RESEARCH ARTICLE

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Expression of CD44v6 and RCAS1 in Uterine Cervical Carcinoma Infected with Human Papillomavirus and Its Effect on Cell Proliferation and Differentiation

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Abstract

Objective: To investigate the expression of CD44v6 and RCAS1 and the presence of HPV in cervical cancer tissues, to determine serum RCAS1 levels, and to evaluate these components in correlation with clinicopathologic features and survival. **Methods:** A total of 52 patients consisting of 28 squamous cell carcinoma (SCC) and 24 adenocarcinoma cases, were studied. RCAS1 and CD44v6 expression was evaluated using immunohistochemical staining. HPV 16 and 18 E6 genes were detected using PCR, and serum RCAS1 concentrations were measured using ELISA. Associations between these factors and clinicopathologic features and survival were analyzed. **Results:** CD44v6 expression was significantly higher in SCC compared with that in adenocarcinoma (P<0.001). It also showed a significant relation to histologic grade (P<0.001) and tumor size (P=0.03). RCAS1 expression was higher in adenocarcinoma than in SCC (P=0.001), and it showed a borderline relation with histological grade (P=0.057). Overall survival was not significantly different in both CD44v6 and RCAS1 expression; however, FIGO stage (P=0.025) and tumor size (P=0.042) resulted statistically different. The pre-surgical treatment serum RCAS1 levels were not associated with any clinicopathological variables. The presence of HPV 16 E6 was higher in SCC, while the presence of HPV 18 E6 was higher in adenocarcinoma (P<0.001). Detection of HPV 16 E6 was significantly associated with expression of CD44v6. The presence of HPV both HPV 16 E6 and HPV 18 E6 was found in cancer tissues with RCAS1 expression, but without any statistical significance. **Conclusion:** CD44v6 and RCAS1 expression seems to be involved in tumor proliferation and differentiation, but it is not implicated in the progression and invasion of cervical cancer infected by HPV. Pre-treatment levels of serum RCAS1 in cervical cancer are not a diagnostic and predictive biomarker.

Keywords: CD44v6- RCAS1- HPV 16 and 18- cervical cancer

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Introduction

Uterine Cervical cancer is recognized as the third most common cancer among women worldwide with an estimated 604,127 new cases and 341,831deaths in 2020 (Bruni et al., 2021). Estimations for 2020 in Thailand, have reported about 9,158 new cases and 4,705 deaths (Bruni et al., 2021). Human Papillomavirus (HPV) infection is the main cause in the etiology of cervical cancer in Thai patients, particularly type 16 and 18 (Siriaunkgul et al., 2008). The oncoprotein E6 and E7, encoded by HPV early genes in the E region, are involved in viral replication and host cell transformation (Moody and Laimins, 2010). The repression of the E6 protein has been demonstrated to activate the p53 pathway and trigger both the senescence and apoptosis of cervical cancer cells (DeFilippis et al., 2003). Cervical cancer is a complex and heterogeneous disease. In addition to well-established risk factors related to HPV infection, additive candidate biomarkers are considered to contribute to the progression and treatment of cervical cancer.

CD44 is a family of transmembrane glycoproteins that function mainly as receptors for hyaluronan and are involved in cell-to-cell or cell-to-extracellular matrix interaction. The complex alternative splicing of CD44 gene transcripts results in the standard isoform (CD44s) and variable isoforms (CD44v) (Goodison et al., 1999). CD44s is encoded by exons 1-5 (s1-s5) and 16-20 (s6-s10), while CD44v is generated by the alternative splicing of variable exons 6-15 (v1-v10). CD44 expresses in a wide variety of cell types in humans, including embryonic stem cells, differentiated cells and cancer cells (Chen et al., 2018). Numerous studies have reported the expression of CD44 isoforms in various types of human malignancies and are considered to associate with tumor invasion, progression and metastasis (Chen et al., 2018; Thapa

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and Wilson, 2016). It has been reported that CD44s and CD44v6 expression in cervical cancer tissues is higher compared with that in normal tissue (Shimabukuro et al., 1997; Xiao et al., 2014). Furthermore, the expression of CD44v6 has been shown to associate with a poor prognostic outcome in cervical cancer (Speiser et al., 1997; Kainz et al., 1995), and that it may be a predictor of lymphatic spread (Biesold et al., 1995; Kainz et al., 1996). In addition, CD44+/CD24+-expressing cervical cancer cells exhibit radiation-resistance and possess the characteristics of stem cells (Zhang et al., 2019). These studies, therefore, suggest that CD44 expression in patients with cervical cancer could be of value as a predictive biomarker for radiation-resistance.

RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) is recognized by monoclonal antibody 22-1-1, generated from the immunization of mice with the human uterine cervical adenocarcinoma cell line SiSo (Sonoda et al., 1996). RCAS1 is a type II membrane protein forming oligomers through the C-terminal coiled-coil structures, but it also exists in soluble form, probably as a result of ectodomain shedding (Sonoda, 2011). Tissue RCAS1 expression is a prognostic factor in various malignant tumors and its expression correlates with tumor aggressiveness (Sonoda et al., 2008; Giaginis et al., 2009). RCAS1 expression was investigated during the carcinogenesis of cervical cancer, and it was detected in carcinomas in situ, microinvasive carcinomas, and invasive carcinomas, but not in dysplasia (Sonoda et al., 1998). Therefore, RCAS1 expression may be associated with tumor progression and invasion in the uterine cervix. RCAS1 has been suggested to relate to the tumor's ability to evade immune surveillance via inducing the growth arrest and apoptosis of T cells and natural killer (NK) cells (Nakashima et al., 1999).

CD44 and RCAS1 might serve as biomarkers that may have useful applications in terms of tumor progression or tumor treatment via therapeutic targeting; however, the evidence for the prognostic significance of CD44 and RCAS1 expression and HPV E6 gene infection in cervical cancer is still scarce. This study aimed to assess the expression of CD44v6 and RCAS1 as well as the presence of HPV 16 and 18 E6 genes in cervical cancer tissues, to determine serum RCAS1 levels in cervical cancer patients and to analyze which of these components is associated with clinicopathologic factors and survival.

Materials and Methods

Patients and serum samples

Sera and tissue samples were collected from patients with cervical cancer undergoing hysterectomy or biopsy at Songklanagarind University Hospital between 2007 and 2009. There were 52 patients included in this study, of which 28 were diagnosed with squamous cell carcinoma (SCC) and the remaining 24 with adenocarcinoma. Three patients received chemotherapy or radiotherapy before undergoing hysterectomy. The participants'clinicopathologic data are summarized in Table 1. Besides, serum samples from 14 healthy female blood donors served as the control group. The median age of these donors was 43 years, with a range of 29 years to 59 years. The blood samples were centrifuged at 3000g for 10 min at room temperature after clot formation, and the separated serum samples were stored at -70 °C. The patients were followed-up for periods between 1 and 167 months. Overall survival (OS) was defined as the time interval between the date of surgery and the date of death due to cervical cancer. The follow-up result revealed that 9 patients had disease recurrence, 6 died of tumor, and 46 were alive and disease-free. This study was approved by the Ethics Committee of Prince of Songkla University, Thailand (EC 50-364-013-4).

Immunohistochemical staining

To detect the expression of CD44v6 and RCAS1, immunohistochemical staining was performed on sections of formalin-fixed paraffin-embedded tissue blocks using the monoclonal antibody CD44v6 (clone VFF-18, Bender MedSystems, Austria) and the monoclonal anti-RCAS1 antibody (MBL, International Co, Nagoya, Japan). Anti-CD44v6 and anti-RCAS1 were applied with concentration 2 µg/ml and 1 µg/ml, respectively. Detection was performed using the biotinylated link and the strepavidin-peroxidase complex (Dako, K0690). Briefly, the sections were deparaffinized in xylene, rehydrated in ethanol and rinsed in PBS. The antigen was retrieved via microwave treatment in a 10 mM sodium citrate buffer (pH 6). Endogenous peroxidase was blocked using 3% H_2O_2 in methanol for 10 min. The nonspecific binding sites of the sections were blocked with protein block serum (Dako, X0909), followed by incubation with anti-CD44v6 (2 µg/ml) and anti-RCAS1 (1 µg/ml) overnight at 4 °C. Subsequently, the biotinylated link and the strepavidin-peroxidase complex (Dako, K0690) were applied to the sections. Immunostaining was visualized using a DAB substrate kit (Dako, K3466). The expression was classified as a score of 0: <10% of immunopositive cells, 1: 10-25% of immunopositive cells, 2: >25-50% of immunopositive cells, and 3: >50% immunopositive cells. Finally, the tissue sections with <10% of tumor cells positive were considered negative, and those with ≥10% of tumor cells positive were defined as positive.

Enzyme-linked immunosorbent assay (ELISA) for the RCAS1 antigen in serum

The amount of serum RCAS1 was measured using commercial ELISA kits (Human receptor-binding cancer antigen expressed on SiSo cells, RCAS1: Cusabio Biotech CO., China) following the manufacturer's instructions. Briefly, the serum samples were added to a 96-well plate coated with avidin, followed by 25 µl of biotin-antibody and 50 µl of HRP-conjugated antibody, and then mixed and incubated for 3 h at 37 °C. After washing, the plates were incubated with 50 µl/well of substrate A and 50 µl/well of substrate B; then they were mixed and incubated for 15 min at 37 °C. The reaction was stopped using 50 µl/well of stop solution. Absorbance was read at 450 nm, and each sample was performed in duplicates. The plates were individually calibrated according to the quantitative RCAS1 reference standard provided by the manufacturer and expressed in arbitrary units (U/ml).

PCR analysis for HPV, HPV 16 E6 and HPV 18 E6

A total of 52 formalin-fixed, paraffin-embedded tumor samples were selected after reviewing the hematoxylin and eosin (H&E)-stained slides. The tumor areas were microdissected on a corresponding unstained slide and transferred to an Eppendorf tube for DNA isolation. The DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The PCR for HPV L1 gene was performed using a GP5+/GP6+ primer set according to a previously published protocol. The amplicon size of 150 bp was detected as a wide range of HPV types. The quality of the obtained DNA was amplified using a PC04/GH20 primer set in order to detect the beta globin gene. The PCR product had a size of 268 bp. DNA samples that resulted positive after the application of GP5+/GP6+ were studied for the amplification of the E6 regions of HPV types 16 and 18. All primer sequences are shown in Table 2. The PCR conditions were as follows: 94 °C for 4 minutes, then 40 cycles at 94 °C for 45 seconds, at 53 °C (GP5+/GP6+) and 55 °C (PC04/GH20, HPV 16 E6, and HPV 18 E6) for 45 seconds, and at 72 °C for 1 minute, with a final extension at 72 °C for 7 minutes. The PCR products underwent 2% agarose gel electrophoresis and were stained with ethidium bromide for size assessment.

Statistical analysis

The statistical analysis was performed using the R program. Pearson's Chi-square test or Fisher's exact test was used to assess the associations of CD44v6 and RCAS1 protein expression with clinicopathologic variables. The differences in serum RCAS1 levels according to clinicopathologic variables as well as between the cervical cancer patients and the control groups were analyzed using the Kruskal-Wallis test. The relationship between the E6 gene of HPV 16 or 18 and the expression of CD44v6 and RCAS1 was analyzed via Fisher's exact test. The correlation between continuous data was assessed using the spearman correlation analysis. The Kaplan-Meier method was used to estimate overall survival and survival differences were analyzed via the log-rank test. Moreover, a multivariate survival analysis was conducted using the Cox proportional-hazards model following the stepwise entering method. Statistical significance was set at a P value of <0.05.

Results

Immunohistochemical detection of CD44v6 and RCAS1 and correlation with clinicopathologic features

CD44v6 expression was detected in 57.7% (30/52) of cervical cancer patients. Regarding SCC and adenocarcinoma, the expression rates of CD44v6 were 85.7% (24/28) and 25% (6/24), respectively. The immunoreactivity of CD44v6 was predominantly detectable on the membrane of the cancer cells (Figure 1A-1B). The membranous CD44v6 staining was also observed in the squamous epithelium adjacent to tumor areas except for the superficial layers (Figure 1C). As shown in Table 3, the expression of CD44v6 was significantly higher in SCC than in adenocarcinoma ($P<0.001$). Furthermore, there was a significant correlation between CD44v6 expression and tumor size ($P=0.03$) and histological grade ($P<0.001$). However, no correlation was found between CD44v6 expression and the other variables such as age, FIGO stage, LVSI, pelvic lymph node metastasis, and recurrence.

RCAS1 expression was observed in 59.6% (31/52) of cervical cancer patients, in 39.3% (11/28) of SCC, and 83.3% (20/24) of adenocarcinoma cases (Table 3). RCAS1 immunoreactivity was mainly observed on the cell membrane of cancer cells, but it was also detected in the cytoplasm (Figure 1D-1E). In contrast to CD44v6 staining, RCAS1 presented as membranous staining mainly in the superficial and intermediate layers of the cervical squamous epithelium adjacent to tumor areas (Figure 1F). In contrast with CD44v6, the expression of RCAS1 was significantly higher in adenocarcinoma than in SCC ($P=0.001$). However, RCAS1 expression was not significantly associated with the other clinicopathologic factors examined; only a borderline positive relationship with histologic grade ($P=0.057$) was observed (Table 3).

Table 1. Characteristics of Patients with Cervical Cancer

Clinicopathologic variables	Total number of patients with SCC	Total number of patients with adenocarcinoma
Number of patients	28	24
Age (years)		
Median	47.5	45
Range	27-70	28-64
Histological grade		
G1	2	18
G2	15	1
G3	11	5
FIGO stage		
IA1	0	1
IA2	1	0
IB1	21	21
IB2	3	1*
IIA	2	0
IIB	1**	1*
Tumor size (mm)		
<25	10	14
≥25	18	10
Lymph-vascular space invasion		
Negative	11	11
Positive	17	13
Pelvic lymph node metastasis		
Negative	24	19
Positive	4	2
None	0	3
Recurrence		
Negative	25	18
Positive	3	6

*Patients who had received radiotherapy or **chemotherapy prior to surgical treatment

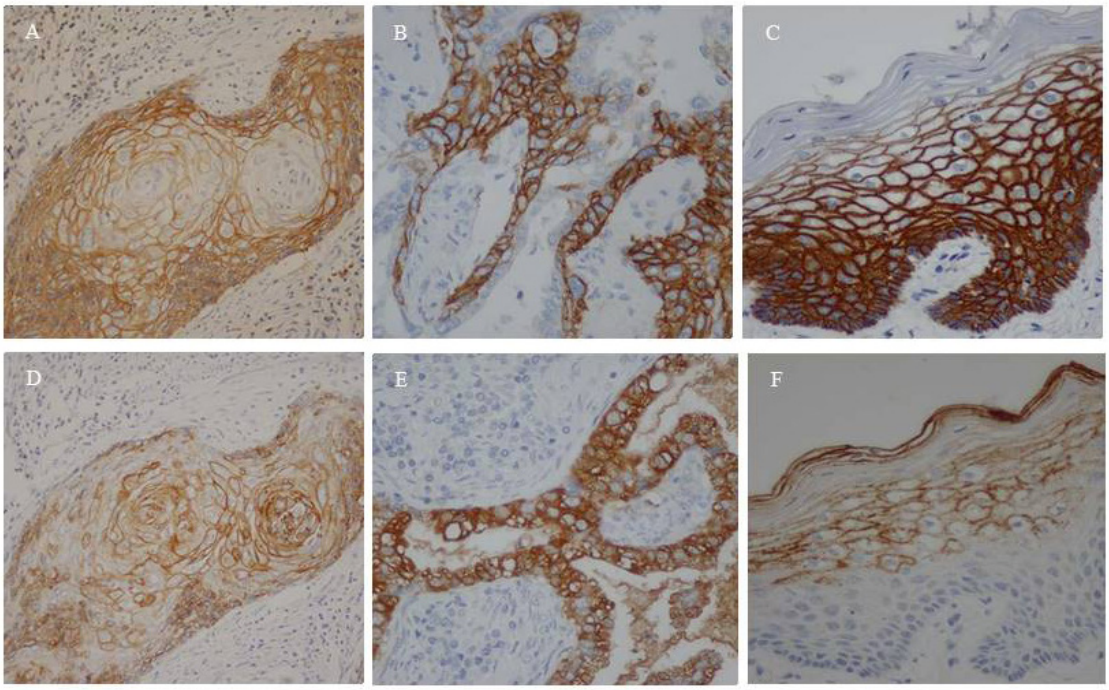

Figure 1. Immunohistochemical Staining for CD44v6 and RCAS1 in Cervical Cancer. The positive expression of CD44v6 was strongly localized on the tumor cell membrane of SCC (A) and adenocarcinoma (B). CD44v6 was present on the cell membrane in the basal, parabasal and intermediate layers of the squamous epithelium (C). The positive staining of RCAS1 was strongly expressed on the tumor cell membrane of SCC (D) and in the cytoplasm of adenocarcinoma cells (E). RCAS1 was detected on the cell membrane of both the intermediate and superficial layers of the squamous epithelium (F). A and D, magnification x 200; B, C, E and F, magnification x 400

Table 2. Primers Used in PCR Amplification of Human Papillomavirus DNA

Primer	Target gene	Sequences (5'-3')	Product size (bp)	Reference
GP5+	HPVL1	TTTGTTACTGTGGTAGATACTAC	150	Siriaunkgul et al., 2008
GP6+	HPVL1	GAAAAATAAACTGTAAATCATATTC		
HPV16E6F	HPV16E6	AAGGGCGTAACCGAAATCGGT	140	Mizobuchi et al., 1997
HPV16E6R	HPV16E6	GTTTGCAGCTCTGTGCATA		
HPV18E6F	HPV18E6	AAGGGAGTAACCGAAAACGGT	140	Mizobuchi et al., 1997
HPV18E6R	HPV18E6	GTGTTCAGTTCCGTGCACA		
PC04	Beta globin	CAACTTCATCCACGTTCACC	268	Saiki et al., 1985
GH20	Beta globin	GAAGAGCCAAGGACAGGTAC		

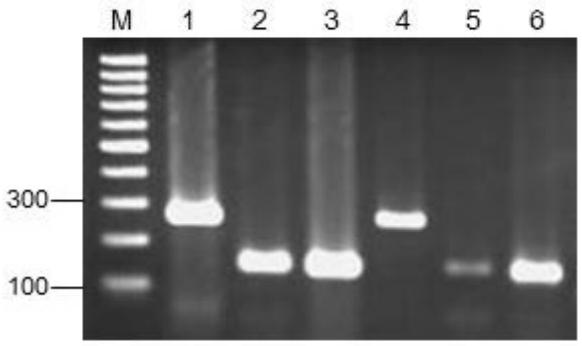

Figure 2. PCR Amplification of Genomic DNA from Cervical Cancer Tissues. M=100 bp DNA ladder marker, Lane 1-3, SCC samples; Lane 4-6, adenocarcinoma samples; Lane 1 and 4, PCR to control quality via beta-globin gene (268 bp); Lane 2 and 5, PCR with GP5+/GP6+ to detect HPV types (150 bp); Lane 3, PCR with primer set for HPV 16 E6 gene (140 bp); Lane 6, PCR with primer set for HPV 18 E6 gene (140 bp)

Serum RCAS1 concentrations and correlation with clinicopathologic features

Serum RCAS1 concentrations were measured in 14 healthy blood donors. The mean value of serum RCAS1 was 1.11 ± 0.35 U/ml (range 0.64-1.45). In cervical cancer patients prior to hysterectomy, the serum RCAS1 values were 2.29 ± 2.84 U/ml (range 0.30-12.52) for SCC and 1.35 ± 0.81 U/ml (range 0.34-4.05) for adenocarcinoma. The serum RCAS1 levels were elevated in patients with SCC but not to a significantly different level when compared to healthy controls (P=0.105). Similarly, there were no significant differences in terms of serum RCAS1 concentrations according to any of the clinicopathologic variables tested. Moreover, no correlation was found between serum RCAS1 levels and RCAS1 expression in the tumor tissues (r=0.186, P=0.2).

Table 3. Association of CD44V6 and RCAS1 Expression with Clinicopathologic Variables of 52 Patients with Cervical Cancer

Clinicopathologic variables	Total	CD44v6		P value	RCAS1		P value
		Positive (%)	Negative (%)		Positive (%)	Negative (%)	
Histologic type				<0.001*			0.001*
SCC	28	24 (80)	4 (18)		11 (35)	17 (81)	
Adenocarcinoma	24	6 (20)	18 (82)		20 (65)	4 (19)	
Age (years)				0.7			0.5
<45	23	14 (47)	9 (41)		15 (48)	8 (38)	
≥45	29	16 (53)	13 (59)		16 (52)	13 (62)	
Histologic grade				<0.001*			0.057
G1	20	4 (13)	16 (73)		16 (52)	4 (19)	
G2	16	16 (53)	0 (0)		7 (23)	9 (43)	
G3	16	10 (33)	6 (27)		8 (26)	8 (38)	
FIGO stage				0.3			0.3
I	48	29 (97)	19 (86)		30 (97)	18 (86)	
II	4	1 (3.3)	3 (14)		1 (3.2)	3 (14)	
Tumor size (mm)				0.030*			0.9
<25	24	10 (33)	14 (64)		14 (45)	10 (48)	
≥25	28	20 (67)	8 (36)		17 (55)	11 (52)	
LVSI				0.13			0.6
Negative	22	10 (33)	12 (55)		14 (45)	8 (38)	
Positive	30	20 (67)	10 (45)		17 (55)	13 (62)	
Pelvic lymph node metastasis				0.4			0.9
Negative	43	24 (83)	19 (95)		25 (89)	18 (86)	
Positive	6	5 (17)	1 (5)		3 (11)	3 (14)	
None	3	1	2		3	0	
Recurrence				0.144			0.708
Negative	43	27 (90)	16 (73)		28 (85)	15 (79)	
Positive	9	3 (10)	6 (27)		5 (15)	4 (21)	

LVSI, Lymph-vascular space invasion; *P<0.05

HPV infection and correlation with CD44v6 and RCAS1 expression

In total, 96.2% (50/52) of the cervical cancer specimens resulted positive for HPV DNA as indicated by the presence of the 150-bp PCR product using the GP5+/GP6+ primer pairing (Figure 2). The HPV infected specimens comprised 93% (26 /28) of the SCC and 100% (24/24) of the adenocarcinoma samples. In regard to the E6 gene, a total of 49 cases were identified, 25 with SCC and 24 with adenocarcinoma. As far as the HPV E6 types in SCC are concerned, 84% (21/25) of the specimens were HPV 16 and 16% (4/25) were HPV 18. Meanwhile in adenocarcinoma, HPV 16, HPV 18, and both HPV 16 and 18 were found in the proportions of 25% (6/24), 70.8% (17/24), and 4.2% (1/24), respectively. A high rate of infection with HPV 16 was noted in SCC, and HPV 18

Table 4. Association of CD44V6 and RCAS1 Expression as well as Histologic Type with HPV E6 Gene Type in Patients with Cervical Cancer

Factors	Total	HPV 16 E6 Positive (%)	HPV 18 E 6 Positive (%)	P value
Histological type				<0.001*
SCC	25	21 (78)	4 (19)	
Adenocarcinoma	23	6 (22)	17 (81)	
CD44v6				0.040*
Positive	27	19 (70)	8 (38)	
Negative	21	8 (30)	13 (62)	
RCAS1				0.133
Positive	30	14 (52)	16 (76)	
Negative	18	13 (48)	5 (24)	

*P<0.05

Table 5. Univariate Analysis of Prognostic Factors for 10-Year Overall Survival Using Log Rank Test

Factors		10-year survival rate (%)	P value
Age	<45 vs. ≥45	90-83	0.8
Histologic type	SCC vs. AD	100-70	0.058
Histologic grade	G1/G2 vs. G3	81-100	0.600
FIGO stage	I vs. II	87-67	0.025*
Tumor size (mm)	<25 vs. ≥25	100-76	0.042*
LVSI	Negative vs. positive	87-84	0.900
Pelvic lymph node metastasis	Negative vs. positive	90-50	0.200
CD44v6	Negative vs. positive	79-90	0.200
RCAS1	Negative vs. positive	92-82	0.200

AD, adenocarcinoma; *P<0.05

infection was identified predominantly in adenocarcinoma samples (P<0.001). CD44v6 expression was significantly associated with HPV16 E6 (P =0.04), whereas RCAS1 expression was not significantly related to the E6 gene of either HPV 16 or HPV 18 (P =0.133) (Table 4).

Patient survival

The 10-year OS of the patients with cervical cancer was assessed using the log rank test. Significant differences in OS related to FIGO stage (P=0.025) and tumor size (P=0.042) were observed, whereas only a borderline difference in OS in terms of histological type was noted (P=0.058) (Table 5). Nevertheless, these factors did not exhibit any significant differences when it comes to survival after the multivariate analysis.

Discussion

CD44 has been reported as a cancer stem cells (CSCs) in different tumors including cervical cancer (Lopez et al., 2012). CSCs play a prominent role in the development of the disease as well as tumor progression and metastasis. The expression of CD44v6 has been shown to associate with LVSI and regional lymph node metastasis in cervical carcinoma (Biesold et al., 1995; Kainz et al., 1996). Poor prognosis among patients with CD44v6 expression in tumor tissues in FIGO stage IB and III has also been reported (Kainz et al., 1995; Speiser et al., 1999), and CD44v6 has been described as an independent prognostic factor in early-stage cervical carcinoma (Speiser et al., 1999; Ayhan et al., 2001). The present findings are in contrast with those of the above studies; CD44v6 expression was correlated with histologic types and grade, and tumor size, but no relationship with LVSI, pelvic lymph node metastasis or survival was detected. Meanwhile, in previous studies, the expression of CD44v6 in SCC was significantly higher than that in adenocarcinoma (Bouda et al., 2005; Saegusa et al., 1999; Tokumo et al., 1998). It has been reported that the CD44v6 expression in cervical cancer with histological grades 1 and 2 was significantly higher than that in grade 3 (Tokumo et al., 1998). In line with those findings, our results showed that CD44v6 expression occurs in histological grade 2 more than that in grade 3. Additionally, the present study shows that the higher expression of CD44v6 is linked to a larger tumor size. The univariate analysis revealed no significant difference in overall survival for present/absent CD44v6 expression in this study. A similar result to ours has been reported in an earlier research (Bouda et al., 2005). All the above findings suggest that CD44v6 might be involved in tumor proliferation and differentiation, whereas it has not yet been implicated in the progression or metastasis of cervical cancer.

RCAS1 expression has been identified as a prognostic factor in various malignant tumors including cervical cancer (Giaginis et al., 2009). The expression of RCAS1 has been found to significantly correlate with LVSI, pelvic lymph node metastasis and tumor volume, but it has not been shown to associate with histological subtype in cervical cancer (Sonoda et al., 2005). Moreover, the overall survival rates of patients with high RCAS1 expression have been reported to be significantly shorter than of those with low RCAS1 expression (Kaku et al., 1999). Our results are in contrast with those of the above studies; RCAS1 expression was not associated with any clinicopathological variable except histologic type. The expression of RCAS1 in adenocarcinoma was significantly higher than that in SCC. This study found that the expression of RCAS1 had only a positive borderline association with histologic grade. In this context, RCAS1 expression was found to be higher in differentiated grade-1 tumors than that in those of grade 2 or 3. In contrast, a previous study indicated that RCAS1 was expressed at significantly higher rates in histological grade 3 (Liu et al., 2007). Our univariate analysis showed that there was no significant difference in overall survival for the present/absent RCAS1 expression in our cervical cancer population. Therefore, these findings suggest that RCAS1 expression might be involved in tumor proliferation or differentiation, but it may not be considered a reliable prognostic factor in cervical cancer.

In this study, immunoreactivity for RCAS1 was detected in nonneoplastic squamous epithelium and seen in normal endocervical epithelium; these findings are in line with those of a previous report (Kawano et al., 2005). That study reported a RCAS1 expression in the cervical squamous epithelium and a strong RCAS1 expression in endocervical glands, particularly around the areas of squamous metaplasia. This suggests that RCAS1 may affect the proliferation and differentiation of cervical

glands or the squamous epithelium through a metaplastic process.

In addition, soluble RCAS1 has been reported as a diagnostic and prognostic factor in several tumors (Sonoda, 2011). RCAS1 is secreted via proteolytic processing and is detected in the vaginal discharge of cervical cancer patients (Sonoda et al., 1998). The serum RCAS1 concentrations were statistically higher in cervical cancer patients than among the healthy blood donors. Additionally, the serum RCAS1 concentration value in adenocarcinoma has been reported to be significantly higher than that in the SCC of the cervix (Sonoda et al., 2006). The findings of this study are in contrast with those of the above studies; the serum RCAS1 levels were not significantly different neither between patients with cervical cancer and healthy blood donors nor between SCC and adenocarcinoma of the cervix. A recent study has reported that serum RCAS1 levels of both pre- and post-intervention were unaffected by the histological type of the cervical cancer (Szubert et al., 2020). Although 60% of tumor tissue samples expressed RCAS1 protein in this study, no direct correlation between serum RCAS1 levels and RCAS1 protein expression in tumor tissue was observed. It is probable that the RCAS1 expressed in tumor samples may be released into the serum with a small amount of soluble protein. According to previous studies, RCAS1 is secreted via ectodomain shedding induced by phorbol ester, pro-inflammatory cytokines, various stress-inducing stimuli and growth factors, but the signaling mechanisms activating this process remains largely unknown (Sonoda et al., 2010). the authors reported that not all RCAS1-expressing cells secreted serum RCAS1 to the blood serum (Enjoji et al., 2005).

In concordance with the results of a previous study (Siriaunkgul et al., 2008), the most common HPV types detected in patients with cervical cancer in this study are HPV 16 and 18. The oncogenic potential of HPV is associated with the binding affinities of E6 and E7 proteins. E6 promotes the degradation of p53 through its interaction with cellular proteins, E6-associated protein (E6-AP) and E3 ubiquitin-protein ligases (E3s), whereas E7 binds to the Rb protein and disrupts its complex formation with E2F transcription factors (Narisawa-Saito and Kiyono, 2007). This study showed a significant relationship between CD44v6 expression and the HPV16 E6 gene in cervical cancer. Similar results have been reported in esophageal squamous cell carcinoma (Liu et al., 2005). Meanwhile, a relationship between CD44v6 and the mutant p53 protein has been reported in colorectal cancer (Mulder et al., 1995). Such concurrence between the published evidence indicates that CD44 is a key tumor-promoting agent in transformed tumor cells lacking the p53 function (Godar et al., 2008). These findings might support what this study found, i.e., that infection with HPV 16 results in the degradation or inactivation of the p53 function effecting the expression of CD44v6 during the carcinogenesis of cervical cancer. On the other hand, no significant correlation between RCAS1 expression and the E6 gene of HPV 16 or 18 in our cervical cancer cases was noticed. It is possible that the pathogenesis of cervical cancer might involve other factors in addition to the E6 gene of HPV 16 and 18. In light of this observation, a previous study lent support to the idea that RCAS1 expression is positively correlated with the HPV 16 E7 gene in cervical carcinoma and suggested that RCAS1 expression in the tumor may promote the apoptosis of immune cells resulting in HPV-positive tumor cells evading immune surveillance (Liu et al., 2007).

However, because of limited number of cases included in our study, the exact prognostic role of CD44v6 and RCAS1 expression and serum RCAS1 in cervical cancer infected with HPV should be verified in prospective cohort large studies. Considering the cut-off scores of immunohistochemical staining should be interpreted cautiously.

In conclusion, this study provides evidence that the expression of CD44v6 is significantly related to tumor size, histologic grade, and type. Meanwhile, RCAS1 expression was found to correlate significantly with histological type, but it showed only a borderline relationship with histological grade. Overall survival was not significantly different between CD44v6 and RCAS1 expression; however, FIGO stage and tumor size had significantly different overall survival rates. Serum RCAS1 levels in pre-surgical treatment cervical cancer patients were not associated with any of the clinicopathologic variables investigated. Moreover, no direct correlation between serum RCAS1 levels and RCAS1 protein expression in tumor tissue was detected. However, the presence of HPV 16 E6 was significantly correlated with SCC, while the presence of HPV 18 E6 was significantly related to adenocarcinoma. Furthermore, the detection of HPV 16 E6 was significantly associated with the expression of CD44v6. In contrast, neither HPV 16 E6 nor HPV 18 E6 was found to significantly correlate with RCAS1 expression. These results may suggest that the CD44v6 and RCAS1 expression could be involved in tumor proliferation and/or differentiation, whereas their expression may not be implicated in the progression and invasion of cervical cancer infected by HPV. Finally, the pre-treatment level of serum RCAS1 in cervical cancer is not a diagnostic or predictive biomarker.

Author Contribution Statement

Study conception and design, methodology and manuscript draft, S.S.; specimen collection, histopathological diagnosis and interpretation, K.T.; supervision, S.S. All authors approved the manuscript.

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Ethics approval

This study was approved by the Ethics Committee of Prince of Songkla University, Thailand (EC 50-364-013-4).

Availability of data

Not applicable.

Conflicts of interest

The authors declare no conflict of interest.

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