 (22)
	Absent	46 (78)
Response (%)		
	CR	44 (75)
	PR	10 (17)
	SD	1 (2)
	PD	4 (7)
Recurrence		
	No	29 (49)
	Yes	25 (42)
	Persistent disease	5 (8)
Recurrent site		
	Inside the irradiated field	13 (22)
	Outside the irradiated field	15 (25)
	Both	2 (3)
PIK3CA mutation		
	Wild type	52 (88)
	Mutated	7 (12)

Lachkar B, et al. Medicine (Baltimore). 2018;97(31):e11392

We found *PIK3CA* mutation in 7 out of 59 patients (12%). Five (71%) of the mutations were mapped on the helical domain, and one (14%) on the kinase domain of the p110α protein (Table 5)[17].

Table 5[17]. Results of *PIK3CA* mutation analysis.

	Nucleotide	**Amino acid**	**Exon**	**Domain**
1	1571G>A	R524K	9	Helical
2	1624G>A	E542K	9	Helical
3	1624G>A	E542K	9	Helical
4	1633G>A	E545K	9	Helical
5	1633G>A	E545K	9	Helical
6	1634A>C	E545A	9	Helical
7	3073A>G	T1025A	20	Kinase

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We next compared various clinicopathologic features according to *PIK3CA* mutational status, finding no significant differences in any of the variables including age, FIGO stage, histologic subtype, and response rates after CCRT (Table 6)[17].

Table 6[17]. Relationship between *PIK3CA* mutational status and clinicopathological variables.

		PIK3CA		
Clinicopathologic variable		**Wild type**	**Mutated**	***P*-value**
Mean age (range)		51.3 (28-75)	51.1 (29-68)	0.97
FIGO stage				
	IIB	1 (2%)	1 (14%)	
	IIIA	2 (4%)	0 (0%)	
	IIIB	46 (88%)	6 (86%)	
	IVA	3 (6%)	0 (0%)	0.43
Histology				
	SCC	46 (88%)	7 (100%)	
	Others	6 (12%)	0 (0%)	1.0
Pelvic node metastasis				
	Present	35 (67%)	4 (57%)	
	Absent	17 (33%)	3 (43%)	0.68
Paraaortic node metastasis				
	Present	12 (23%)	1 (14%)	
	Absent	40 (77%)	6 (86%)	1.0
Response				
	CR/PR	47 (90%)	7 (100%)	
	SD/PD	5 (10%)	0 (0%)	1.0
Recurrence				
	No	27 (52%)	2 (29%)	
	Yes	20 (38%)	5 (71%)	
	Persistent disease	5 (10%)	0 (0%)	0.30
Recurrent site				
	Inside the irradiated field	12 (48%)	1 (20%)	
	Outside the irradiated field	11 (44%)	4 (80%)	
	Both	2 (8%)	0 (0%)	0.54

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We subsequently compared cancer-specific survival according to various prognostic factors including *PIK3CA* mutational status (Figure 8)[17]. Pelvic node metastasis showed a difference without statistical significance (p=0.053; Figure 8E).

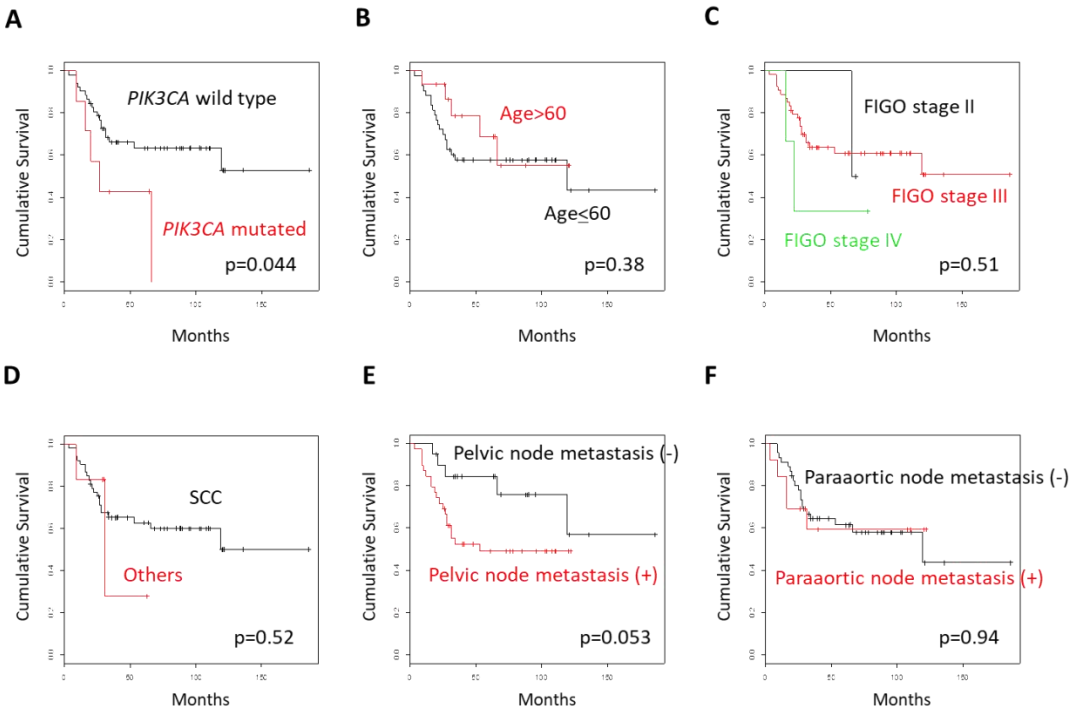

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Figure 8[17]: Kaplan-Meier curves for cancer-specific survival in cervical cancers treated by CCRT. A, cases with wild-type *PIK3CA* (n=52) vs. mutatnt *PIK3CA* (n=7); B, cases with age≤60 (n=43) vs. age>60 (n=16); C, cases with FIGO stage II (n=2) vs. III (n=54) vs. IV (n=3); D, cases with squamous cell carcinomas (n=53) vs. other histologic types (n=6); E, cases with negative pelvic node metastasis (n=20) vs. positive pelvic node metastasis (n=39); F, cases with negative paraaortic node metastasis (n=46) vs. positive paraaortic node metastasis (n=13).

Interestingly, *PIK3CA* mutation was the only prognostic factor showing a significant association with poor cancer-specific survival (5-year survival rate: 64% vs. 43%; p=0.044; Figure 8 A). We also compared overall survival and progression-free survival according to *PIK3CA* mutational status, but both differences were not significant (5-year survival rate: 64% vs. 43% and 52% vs. 21%; p=0.055 and 0.29,

respectively; Figures 9A and 9B)[17].

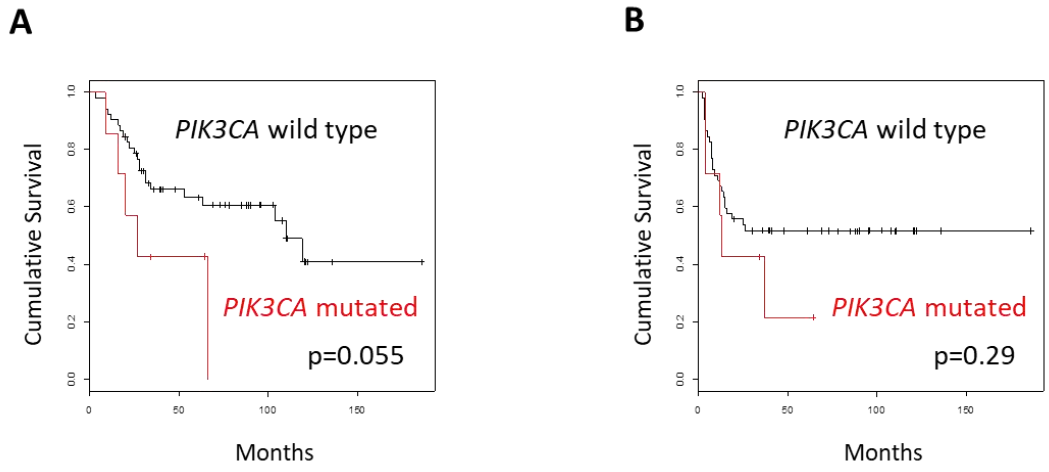

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Figure 9[17]: Kaplan-Meier curves in cervical cancers treated by CCRT. A, overall survival in cases with wild-type *PIK3CA* (n=52) vs. mutant *PIK3CA* (n=7); B, progression-free survival in cases with wild-type *PIK3CA* (n=52) vs. mutant *PIK3CA* (n=7).

Next we conducted univariate and multivariate analyses of *PIK3CA* mutation for survival (Table 7)[17]. Notably, adjusted multivariate analysis demonstrated that *PIK3CA* mutation was significant for poor overall survival (hazard ratio [HR], 3.9; 95% confidence interval [CI], 1.3-11.8; p=0.017; Table 7) and cancer-specific survival (HR, 3.6; 95% CI, 1.2-11.0; p=0.024; Table 7), but not for poor progression-free survival (HR, 2.3; 95% CI, 0.81-6.5; p=0.12; Table 7).

Table 7[17]. Univariate and multivariate analyses of *PIK3CA* mutational status for survival.						
	Univariate			**Multivariate adjusted**		
	HR	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Progression-free survival	1.68	0.64-4.38	0.29	2.29	0.81-6.50	0.12
Overall survival	2.57	0.95-6.90	0.062	3.88	1.28-11.78	0.017
Cancer-specific survival	2.69	0.99-7.27	0.051	3.60	1.19-10.95	0.024

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Finally we performed cell viability assay to examine the effect of a PI3-kinase inhibitor copanlisib (BAY80-6946) in combination with cisplatin on cell proliferation of CaSki cells harboring heterozygous *PIK3CA* mutation. We first confirmed the mutation in CaSki cells by DNA sequencing of the cells (Figure 10).

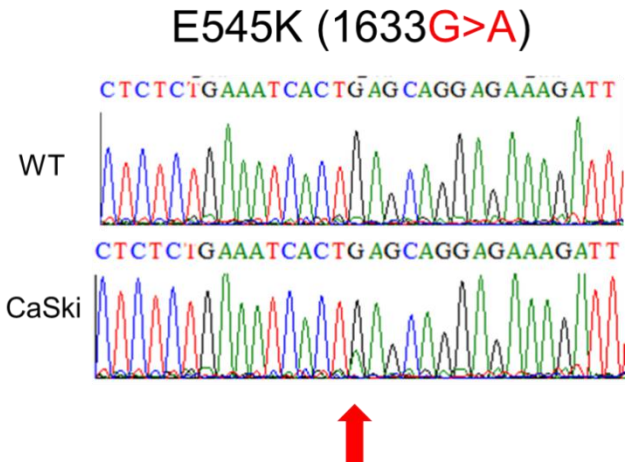

Figure 10: Heterozygous *PIK3CA* mutation E545K in CaSki cells.

The ratio of viable cells was further reduced by the combination treatment compared to single-agent treatments (Figure 11).

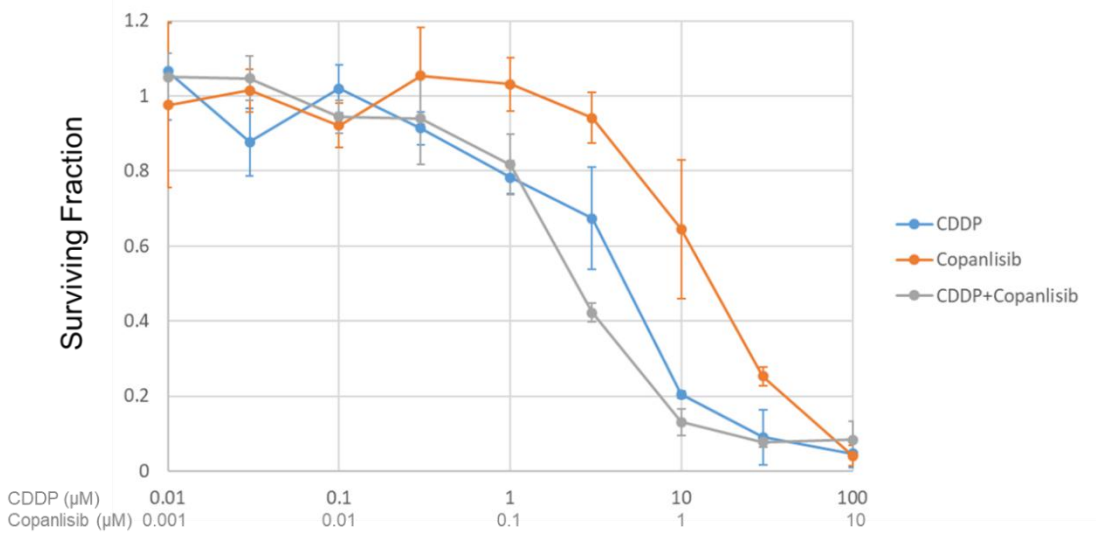

Figure 11: The ratio of viable cells was reduced by the combination treatment compared to single-agent treatments.

By treatment with copanlisib in combination with cisplatin, IC_{50} decreased from 7.32 μM to 6.04 μM (Table 8). From the numerical value of the calculated Combination Index, this effect was found to be synergistic rather than additive effect (Table 9).

Table 8: IC_{50} of cisplatin, copanlisib, and the combination treatment in CaSki cells.

	Cisplatin	**Copanlisib**	**Cisplatin + Copanlisib**
IC_{50}	7.32 μM	8.80 μM	6.04 μM (Cisplatin) 0.60 μM (Copanlisib)

Table 9: Combination index by cisplatin plus copanlisib in CaSki cells.			
Combination index	**<0.9**	**0.9-1.0**	**1.1<**
	synergistic	additive	antagonistic
Cisplatin + Copanlisib	0.89 (IC_{50})		

4. DISCUSSION

Our mutational analysis found *PIK3CA* mutations in 12% of the patients, which is relatively lower than the results previously reported (13-36%), possibly due to difference in quality of samples for DNA extraction, as we used archival FFPE biopsy specimens from patients with mostly locally advanced cancers before treatment. PI3-kinase is a heterodimer of the catalytic subunit p110α and the regulatory subunit p85α. P110α is composed of five domains: an adapter-binding domain (ABD; residues 16-105), a Ras-binding domain (RBD; residues 187–289), a C2 domain (residues 330-487), a helical domain (residues 517-694), and a kinase domain (residues 797-1068). P85α also comprises five domains: an SH3 domain, a GAP domain, an N-terminal SH2 (nSH2) domain, an inter-SH2 domain (iSH2), and a C-terminal SH2 domain (cSH2). All of those mutations found in our study were mapped inside the helical or kinase domains of p110α (Table 5). E542 and E545 on the helical domain are suggested to biochemically interact with K379 and R340 of the nSH2 of p85[19,20]. Moreover, the crystal structure of p110α/p85α complex reportedly showed that E542 and E545 are located at the interface with nSH2 in close proximity to the nSH2-kinase

domain interface, suggesting a mechanism whereby E542K and E545K mutations can affect the enzyme activity of p110α[21]. T1025 was shown to be located close to the N-terminus of the catalytic loop, and may therefore alter the enzyme activity through changing the conformation of the catalytic loop[21]. Based on these structural information, we regard that considerable translational significance lies in our subsequent analyses on the associations between *PIK3CA* mutational status and clinicopathologic data.

Our subsequent survival analyses revealed that patients with mutant *PIK3CA* had significantly worse cancer-specific survival than those with wild type, and that *PIK3CA* mutation was a significant prognostic factor for poor overall and cancer-specific survival. As regards prognostic impact of *PIK3CA* mutation, McIntyre et al. previously reported that *PIK3CA* mutational status was strongly associated with overall survival in FIGO stage IB/II patients, but not in stage III/IVA patients ($p=0.0002$ vs. 0.98)[6]. However, most of the patients in our study were stage III/IVA (97%; Table 4). Likewise, Wang et al. reported that patients without genetic alterations (mutations or amplification) of *PIK3CA* had a significantly higher response rate than those with the alterations ($p=0.006$)[12], however our study did not find any difference in response rate (Table 6). These discrepancies among studies may be attributed to differences in analyzed genetic alterations (including or not amplification), constitution of histological subtypes (including or not other than SCC) and FIGO stages, patient follow-up durations, and/or recurrence treatment strategies. In any case, the current findings further support the significance of *PIK3CA* genetic

aberrations on outcome of cervical cancers treated by CCRT.

The results of our survival analyses are suggestive of a possibility that inhibiting PI3-kinase by molecular targeting agents may improve outcome by CCRT with cisplatin. Regarding the effect of PI3-kinase inhibitor combined in the treatment of cervical cancer, there have been some preclinical studies reported. Xie et al. have recently reported that a dual PI3-kinase/mTOR inhibitor NVP-BEZ235 treatment in combination with cisplatin or carboplatin induced a synergistic anti-tumor response in cervical carcinoma cells in vitro[21]. Likewise, PI3-kinase inhibitor LY294002 reportedly radiosensitized cervical cancer cell lines in vitro[22] and in vivo[23].

Moreover, Arjumand et al. recently examined in vitro whether mutated *PIK3CA* confers cervical cancer cells higher resistance to cisplatin and/or radiation, and whether this phenotype is reversed by inhibiting PI3-kinase[24]. They reported that CaSki cells harboring heterozygous E545K were more resistant to cisplatin/cisplatin + radiation than HeLa or SiHa cells with wild-type *PIK3CA*, and that HeLa cells stably expressing E545K were more resistant to cisplatin/cisplatin + radiation than cells with wild-type/depleted *PIK3CA*. Cells expressing E545K showed constitutively activated PI3-kinase pathway and augmented cell migration and Pictilisib (GDC-0941) PI3-kinase inhibitor reversed these phenotypes. In our cell viability assay as well, mild synergistic effect on cell proliferation was likewise observed by copanlisib (BAY80-6946) treatment combined with cisplatin.

As mentioned above, *PIK3CA* mutation can be theoretically involved both in radioresistance and cisplatin resistance[14,25]. Our next question was which mechanism

is primarily contributing to the observed poor survival in our patients. If radioresistance by mutated *PIK3CA* is the major mechanism, there should have been more recurrences inside the irradiated fields in patients with mutant *PIK3CA* than in those with wild type, but the result was reverse (20% vs. 56%; Table 6). Conversely, there were more recurrences outside the irradiated fields in mutant than in wild type (80% vs. 52%; Table 6), hence we presume that cisplatin resistance may be the major mechanism for the survival impact of *PIK3CA* mutation. Patients with cisplatin-resistant tumors will have more recurrences outside the irradiated fields which should be more critical to prognosis than recurrences inside the irradiated fields, and their recurrence therapies are mostly platinum-based chemotherapies such as paclitaxel plus carboplatin. This can explain why wild-type group had better survival even though they included more of persistent diseases with no response to CCRT (10% vs. 0%; Table 6) who must have responded more to platinum-based chemotherapy because of platinum-sensitive recurrences. Indeed by contrast with cancer-specific survival and overall survival, when we conducted adjusted multivariate analyses of *PIK3CA* mutation for progression-free survival, only trends without statistical significance was observed (Table 7), most likely reflecting prognostic impact of *PIK3CA* mutation on sensitivity to recurrence therapies. These findings may suggest that patients would benefit from PI3-kinase inhibitors combined with not only CCRT but also with systemic chemotherapies for recurrence. Further clinical and basic studies are required to clarify this issue.

In conclusion, we have demonstrated here that *PIK3CA* mutation is a significant

prognostic factor for poor overall and cancer-specific survival in cervical cancers treated by CCRT with weekly cisplatin. In our in-vitro study, we further found that a PI3-kinase inhibitor copanlisib in combination with cisplatin showed a synergistic effect on proliferation of CaSki cells harboring *PIK3CA* mutation. Together with the previously published findings, the current observations further suggest that molecular inhibitors targeting the PI3-kinase/AKT pathway may improve the outcome by cisplatin-based CCRT in locally advanced cervical cancers harboring *PIK3CA* mutation. Further basic and clinical studies are warranted to confirm our findings.

5. STUDY LIMITATIONS

We acknowledge that our study contains a couple of limitations. First, the total sample size is small, which may have also impacted on the small number of *PIK3CA* mutations found. These limit the significance of our survival analyses, and further accumulation of evidence is necessary to confirm our observations. Secondly, only one cell line, *PIK3CA*-mutated CaSki, was used in the cell viability assay. We are next going to perform cell viability assay using other cervical cancer cell lines including *PIK3CA* wild-type SiHa. Thirdly, the effect of PI3-kinase inhibitor to the signaling pathway was not examined in the cell viability assay. Therefore, we are going to next examine the phosphorylation of downstream proteins by western blotting in order to confirm the inhibition of the PI3-kinase/AKT pathway by the inhibitors.

6. Bibliography

1. Ma YY, Wei SJ, Lin YC, et al. PIK3CA as an oncogene in cervical cancer. *Oncogene*. 2000;19(23):2739-2744. doi:10.1038/sj.onc.1203597.
2. Miled N, Yan Y, Hon W-C, et al. Mechanism of Two Classes of Cancer Mutations in the Phosphoinositide 3-Kinase Catalytic Subunit. *Science*. 2007;317(5835):239-242. doi:10.1126/science.1135394.
3. Zhao L, Vogt PK. Class I PI3K in oncogenic cellular transformation. *Oncogene*. 2008;27(41):5486-5496. doi:10.1038/onc.2008.244.
4. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. *Oncogene*. 2008;27(41):5497-5510. doi:10.1038/onc.2008.245.
5. Janku F, Tsimberidou AM, Garrido-Laguna I, et al. PIK3CA Mutations in Patients with Advanced Cancers Treated with PI3K/AKT/mTOR Axis Inhibitors. *Mol Cancer Ther*. 2011;10(3):558-565. doi:10.1158/1535-7163.MCT-10-0994.
6. McIntyre JB, Wu JS, Craighead PS, et al. PIK3CA mutational status and overall survival in patients with cervical cancer treated with radical chemoradiotherapy. *Gynecol Oncol*. 2013;128(3):409-414. doi:10.1016/j.ygyno.2012.12.019.
7. Bauer TM, Patel MR, Infante JR. Targeting PI3 kinase in cancer. *Pharmacol Ther*. 2015;146:53-60. doi:10.1016/j.pharmthera.2014.09.006.
8. Liu S, Knapp S, Ahmed AA. The structural basis of PI3K cancer mutations: From mechanism to therapy. *Cancer Res*. 2014;74(3):641-646. doi:10.1158/0008-5472.CAN-13-2319.
9. Ma Y-Y, Wei S-J, Lin Y-C, et al. PIK3CA as an oncogene in cervical cancer.
10. Burk RD, Chen Z, Saller C, et al. Integrated genomic and molecular characterization of cervical cancer. *Nature*. 2017;228. doi:10.1038/nature21386.
11. Wieringa HW, van der Zee AGJ, de Vries EGE, van Vugt MATM. Breaking the DNA damage response to improve cervical cancer treatment. *Cancer Treat Rev*. 2016;42:30-40. doi:10.1016/j.ctrv.2015.11.008.
12. Wang J, Chai Y-L, Wang T, Liu J-H, Dai P-G, Liu Z. Genetic alterations of PIK3CA and tumor response in patients with locally advanced cervical squamous cell carcinoma treated with cisplatin-based concurrent chemoradiotherapy. 2015. doi:10.1016/j.yexmp.2015.03.014.

13. Marth C, Landoni F, Mahner S, McCormack M, Gonzalez-Martin A, Colombo N. Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2017;28(suppl_4):iv72-iv83. doi:10.1093/annonc/mdx220.

14. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*. 2003;22(47):7265-7279. doi:10.1038/sj.onc.1206933.

15. Barker HE, Paget JTE, Khan AA, Harrington KJ. The Tumour Microenvironment after Radiotherapy: Mechanisms of Resistance and Recurrence. doi:10.1038/nrc3958.

16. Patnaik A, Appleman LJ, Tolcher AW, et al. First-in-human phase I study of copanlisib (BAY 80-6946), an intravenous pan-class I phosphatidylinositol 3-kinase inhibitor, in patients with advanced solid tumors and non-Hodgkin's lymphomas. *Ann Oncol*. 2016;27:1928-1940. doi:10.1093/annonc/mdw282.

17. Lachkar B, Minaguchi T, Akiyama A, Liu S, Zhang S, Xu C, Shikama A, Tasaka N, Sakurai M, Nakao S, Ochi H, Yoshikawa H, Satoh T. Prognostic significance of PIK3CA mutation in stage IIB to IVA cervical cancers treated by concurrent chemoradiotherapy with weekly cisplatin. *Medicine (Baltimore)*. 2018;97(31):e11392. https://journals.lww.com/md-journal/Fulltext/2018/08030/Prognostic_significance_of_PIK3CA_mutation_in.15.aspx.

18. Abe A, Minaguchi T, Ochi H, Onuki M, Okada S, Matsumoto K, Satoh T, Oki A, Yoshikawa H. PIK3CA overexpression is a possible prognostic factor for favorable survival in ovarian clear cell carcinoma. *Hum Pathol*. 2013 Feb;44(2):199-207. doi: 10.1016/j.humpath.2012.05.005.

19. Yu J, Wjasow C, Backer JM. Regulation of the p85/p110 alpha phosphatidylinositol 3'-kinase. Distinct roles for the n-terminal and c-terminal SH2 domains. 1998;273(46):30199-30203.

20. Huang CH, Mandelker D, Gabelli SB, Amzel LM. Insights into the oncogenic effects of PIK3CA mutations from the structure of p110α/p85α. *Cell Cycle*. 2008;7(9):1151-1156. doi:10.4161/cc.7.9.5817.

21. Xie G, Wang Z, Chen Y, et al. Dual blocking of PI3K and mTOR signaling by NVP-BEZ235 inhibits proliferation in cervical carcinoma cells and enhances therapeutic response. *Cancer Lett*. 2017;388:12-20. doi:10.1016/j.canlet.2016.11.024.

22. Lee CM, Fuhrman CB, Planelles V, et al. Phosphatidylinositol 3-kinase inhibition

by LY294002 radiosensitizes human cervical cancer cell lines. *Clin Cancer Res*. 2006;12(1):250-256. doi:10.1158/1078-0432.CCR-05-1084.

23. Liu Y, Cui B, Qiao Y, et al. Phosphoinositide-3-kinase inhibition enhances radiosensitization of cervical cancer in vivo. *Int J Gynecol Cancer*. 2011;21(1):100-105. doi:10.1097/IGC.0b013e3182021bfd.

24. Arjumand W, Merry CD, Wang C, et al. Phosphatidyl inositol-3 kinase (PIK3CA) E545K mutation confers cisplatin resistance and a migratory phenotype in cervical cancer cells. *Oncotarget*. 2016;7(50):82424-82439. www.impactjournals.com/oncotarget.

25. Bussink J, van der Kogel AJ, Kaanders JH. Activation of the PI3-K/AKT pathway and implications for radioresistance mechanisms in head and neck cancer. *Lancet Oncol*. 2008;9(3):288-296. doi:10.1016/S1470-2045(08)70073-1