รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาการแสดงออกของยีนและโปรตีนของ RCAS1 ในมะเร็งปากมดลูก

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พฤษภาคม 2553

สัญญาเลขที่ MRG5180290

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บทคัดย่อ

รหัสโครงการ: **MRG5180290**

ชื่อโครงการ: **การศึกษาการแสดงออกของยีนและโปรตีนของ RCAS1 ในมะเร็งปากมดลูก**

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บทคัดย่อ:

RCAS1 เป็น transmembrane protein ที่เซลล์มะเร็งหลายชนิดสร้างและหลั่งออกมาจากเซลล์ ทำหน้าที่เกี่ยวข้องกับการเกิด apoptosis ของ lymphocyte และ NK cell ทำให้เซลล์มะเร็งไม่ถูกทำลายด้วยระบบภูมิคุ้มกัน CD44 เป็น transmembrane protein จัดอยู่ในกลุ่มโปรตีน adhesion, CD44v6 ซึ่งเกิดจาก alternative splicing ของ CD44 พบได้ในมะเร็งหลายชนิด และมีความสัมพันธ์กับการเจริญและการลุกลามของเซลล์มะเร็ง การศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อหาปริมาณของ RCAS1 ในซีรั่มของผู้ป่วยมะเร็งปากมดลูกเปรียบเทียบกับในคนที่มีสุขภาพสมบูรณ์ และวิเคราะห์ปริมาณของ RCAS1 mRNA ปริมาณของโปรตีน RCAS1 และ CD44v6 ในชิ้นเนื้อมะเร็งปากมดลูก และศึกษาความสัมพันธ์กับ E6 DNA ของ HPV-16, -18 และ clinicopathologic variables การศึกษาทำในผู้ป่วย 52 รายซึ่งประกอบด้วย 28 รายเป็นมะเร็งชนิด squamous cell carcinoma (SCC) และ 24 รายเป็นมะเร็งชนิด adenocarcinoma ปริมาณของ RCAS1 ในซีรั่มวัดด้วยเทคนิค ELISA ปริมาณโปรตีนของ RCAS1 และ CD44v6 ในชิ้นเนื้อวิเคราะห์ด้วยเทคนิค immunohistochemical staining ตรวจ E6 DNA ของ HPV-16 และ 18 ด้วยวิธี PCR, วัดปริมาณ RCAS1/GADPH mRNA ด้วย real-time RT-PCR ผลการศึกษา ปริมาณ RCAS1 ในซีรั่มของมะเร็งชนิด SCC สูงกว่าในคนปรกติประมาณหนึ่งเท่า และไม่มีความแตกต่างในซีรั่มของมะเร็งชนิด adenocarcinoma เมื่อเทียบกับคนปกติ ปริมาณโปรตีน RCAS1 พบมีความสัมพันธ์กับ histological grade และ lymph-vascular space ปริมาณโปรตีน CD44v6 มีความสัมพันธ์กับ histological grade และมีแนวโน้มที่จะสัมพันธ์กับ lymph-vascular space แม้ตรวจพบ E6 DNA ของ HPV-16 และ 18 ในปริมาณมาก แต่ก็ไม่มีความสัมพันธ์อย่างมีนัยสำคัญกับปริมาณโปรตีนของ RCAS1 และ CD44v6 ปริมาณ RCAS1 /GADPH ratio ในชิ้นเนื้อมะเร็งไม่มีความสัมพันธ์กับโปรตีน RCAS1 ปริมาณโปรตีน RCAS1 และ CD44v6 ในชิ้นเนื้อเป็นเพียงปัจจัยเสริมที่สัมพันธ์กับกระบวนการเจริญและลุกลามของเซลล์มะเร็งปากมดลูก

คำหลัก: RCAS1 (receptor-binding cancer antigen expressed on SiSo cells), CD44v6, Human papillomavirus (HPV) 16 and 18, cervical cancer, squamous cell carcinoma (SCC), real-time RT-PCR

Abstract

Project Code : **MRG5180290**

Project Title : **Protein and gene expression of RCAS1 in uterine cervical cancer**

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Project Period : **2 years**

Abstract:

RCAS1 is a transmembrane protein expressed in various human cancer cells and is related to tumor escape from host immune surveillance. CD44v6 is a cell surface adhesion protein which may promote tumor progression and metastasis. This study was to investigate serum RCAS1 concentrations in cervical cancer patients and to determine RCAS1 gene, expression of RCAS1 and CD44v6, and the presence of HPV-16 and-18 E6 gene in cervical cancer tissues in correlation with clinicopathologic features. A total of 52 patients were studied, including 28 cases of squamous cell carcinoma (SCC), and 24 cases of adenocarcinoma. Serum RCAS1 concentrations were measured using ELISA. Expression of RCAS1 and CD44v6 was evaluated using immunohistochemical staining. HPV-16 and -18 E6 DNA were detected using PCR. Expression of RCAS1 and GAPDH mRNA was measured using real-time RT-PCR. The serum RCAS1 levels were one fold higher in SCC than healthy controls, but was not different between adenocarcinoma cases and controls. RCAS1 expression was significant correlation with lymph-vascular space invasion and histological grade. CD44v6 expression was significant relation to histological grade, with a trend toward the presence of lymphovascular space invasion. Detection of HPV was not associated with expression of RCAS1 and CD44v6. The RCAS1/GAPDH ratio of tumors with RCAS1 protein positive (range, 0.36-1.03 of SCC and 0.48-1.29 of adenocarcinoma) did not differ from tumors without RCAS1 expression (range, 0.31-1.85 of SCC and 0.69-1.62 of adenocarcinoma). Our results suggest that RCAS1 and CD44v6 expression are selective factors that may contribute progression and invasion of cervical cancer.

Keywords: RCAS1 (receptor-binding cancer antigen expressed on SiSo cells), CD44v6, Human papillomavirus (HPV) 16 and 18, cervical cancer, squamous cell carcinoma (SCC), real-time RT-PCR

Introduction

Cervical cancer is the most common cancer among Thai women with an age-standardized incidence rate (ASR) per 100,000 of 19.5 and approximately 6300 new cases per year (Sriplung et al., 2003). Human Papillomavirus (HPV) infection is the main cause in the etiology of cervical cancer, particularly type 16 and 18 (Siriaunkgul et al., 2008 and Chichareon et al., 1998). Oncoprotein, E6 encoded by HPV early gene E region is responsible for host cell transformation and cancer progression. The E6 protein originating from HPV types 16 and 18 has been shown to bind to target the tumor suppressor p53 for degradation with the ubiquitin-proteasome pathway (Hengstermann et al., 2001, Pim et al., 1997). Repression of the E6 protein has been demonstrated to activate the p53 pathway and trigger both senescence and apoptosis of cervical cancer cells (DeFilippis et al., 2003).

Cervical cancer remains the leading cause of cancer mortality in Thai women. Cervical cancer is a complex and heterogeneous disease. In addition to well-established risk factors being related to HPV infection, there are other candidate biomarkers such as receptor-binding cancer antigen expressed on SiSo cell (RCAS1) and cell adhesion molecules that probably contribute to disease differences.

RCAS1 was recognized by monoclonal antibody, 22-1-1, generated from immunization of mice with the human uterine cervical adenocarcinoma cell line SiSo (Sonoda et al.,1996). RCAS1 is a type II membrane protein, consisting of 213 amino acid with a transmembrane region at N-terminus and a coiled-coil structure at the C-terminus that is able to form oligomers through the C-terminal coiled-coil structure, whereas it also exists in soluble form (Yamaguchi et al., 2005). RCAS1 expression has been suggested to be related to tumor escape from immune surveillance due to inducing growth arrest and apoptosis of T cells and natural killer (NK) cells in vitro (Nakashima et al., 1999). RCAS1 expression has been correlated with aggressive characteristics and poor overall survival in various human cancers such as those of lung, colon, stomach, breast and oral cavity (Iwasaki et al., 2000, Leelawat et al., 2003, Kubokawa et al., 2001, Rousseau et al., 2002, Tsai et al., 2008). In female genital organ, RCAS1 was expressed in malignant tissues arising in the uterine cervix, endometrium and ovary (Sonoda et al., 2003, Kaku et al., 1999, Akahira et al., 2004). RCAS1 was detectable in both squamous cell carcinoma and

adenocarcinoma of uterine cervix (Sonoda et al., 2005a). There were significant relationship between RCAS1 expression and progression and invasion of uterine cervical cancers (Sonoda et al., 2005b). It has been revealed that serum RCAS1 concentrations in patients with cervical cancer and endometrial cancer were significantly higher than in healthy blood donors (Sonoda et al., 2006). RCAS1 was also found in normal and pre-cancerous lesions of uterine cervix and endometrium. Specifically, it revealed a gradually increased incidence for RCAS1 expression from normal endometrium to hyperplasia and uterine carcinoma (Sonoda et al., 2000). The superficial area of uterine cervical glands, the vicinity of areas with squamous metaplasia, and uterine cervical squamous epithelium were shown positive for RCAS1 immunoreactivity (Kawano et al., 2005). In addition, individual report has been cited the significant association between RCAS1 expression and matrix metalloproteinase 1 (MMP-1) and laminin-5 in cervical cancer (Sonoda et al., 2005b). Furthermore, RCAS1 expression was associated significantly with VEGF expression and with microvessel density in uterine cervical cancer (Sonoda et al., 2007). Collectively, these findings support role of RCAS1 in tumor progression by modifying the characteristics of connective tissue around tumor cells.

CD44 is a family of transmembrane glycoproteins that function mainly as receptors for hyaluronan. The CD44 molecule is encoded by a single gene, comprising 20 exons and forming two groups. One group contains exons 1-5 (s1-s5) and exons 16-20 (s6-s10) spliced together to form a single transcript known as the standard isoform (ie CD44s) (Figure 1). The other group contains the variable exons 6-15 (frequently referred to as v1-v10) that are alternatively spliced between the standard exons, ie at an insertion site between exon5 and 16 (Goodison et al., 1999). The products containing the variable exons are designated CD44v. As a result of this alternative splicing, a myriad of CD44 variants can be generated.

Expression of the CD44 has been found in a wide variety of normal cell types including haemopoietic cells, transitional epithelial cells, dermal fibroblasts, and neural glial cells. Most normal epithelial and non-epithelial tissues express the CD44s isoform. Expression of CD44 variant isoforms is far more restricted in normal than in malignant tissues. It has been reported the expression of CD44 isoforms correlated

with tumor progression and metastasis in malignant diseases, such as colorectal cancer (Mulder et al.,1994), breast cancer (Kaufmann et al.,1995), lung cancer (Shimbori et al.,2003), and non-Hodgkin's lymphoma (Lockhart et al.,1999). The overexpression of CD44v6 was identified in cervical, endometrial and ovarian carcinomas and may be involved in stromal invasion of early squamous cervical carcinomas and in the cellular differentiation of endometrial cancer (Hong et al., 2006). In addition, the expression of CD44v6 has been shown to be associated with significantly poorer prognosis in stage III cervical cancer patients (Kainz et al., 1995). The assessment of CD44v6 expression has been implicated in additional prognostic marker in surgically treated cervical cancer (Speiser et al., 1997, and Costa et al., 2001).

The specific aims of this study were:

1. To determine serum RCAS1 concentrations for cervical cancer patients compared with those for healthy blood donors, and to determine relationship between serum RCAS1 values and clinicopathological factors.

2. To determine expression of RCAS1 and CD44 and presence of HPV E6 of type 16 and 18 in cervical cancer tissue, and to determine which of these components is associated with clinicopathological factors.

3. To determine correlation between expression of RCAS1 gene and protein expression levels in cervical cancer.

Materials and methods

Specimen collection

Sera and tissue samples were collected from patients with cervical cancer undergoing hysterectomy or biopsy at Songklanagarind University Hospital between 2007- 2009. There were 52 patients included in this study, of which 28 patients were squamous cell carcinoma and 24 were adenocarcinoma. Clinicopathologic data for these patients was summarized in Table 1. Serum samples from 14 healthy female blood donors were recruited as a control group. The median age of these donors was 43 years, with a range of 29 years to 59 years. This study was assembled with approval from the Hospital Ethics Committee. The fresh tissues were divided into two parts: one was placed in liquid nitrogen and stored frozen at -70^{O}C for real – time quantitative RT-PCR, the other was embedded by paraffin for immunohistochemistry. Some paraffin blocks were obtained from patients who underwent surgery in 2006.

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

Serum samples were stored at -70^{O}C until analysis. The amount of serum RCAS1 was measured by using commercial ELISA kits (Human receptor-binding cancer antigen expressed on SiSo cell, RCAS1: Cusabio Biotech CO., China), according to the manufacturers' instructions. For assay, 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, then mixed and incubated for 3 h at 37^{O}C. After washing, the plates were incubated with 50 µl/well of substrate A and 50 µl/well of substrate B, then mixed and incubated for 15 min at 37^{O}C. The reaction was stopped by 50 µl/well of stop solution. Absorbance was read at 450 nm. Each sample was performed in duplicate. The plates were individually calibrated by quantitative RCAS1 reference standard provided by the manufacturer and expressed in arbitrary units (U/ml).

Immunohistochemical staining

To detect expression of RCAS1 and CD44v6, immunohistochemical staining was performed in sections from formalin-fixed paraffin-embedded tissue blocks of patients using monoclonal antibody 22-1-1 (MBL, Nagoya, Japan)and monoclonal antibody CD44v6 (clone VEF-18, Bender MedSystems, Austria). Sections (4 µm) were baked for 60 min at 50-60^{O}C. They were then deparaffinized in xylene, rehydrated

in ethanol and rinsed in PBS, pH 7.4. Antigen was retrieved with microwave by placing the slides in 0.01 M sodium citrate buffer pH 6.0. Endogenous peroxidase was blocked using 3%H_2O_2 in methanol for 10 min. The sections were blocked nonspecific binding sites with protein block serum (Dako, X0909), followed by incubation with anti-RCAS1 (1 μg/ml) and anti-CD44v6 (2 μg/ml) overnight at 4°C. Subsequently, biotinylated link and strepavidin-peroxidase complex (Dako, K0690) were applied to the sections. Immunostaining was visualized by using 3,3'diaminobenzidine tetrahydrochloride and hydrogen peroxide (Dako, K3466). The intensity was graded visually as negative = 0, weak = 1, moderate = 2, and strong = 3. The expression was classified as a score:

0 = < 5% immunopositive cells, 1 = 5-25% immunopositive cells, 2 = ＞ 25-50% immunopositive cells, 3 = > 50% immunopositive cells.

PCR analysis for HPV-16E6 and HPV-18E6 DNA

For DNA extraction, three 5-μm sections were cut from the paraffin block. After deparaffinization in xylene and rehydration in ethanol, tumor area was scraped by surgical blade and then was extracted DNA using QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer's protocol. A final volume of 40 μl was stored to the PCR reaction.

All samples were screened for the first PCR amplification with primers MY09/MY11 and the amplified DNA samples were re-amplified using nested PCR and primers GP5+ and GP6+ (Table 2) according to a previously published protocol (Remmerbach et al., 2004). For the first step PCR, 4 μl of DNA solution was used with a final volume of 25 μl. The nested PCR was amplified using 1 μl of the first PCR product as a template. To determine that all samples originally contained DNA of sufficient quality and quantity, samples were co-amplification for the presence of an internal standard, in this case, beta globin. DNA samples that were positive by GP5+/GP6+ were studied for amplification of the E6 regions of HPV-16 and HPV-18 according to published sequences (Table 2). The amplification reaction was performed with initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 45 s and with a final extension at 72°C for 7 min. The amplified products were run on a 2% agarose gel and stained with ethidium bromide for size verification

PCR amplification analysis

Total RNA was extracted from frozen tissue blocks using RNA extraction tissue kit (Geneaid), according to the manufacturer's protocol. The RNA was reverse transcribed using SuperscriptTMII (Invitrogen) and PCR amplification of RCAS1 was performed using the oligonucleotide primers as shown in Table 2. The amplification reaction for RCAS1 (802 bp, product) included an initial denaturation at 95^{O}C for 7 min, followed by 40 cycles of denaturation at 95^{O}C for 1 min, annealing at 60^{O}C for 1 min, and extension at 72^{O}C for 1 min and with a final extension at 72^{O}C for 7 min. For other RCAS1 and GADPH (100 bp, and 227 bp, product, respectively), the PCR reaction was amplified with initial denaturation at 95^{O}C for 7 min, followed by 40 cycles of denaturation at 95^{O}C for 30 s, annealing at 60^{O}C for 45 s, and extension at 72^{O}C for 45 s and with a final extension at 72^{O}C for 7 min. The amplified products were run on a 2% agarose gel and stained with ethidium bromide for size verification

Real-Time Quantitative RT-PCR

The RNA was reverse transcribed using SuperscriptTMII (Invitrogen) and PCR amplification of RCAS1 was performed in a LightCycler 480 (Roche, USA). The reaction mixture contained 2 µl of cDNA, 10 µl of QuantiSYG (Quantimix Easy SYG Kit, Biotools Spain), 0.5 µM each primer (RCAS2F and RCAS2R, Table 2), and nuclease-free water to a final volume of 20 µl. The amplification reaction consisted of a preincubation step at 95^{O}C for 5 min, followed by 40 cycles of amplification including denaturation at 95^{O}C for 15 s, annealing at 60^{O}C for 30s, and extension at 72^{O}C for 30s, melting curve program (60-95^{O}C with a heating rate of 0.05^{O}C per second and a continuous fluorescence measurement) and finally a cooling step to 40^{O}C. Quantification of gene expression was performed by standard curve for RCAS1 and GADPH were generated using serial dilutions of cDNA (containing 100, 50, 25, 12.5 ng reverse transcribed total RNA) derived from the tissue of one of tumor. Each sample, the amounts of RCAS1 and GAPDH transcripts were determined from the standard curves. The expression level of RCAS1 was evaluated as the ratio RCAS1/GAPDH.

Statistical analysis

The chi-square test and Fisher's exact test were employed appropriately. Probability values of less than 0.05 were set as the significance level. All analyses were performed with SPSS 17.0 software.

Results

Serum RCAS1 concentrations

The result of serum RCAS1 concentrations in healthy blood donors and patients with cervical cancer was shown in Table 3. The serum RCAS1 levels were 1.11 ± 0.35 U/ml (range, 0.64-1.45 U/ml) for healthy donors. In cervical patients, serum RCAS1 values were 2.57 ± 3.66 U/ml (range, 0.30-17.11 U/ml) for squamous cell carcinoma and 1.44 ± 0.65 U/ml (range, 0.52-4.05 U/ml) for adenocarcinoma. The serum RCAS1 levels were elevated in patients with squamous cell carcinoma, but not significant difference when compared to healthy controls. There was no significant correlation between the serum RCAS1 concentrations and any of clinicopathologic variables tested for patients with cervical cancer.

Immunolocalization and expression of RCAS1 and CD44v6

Staining for RCAS1 and CD44v6 was performed in 52 cervical cancer patients. RCAS1 expression was positive in 33/52 (63%) of patients; in 13/28 (46%) of squamous cell carcinoma; and in 20/24 (83%) of adenocarcinoma (Table 4). RCAS1 immunoreactivity was observed both in the cytoplasm and on the cell membrane of cancer cells (Figure 2, 3) with percentage of positive cells ranging from 5-100%. In non-malignant epithelium areas of cervical cancer specimens, RCAS1 was presented as membranous staining mainly in superficial and intermediate layer of epithelium (Figure 4). Furthermore, RCAS1 staining was found in cytoplasm as granular type of normal endocervical gland and simple columnar endocervical epithelium (Figure 4).

CD44v6 expression was noted in 32/52 (62%) of cervical patients; in 25/28 (89%) of squamous cell carcinoma; and in 7/24 (29%) of adenocarcinoma (Table 4). Immunoreactivity of CD44v6 was predominantly detectable on the membrane of the cervical cells (Figure 2, 3). In contrast to RCAS1 staining, the membranous CD44v6 staining was found mainly in the basal and intermediate layer of non-malignant cervical epithelium (Figure 4). CD44v6 was also present in the apical area of adjacent non-malignant both endocervical gland and simple columnar endocervical epithelium (Figure 4).

Relation between RCAS1 and CD44v6 overexpression and clinicopathologic variables in cervical cancer

RCAS1 overexpression in cervical carcinomas was significant correlation with lymph-vascular space invasion (P = 0.042) (Table 4). The level of RCAS1 expression in well and moderately differentiated carcinoma was significantly higher than that in poorly differentiated carcinoma (P = 0.03) and the percentage of RCAS1 positive cells in cervical carcinomas showed a trend to be related to histological grade (P = 0.061) (Table 5). RCAS1 immunoreactivity revealed no statistically significant correlation with age, clinical stage, tumor size, and pelvic node metastases in patients with cervical carcinoma (Table 4). There was significant difference in RCAS1 expression between squamous cell carcinoma and adenocarcinoma (P = 0.006). RCAS1 expression was up-regulated in patients with adenocarcinoma, but not statistically significant relation to any of clinicopathological factors examined (Table 6). No significant correlation was found between RCAS1 expression and any of clinicopathological factors tested in cervical patients with squamous cell carcinoma (Table 7). However, the percentage of RCAS1 positive cells in squamous cell carcinomas showed a trend to be related to histological grade (P = 0.063).

CD44v6 expression and the percentage of CD44v6 positive cells in cervical cancer were significantly related to histological grade (P = 0.000 and P = 0.000, respectively) (Table 4 and 5). Expression of CD44v6 showed a trend to be correlated with lymph-vascular space invasion (P = 0.058) in patients with cervical cancer, but it revealed no statistically significant relation to age, clinical stage, tumor size, and pelvic node metastasis (Table 4). There was obviously significant difference in CD44v6 expression between squamous cell carcinoma and adenocarcinoma (P = 0.000). CD44v6 expression was up-regulated in squamous cell carcinoma and the percentage of CD44v6 positive cells was significantly related to histological grade (P = 0.022) (Table 5). No significant correlation was noted between CD44v6 expression and the other clinicopathological factors examined in squamous cell carcinoma (Table 7). CD44v6 expression in patients with adenocarcinoma revealed a significant correlation with tumor size (P = 0.007), and it showed a trend to be correlated with lymph-vascular space invasion of the tumor

(P = 0.077) (Table 6). Overexpression of CD44v6 was not statistically significant correlation with other clinicopathologic variables examined (Table 6).

Cervical cancers were associated with HPV infection.

Among the 52 cervical cancer specimens, the presence of HPV DNA was detected in 26/28 (93%) of squamous cell carcinomas, and in 24/24 (100%) of adenocarcinomas using amplification with two consensus primer systems GP5+/GP6+ and MY09/MY11 (Figure 5). The specimens from 26 patients with squamous cell carcinoma, 21 were HPV-16 E6, 3 were HPV-18 E6, and 2 were presence of HPV, but absence of HPV-16 E6 and HPV-18 E6 (Table 8). The specimens from 24 patients with adenocarcinoma, 6 were HPV-16 E6, 17 were HPV-18 E6, and 1 was both HPV-16 E6 and HPV-18 E6.

Correlation of RCAS1 and CD44v6 expression to HPV infection and correlation between HPV and clinicopathological features of cervical cancers

Overexpression of both RCAS1 and CD44v6 was not significantly correlated to E6 gene of HPV-16 and HPV-18 in all cervical specimens. The presence of HPV-16 E6 gene was not statistically significantly associated with any of the clinicopathological features tested in squamous cell carcinoma. The presence of HPV-18 E6 gene in cervical adenocarcinoma was not statistically significantly correlated with any of the clinicopathlological factors tested.

RT-PCR and Real-time PCR of RCAS1 mRNA expression

RCAS1 mRNA expression with product size of 802 bp using RT-PCR was detectable in 40/52 (76.9%) of cervical cancer patients; in 20/28 (71.4%) of squamous cell carcinomas; and in 20/24 (83.3%) of adenocarcinomas (Table 9). A PCR product size of 100 bp was indentified in 24 samples that expressed RCAS1 with PCR product size of 802 bp (Figure 6). The RCAS1/GAPDH ratio of these 24 samples was shown in Table 10. The RCAS1/GAPDH ratio of 8 squamous cell carcinoma cases with RCAS1 protein positive (range 0.36-1.03) was not different from that of the 2 squamous cell carcinoma cases without RCAS1 protein expression (range 0.48-1.29). In according to squamous cell carcinoma, the RCAS1/GAPDH ratio of 12 adenocarcinoma cases with RCAS1 protein positive (range 0.31-1.85) was

not different from that of the 2 adenocarcinoma cases without RCAS1 protein expression (range 0.69-1.62).

Discussion

RCAS1 is a type II membrane protein expressed on human uterine cervical adenocarcinoma cell line SiSo. It was initially recognized by the mouse monoclonal antibody, 22-1-1 in 1996 (Sonoda et al., 1996). Similarly as other type II membrane protein, RCAS1 also existed in soluble form (Yamaguchi, 2005). Both SiSo and MCF-7 cancer cell lines expressed RCAS1, but RCAS1 secretion was detectable in only SiSo cell lines (Sonoda et al., 2010). Additionally, RCAS1 secretion acts as a ligand for a putative receptor-expressing cells, including peripheral lymphocytes, NK cells and various human cell lines and induces apoptosis of these cells. It has been reported that RCAS1 serum concentration levels were significantly lower in patients in the early stages of cancer than those patients with recurrent cancers and disseminated tumors (Dutsch-Wicherek M and Wicherek L, 2008). The serum RCAS1 levels were significantly higher in patients with uterine, ovarian, pancreatic and gastrointestinal tract cancers than in healthy blood donors. In addition, RCAS1 protein was detectable in vaginal discharge of patients with uterine cervical carcinoma (Sonoda et al., 1998). The amount of RCAS1 in serum of uterine cancer patients was significantly higher than that of healthy blood donors (Sonoda et al., 2006). RCAS1 serum concentration levels were significantly higher in patients with cervical adenocarcinoma in comparison to patients with cervical squamous cell carcinoma (Sonoda et al., 2006). Our results were in contrast with those of the above studies, the average of serum RCAS1 levels were not different between patients with cervical adenocarcinoma and healthy blood donors. The average of serum RCAS1 levels in patients with cervical squamous cell carcinoma was slightly higher than that of serum RCAS1 levels in healthy controls. Although 63% of tumor tissue samples expressed RCAS1 protein, we could not find a correlation between serum levels and protein expression. It is probably that RCAS1 expression in the tumor samples may be released into the serum with a small amount of the soluble protein. According to previous studies, RCAS1 was secreted by ectodomain shedding induced by phorbol ester, pro-inflammatory cytokines, various stress-inducing stimuli, growth factors and G-protein-coupled receptor (GPCR)

ligands, but the signaling mechanisms activating this process are largely unknown (Sonoda et al., 2010).

As previously reported, RCAS1 expression was detected in patients with uterine cervical intraepithelial cancer and invasive cancer but not in patients with uterine cervical dysplasia (Sonoda et al., 2005b). In addition, the expression of RCAS1 in patients with invasive cancer was significantly correlated with lymph-vascular space invasion, lymph node metastasis and tumor volume. The survival time of patients with high RCAS1-positive tumor was significantly shorter than that with low RCAS1-positive ones. Moreover, RCAS1 expression was associated with increased of MMP-1 and laminin-5 levels in uterine cervical tumor cells, but was also correlated with a loss of vimentin in stromal tissue (Sonoda et al., 2005a). Thus, these findings may contribute the aggressive characteristics of uterine cervical cancer, including invasion, metastasis, and tumor growth via connective tissue remodeling. In support of above findings, RCAS1 expression in our study was detectable in 33 of 52 patients with cervical invasive cancers and significantly correlated with lymph-vascular space. We also detected the RCAS1 expression that was present in intermediate and superficial layer of adjacent non-tumor cervical squamous epithelium and was rarely seen in normal endocervical epithelium. Kawano et al. (2005) demonstrated RCAS1 expression in normal female genital organ including ednocervical glands, cervical squamous epithelium, and endometrium. Low level expression of RCAS1 has been reported to exist in normal ciliated columnar epithelial cells of the lung (Iwasaki et al., 2000) and in mucosal cells of stomach (Kubokawa et al., 2001). However, the biological functions of RCAS1 expression in noncancerous tissues need to be studied more extensively.

HPV16 and 18 have been reported to be the most common type associated with cervical cancer among Thai women. The E6 and E7 genes of HPV have been shown to be the main contributors to the development of HPV-induced cervical cancer. The E6 promotes the degradation of p53 through its interaction with cellular protein, E6 associated protein (E6AP) and E3 ubiquitin ligase, whereas the E7 binds to Rb protein and disrupts its complex formation with E2F transcription factors

(Narisawa-Saito M and Kiyono T, 2007). Our study showed that the presence of E6 gene of HPV 16 and 18 in cervical cancer reached 92%. HPV 16 E6 gene mainly expressed in squamous cell carcinoma (75%), but HPV 18 E6 gene mainly existed in adenocarcinoma (75%). These findings support the notion that E6 gene of high-risk HPV infection may play causative roles in the pathogenesis of cervical cancer.

To date, RCAS1 acts as a ligand and induces apoptosis of immune cells expressing RCAS1 receptor such as T cells, B cells, and natural killer cells (NK cell). After RCAS1 interacts with RCAS1 receptor, caspase-8 would be activated and induce apoptosis of immune cells (Nakashima et al., 1999). Therefore, tumor cells may escape the host immune surveillance. Rong et al. (2007) showed that RCAS1 expression was positively correlated to HPV 16 E7 in cervical carcinoma and suggested that RCAS1 expression in the tumor may promote the apoptosis of immune cells resulting in HPV positive tumor cells escape from immune surveillance. There is no correlation between RCAS1 expression and E6 gene of HPV 16 and 18 in cervical cancer cases of our this study. It is possible that the sample size was limited in this study or the pathogenesis of cervical cancer might involve other factors in addition to the E6 gene of HPV 16 and 18.

CD44 glycoproteins are cell adhesion molecules. Apart from facilatating tumor cell migration and invasion of extracellular matrix by binding with ligand, hyaluronan, CD44 is also reported to be associated with cell proliferation and angiogenesis (Costa et al., 2001). Expression of CD44 variants, CD44v6, arising from alternative splicing of the CD44 gene has shown to be associated with pelvic lymph node metastasis in cervical cancer (Biesold et al., 1995). In addition, the expression of CD44v6 was up-regulated in cervical cancers and correlated with clinical stages, histopathological grades, and lymph node metastasis (Yaqin et al., 2007). CD44v6 was also described as an independent prognostic factor for overall survival of patients with cervical cancer FIGO stage Ib (Speiser et al., 1997) and a poor prognosis in patients with cervical cancer FIGO stage Ib and stage III whose tumors expressed CD44v6 was reported (Speiser et al., 1997, Kainz et al., 1995). However, Bouda et al. (2005) reported that the CD44v6 expression was not a statistically significant

prognostic factor for overall survival or disease-free interval (DFI) in cervical carcinoma FIGO stage Ib. Similar to a previous study (Bouda et al., 2005), the present study showed the percentage of cervical cancer samples expressing CD44V6 in squamous cell carcinomas was significantly higher than that in adenocarcinoma. Expression of CD44v6 was positively correlated with histological grade of the cervical cancers. In cervical squamous cell carcinomas, the CD44v6 positive tumors were higher in moderate or poor differentiation than those in well differentiation. These results may be supported from our notation to find the presence of CD44v6 staining in basal and parabasal cells, but not in superficial cells in adjacent normal tissues. Consistent with our study, several reports showed the CD44v6 immunostaining in normal cervical epithelium (Hong et al., 2006, Shimabukuro et al.,1997, Soukka et al., 1997). There was no correlation between expression of CD44v6 and histological grades in adenocarcinoma in this study. These facts suggest that the regulation of CD44v6 seems to be different between different histological cell types. Although the expression of CD44v6 were not correlated with other clinicopathological factors in our study including tumor size, clinical stage, and lymph node metastasis, it showed a trend to be correlated with lymph-vascular space invasion (P= 0.058). This fact might be shown to confer metastatic behaviour to cervical cancer. Therefore, further investigation of CD44v6 in a large number of sample size is still needed for analysis.

There was no significant association between expression of CD44v6 and E6 gene in this study. All cases of adenocarcinoma were present of E6 and expression of CD44v6 were found 30% while 86% of squamous cell carcinomas expressed CD44v6 and presence of E6 gene was detectable 83%. Changes in CD44v6 expression, which might be the result of HPV infection, remain to be investigated.

In previous reports, RCAS1 protein expression was detected in 80% of lung adenocarcinomas (Oizumi et al., 2002), 34% of esophageal squamous cell carcinomas (Nakakubo et al., 2002), 27% of hepatocellular carcinomas (Noguchi et al., 2001), 74% of skin squmous cell carcinomas (Takahashi et al., 2001), 78% of uterine cervical squamous cell carcinomas, 76% of uterine cervical

adenocarcinomas (Rong et al., 2007), and 100% of pancreas cancers (Akashi et al., 2003). In our study, RCAS1 protein expression was detected in 46% of uterine cervical squamous cell carcinomas and 83% of uterine cervical adenocarcinomas. These findings indicate that the incidence of RCAS1 protein expression differs between organs. Ikeguchi et al. (2003a) reported the significant correlation between mRNA expression and RCAS1 protein expression in hepatocellular carcinomas, whereas the expression levels of RCAS1 mRNA were not associated with RCAS1 protein expression in esophageal squamous cell carcinomas (Ikeguchi et al., 2003b) . In our findings, the RCAS1/GAPDH ratio of tumors with RCAS1 protein positive did not differ from tumors without RCAS1 protein expression. Although our number of samples was limited, our results may suggest that RCAS1 protein production would be controlled after mRNA transcription.

In conclusion, this study showed serum RCAS1 levels were not different between patients with uterine cervical cancer and healthy donors. RCAS1 protein expression in patients with uterine cervical adenocarcinoma was significant higher than that in patients with uterine cervical squamous cell carcinoma. Whereas CD44v6 was significantly expressed in patients with uterine cervical squamous cell carcinoma when compared to patients with uterine cervical adenocarcinoma. Both RCAS1 and CD44v6 protein expression were detected in adjacent non-cancerous tissue epithelium. Expression of RCAS1 in uterine cervical cancers was significantly correlated with histological grade and lymph-vascular space while expression of CD44v6 in uterine cervical cancers was significantly correlated with histological grade, with a trend toward the presence of lymph-vascular space. However, RCAS1 mRNA expression levels were not associated with RCAS1 protein expression in cancer tissues in this study. Detection of HPV E6 was not significantly associated with expression of RCAS1 and CD44v6. Based on these results may suggest that RCAS1 and CD44v6 expression are selective factors that may contribute progression and invasion of cervical tissue.

Future prospects

In this study, the small number of cases examined in separated tumor type results in a poor ability to detect statistical significance. Thus, we plan to add more cases for immunohistochemical staining of RCAS1 and CD44 expression. This may contribute examining the relation of these antigens to clinicopathological variables to be more reliable diagnostic and prognostic tool.

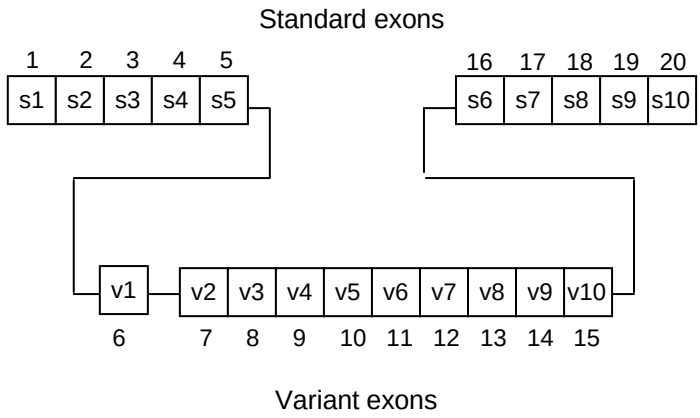

Figure 1. Schematic representation of the structure of the CD44 gene. The standard exons (s1-s10) encode the standard protein isoform, CD44s. The exons s1-s5 is in the extracellular domain and contain HA-binding domain, whereas exon s18 is in the transmembrane domain and exon s19 and exon 20 are in the cytoplasmic domain. Variably spliced exons v1-v10 in the proximal extracellular domain can be inserted between exons s5 and s6 (Goodison et al., 1999).

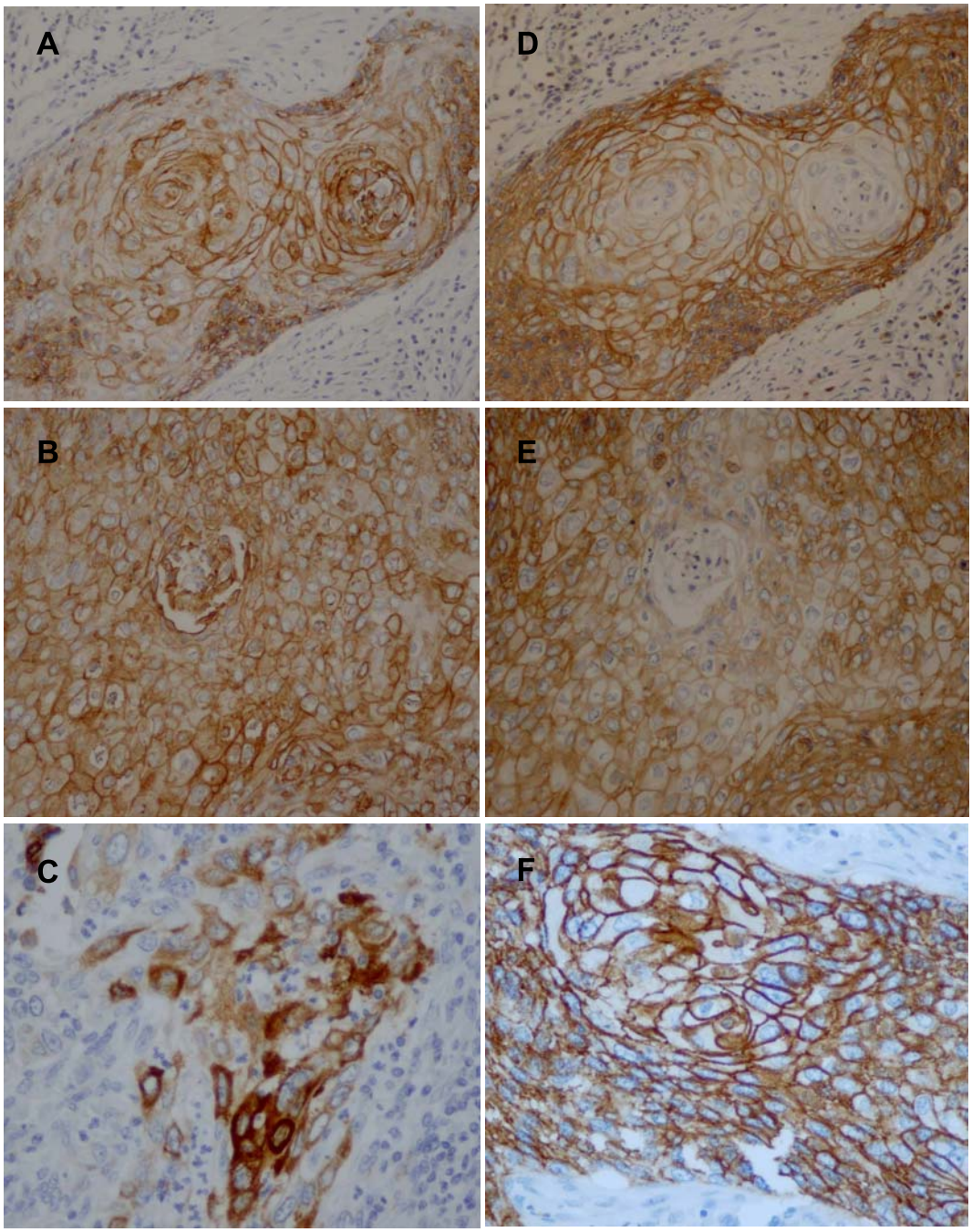

Figure 2. Immunohistochemical staining for RCAS1 and CD44v6 in cervical squamous cell carcinoma. Positive staining of RCAS1 was strongly expressed on cell membrane (A,B) and in the cytoplasm of cancer cells (C). Positive expression of CD44v6 was strongly localized on cell membrane of cancer cells (D,E,F). A-F, magnification x 400

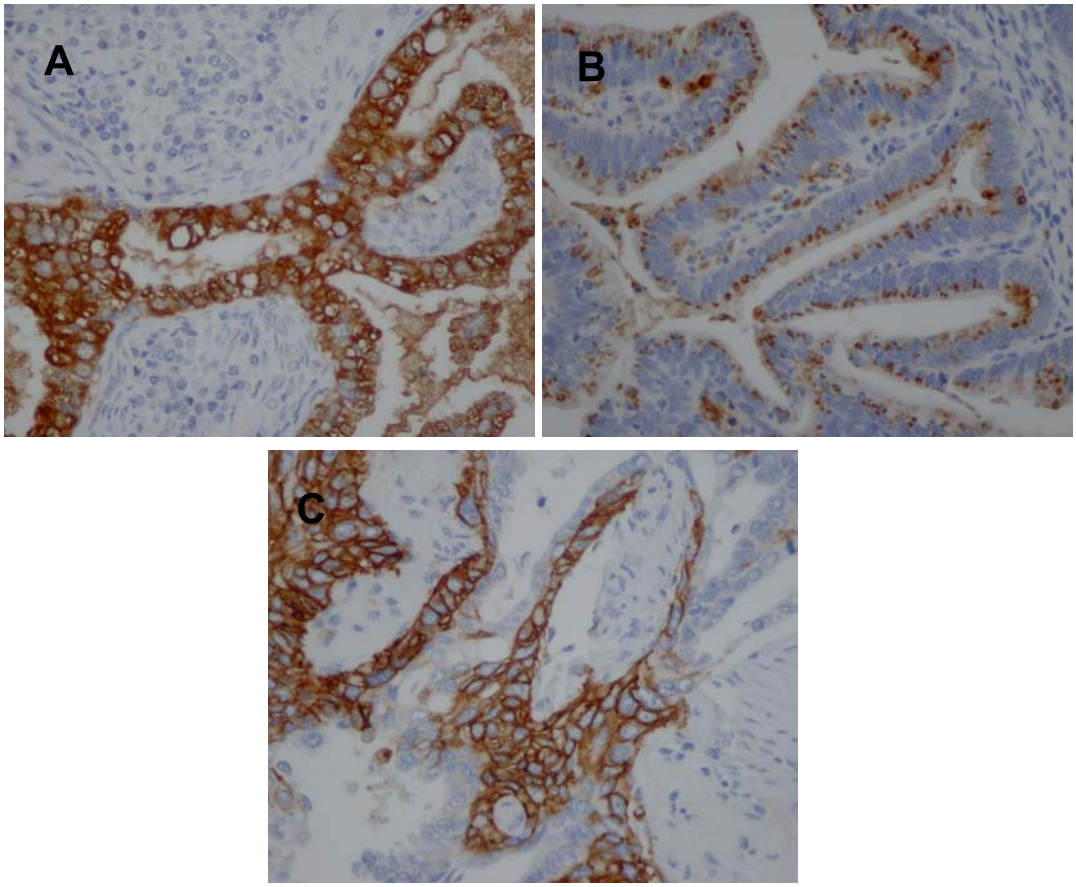

Figure 3. Immunohistochemical staining for RCAS1 and CD44v6 in cervical adenocarcinoma. Immunoreactivity of RCAS1 was strongly expressed on cell membrane and in the cytoplasm of cancer cells (A) and RCAS1 staining in cancer cells was also present as supranuclear fine granular pattern (B). Positive staining of CD44v6 was strongly localized on cell membrane of cancer cells (C). A-C, magnification x 400

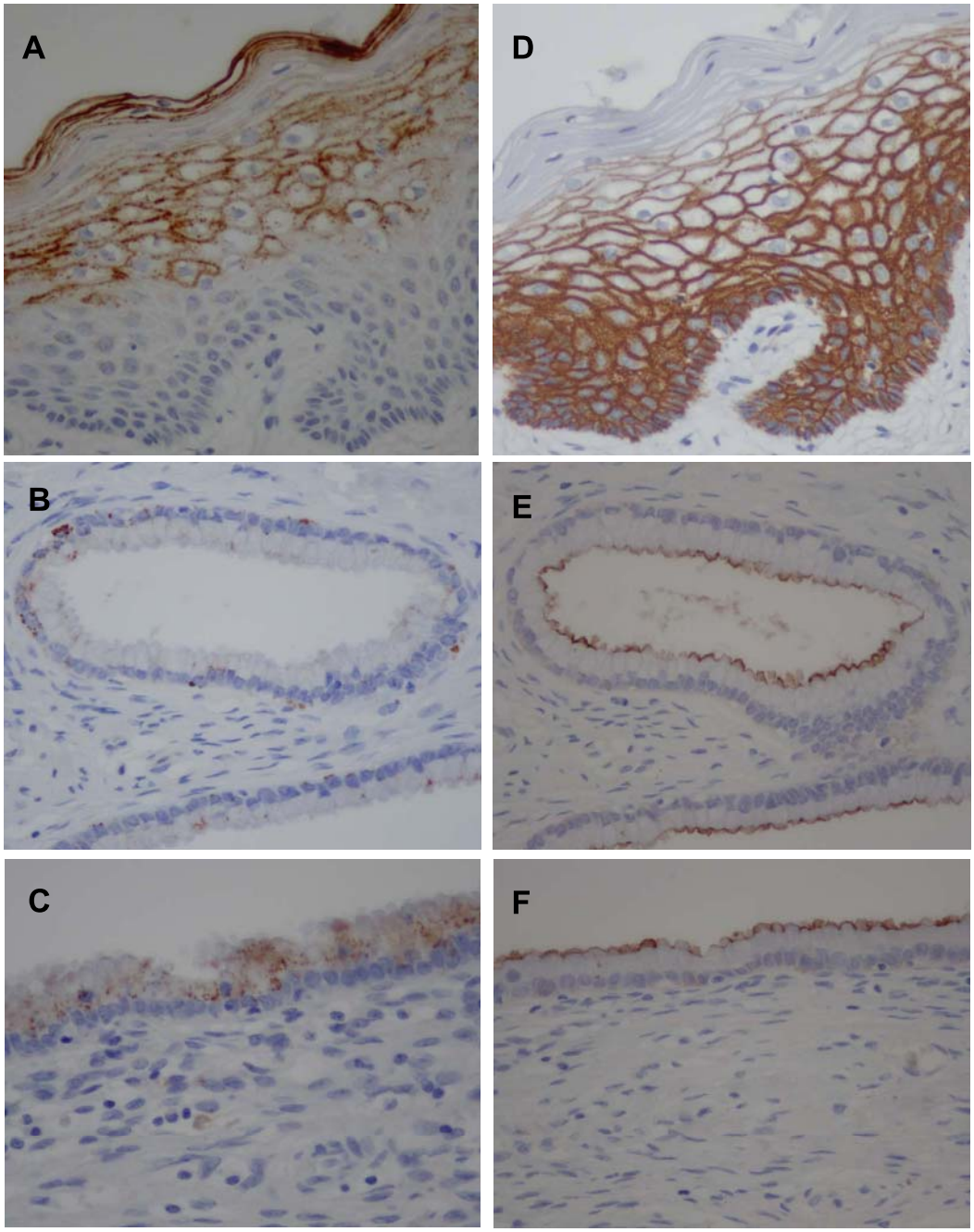

Figure 4. Immunohistochemical staining for expression of RCAS1 and CD44v6 in non-malignant cervical tissue. (A) RCAS1 was present on cell membrane in both intermediate and superficial layers of squamous epithelium. (D) CD44v6 was present on cell membrane in both basal and intermediate layers of squamous epithelium. (B) RCAS1 was rarely present in cytoplasm of endocervical gland. (C) RCAS1 was present as fine granular pattern at supranuclear region of endocervical epithelium. (E and F) CD44v6 was present at the apical region of normal endocervical gland and endocervical epithelium.

A-F, magnification x 400

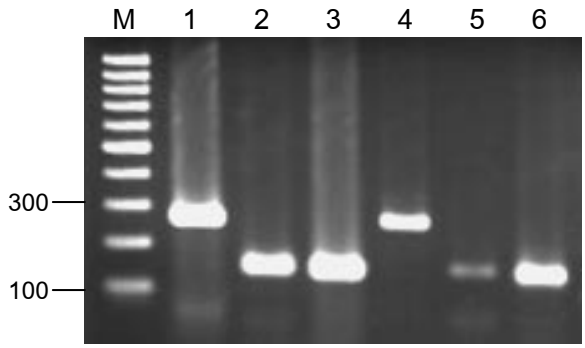

Figure 5. PCR amplification of cervical cancer tissues for presence of HPV L1 gene using primers GP5+/GP6+, and E6 of HPV-16 and HPV-18. M =100 bp ladder marker, Lanes 1-3, squamous cell carcinoma samples; Lanes 4-6, adenocarcinoma samples; Lanes 1 and 4 , primer set GH20/PC04 detecting beta globin (268 bp); Lanes 2 and 5, primer set GP5+/GP6+ detecting HPV types (150 bp); Lane 3, primer set HPV-16 E6 detecting HPV-16 (140 bp); Lane 6, primer set HPV-18 E6 detecting HPV-18 (140 bp).

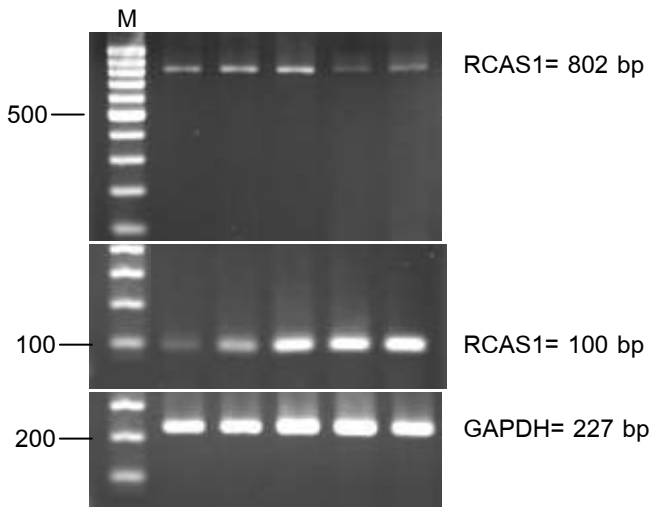

Figure 6. RT-PCR showing amplified mRNA of RCAS1 (802 and 100 bp) and GAPDH (227 bp) in cervical cancer tissues. M= 100 bp ladder marker

Table 1. Characteristics of patients with cervical cancer

Variable	No of patients
Cervical cancer: squamous cell carcinoma	28
Median age (range)	47.5 (27-70) years
Histological grade	
Well differentiated	2
Moderately differentiated	15
Poorly differentiated	11
Clinical stage	
I_{a2}	1
I_{b1}	22
I_{b2}	2
II_{a}	1
II_{b}*	2
Tumor size (mm)	
< 25	11
≥ 25	17
Cervical cancer: adenocarcinoma	24
Median age (range)	45 (28-64) years
Histological grade	
Well differentiated	19
Moderately differentiated	1
Poorly differentiated	4
Clinical stage	
I_{a1}	1
I_{a2}	1
I_{b1}	19
I_{b2}	2
II_{b}	1

*Patient who had received chemotherapy prior to surgery

Table 2. PCR primer specifications

Primer	Target gene	Sequences(5'-3')	Product size (bp)	Ref
RCAS1F	RCAS1	ACC TTA CTG CCC TCC GTC TA	802	Leelawat et al., 2003
RCAS1R	RCAS1	CTT CTT CAT TAG CCG TTG TG		
RCAS2F	RCAS1	ATA ACT TTG CCA ACTACA GTT GAT	100	Ikeguchi et al., 2003
RCAS2R	RCAS1	TCT TTA CAC TGG TGG GTG CAT CTT		
GAPDH-F	GAPDH	GAA GGT GAA GGT CGG AGT C	227	IKeguchi et al., 2003
GAPDH-R	GAPDH	GAA GAT GGT GAT GGG ATT TC		
MY11	HPVL1	GCM CAG GGW CAT AAY AAT GG	450	Remmerbach et al., 2004
MY09	HPVL1	CGT CCM ARR GGA WAC TGA TC		
GP5+	HPVL1	TTT GTT ACT GTG GTA GAT ACT AC	150	Remmerbach et al., 2004
GP6+	HPVL1	GAA AAA TAA ACT GTA AAT CAT ATT C		
H16E6F	HPV16E6	AAG GGC GTA ACC GAA ATC GGT	140	Mizobuchi et al., 1997
H16E6R	HPV16E6	GTT TGC AGC TCT GTG CAT A		
H18E6F	HPV18E6	AAG GGA GTA ACC GAA AAC GGT	140	Mizobuchi et al., 1997
H18E6R	HPV18E6	GTG TTC AGT TCC GTG CAC A		
PC04	globin	CAA CTT CAT CCA CGT TCA CC	268	Saiki et al., 1985
GH20	globin	GAA GAG CCA AGG ACA GGT AC		

M=A or C, R=A or G, W=A or T, Y=C or T

Table 3. Serum RCAS1 concentrations using ELISA in healthy controls and in patients with cervical cancer

	Serum RCAS1 concentrations		
	Healthy controls (n = 14)	Squamous cell carcinoma (n = 28)	Adenocarcinoma (n = 24)
Mean (U/ml)	1.11	2.57	1.44
SD	0.35	3.66	0.65
Range (U/ml)	0.64-1.45	0.30-17.11	0.52-4.05
Median age (range) years	43 (29-59)	47 (27-70)	45 (28-64)

Table 4. Correlations between RCAS1 and CD44 staining positivity and clinicopathologic variables of the 52 patients with cervical cancer

Clinicopathologic variables	total	RCAS1 positive (%)	negative (%)	*P* value[a]	Cd44 positive (%)	negative (%)	*P* value[a]
Age							
＜45	23	16 (31)	7 (13)	0.301	14 (27)	9 (17)	0.578
≥45	29	17 (33)	12 (23)		18 (35)	1 (21)	
Histological grade							
Well	21	17 (33)	4 (8)	0.030[b]*	5 (10)	16 (31)	0.000 *
Moderate	16	8 (15)	8 (15)		16 (31)	0	
poor	15	8 (15)	7 (14)		11 (21)	4 (8)	
Clinical stage							
I	48	32 (62)	16 (31)	0.132	30 (58)	18 (35)	0.501
II	4	1 (2)	3 (6)		2 (4)	2 (4)	
Tumor size							
＜25	26	17 (33)	9 (17)	0.520	22 (42)	4 (8)	0.185
≥25	26	16 (31)	10 (19)		10 (19)	16 (31)	
Lymph-vascular space invasion							
Absent	21	14 (27)	7 (14)	0.042 *	10 (20)	11 (22)	0.058
Present	30	18 (35)	12 (24)		22 (43)	8 (16)	
Pelvic lymph node metastasis							
Negative	41	26 (55)	15 (32)	0.418	25 (53)	16 (30)	0.281
Positive	6	3 (6)	3 (6)		5 (11)	1 (2)	
Histological type							
Squamous cell carcinoma		13 (25)	15 (29)	0.006 *	25 (48)	3 (6)	0.000 *
Adenocarcinoma		20 (38)	4 (8)		7 (14)	17 (33)	

[a] Fisher's exact test

[b] Comparison between well and moderate, and poor differentiated cervical carcinoma

*=statistically significant at $P < 0.05$

Table 5. Correlations between RCAS1 and CD44 staining positivity and clinicopathologic variables of the 52 patients with cervical carcinoma

Clinicopathological Variables	total	RCAS1 positive cells (%)			Cd44 positive cells (%)		
		< 25	≥ 25	*P* value [a]	< 25	≥ 25	*P* value [a]
Histological grade							
Well	21	8 (38)	13 (62)	0.061	19 (90)	2 (10)	0.000*
Moderate	16	11 (69)	5 (31)		0	16 (100)	
Poor	15	11 (73)	4 (27)		8 (53)	7 (47)	
Histological type							
Squamous cell carcinoma							
Well	2	1 (50)	1 (50)	0.063 [b]	1 (50)	1 (50)	0.022 [b] *
Moderate	15	11 (73)	4 (27)		0	15 (100)	
Poor	11	11 (100)	0		5 (45)	6 (55)	
Adenocarcinoma							
Well	19	7 (37)	12 (63)	0.224 [b]	18 (95)	1 (5)	0.437 [b]
Moderate	1	0	1 (100)		0	1	
Poor	4	0	4 (100)		3 (75)	1 (25)	

[a] Fisher's exact test

[b] Comparison between well, and moderate and poor differentiated cervical carcinoma

*=statistically significant at $P < 0.05$

Table 6. Correlations between RCAS1 and CD44 staining positivity and clinicopathologic variables of the 24 patients with cervical adenocarcinoma

Clinicopathologic variables	total	RCAS1 positive (%)	negative (%)	*P* value[a]	Cd44 positive (%)	negative (%)	*P* value[a]
Age							
＜45	11	10 (91)	1 (9)	0.363	3 (27)	8 (73)	0.605
≥45	13	10 (77)	3 (23)		4 (31)	9 (69)	
Histological grade							
Well	19	15 (79)	4 (21)		4 (21)	15 (79)	
Moderate	1	1(100)	0		1 (100)	0	
poor	4	4(100)	0		2 (50)	2 (50)	
Clinical stage							
Ⅰa1	1	1 (100)	0		0	1 (100)	
Ⅰa2	1	1(100)	0		0	1 (100)	
Ⅰb1	19	6 (84)	3 (16)		5 (26)	14 (74)	
Ⅰb2	2	1 (50)	1 (50)		2 (100)	0	
Ⅱb	1	1 (100)	0		0	1 (100)	
Tumor size							
＜25	13	11 (85)	2 (15)	0.642	1 (8)	12 (92)	0.007*
≥25	9	8 (89)	1 (11)		6 (67)	3 (33)	
Lymph-vascular space invasion							
Absent	10	8 (80)	2 (20)	0.596	1 (10)	9 (90)	0.077
Present	13	11 (85)	2 (15)		6 (46)	7 (54)	
Pelvic lymph node metastasis							
Negative	18	15 (83)	3 (17)	0.368	5 (28)	13 (72)	0.521
Positive	2	1 (50)	1 (50)		1 (50)	1 (50)	

[a] Fisher's exact test

*= statistically significant at $P < 0.05$

Table 7. Correlations between RCAS1 and CD44 staining positivity and clinicopathologic variables of the 28 patients with cervical squamous cell carcinoma

Clinicopathologic variables	total	RCAS1 positive (%)	negative (%)	*P* value[a]	Cd44 positive (%)	negative (%)	*P* value[a]
Age							
＜46	13	7 (54)	6 (46)	0.464[b]	11 (85)	2 (15)	0.444
≥46	15	6 (40)	9 (60)		14 (93)	1 (17)	
Histological grade							
Well	2	2 (100)	0		1 (50)	1 (50)	
Moderate	15	7 (47)	8 (53)		15 (100)	0	
poor	11	4 (36)	7 (67)		9 (82)	2 (18)	
Clinical stage							
Ⅰa2	1	1 (100)	0		0	1 (100)	
Ⅰb1	22	10 (45)	12 (55)		21 (95)	1 (5)	
Ⅰb2	2	2 (100)	0		2 (100)	0	
Ⅱa	1	0	1 (100)		1 (100)	0	
Ⅱb	2	0	2 (100)		1 (50)	1 (50)	
Tumor size							
＜25	11	4 (36)	7 (67)	0.402	9 (82)	2 (18)	0.303
≥25	17	9 (53)	8 (47)		6 (94)	1 (6)	
Lymph-vascular space invasion							
Absent	11	6 (55)	5 (45)	0.380	9 (82)	2 (18)	0.336
Present	17	7 (41)	10 (59)		16 (94)	1 (6)	
Pelvic lymph node metastasis							
Negative	23	11 (48)	12 (52)	0.673	20 (87)	3 (13)	0.605
Positive	4	2 (50)	2 (50)		4 (100)	0	

[a] Fisher's exact test except for [b], Chi-square test

Table 8. Presence of HPV DNA in cervical cancer tissues (%)

Factor	Histologic subtype	
	Squamous cell carcinoma (n=28)	Adenocarcinoma (n=24)
HPV DNA		
Positive	26 (93)	24 (100)
Negative	2 (7)	0
HPV subtypes E6		
HPV-16 E6	21 (88)	6 (25)
HPV-18 E6	3 (12)	17 (71)
HPV-16,18 E6	0	1 (4)

Table 9. Expression of RCAS1 mRNA in patients with cervical cancer

Type of tumor	total	Expression of RCAS1 mRNA (802 bp)
Squamous cell carcinoma	28	
Presence of immunostaining RCAS1	13	11
Absence of immunostaining RCAS1	15	9
Adenocarcinoma	24	
Presence of immunostaining RCAS1	20	18
Absence of immunostaining RCAS1	4	2

Table 10. The RCAS1/GAPDH ratio of patients with cervical cancer using Real-Time RT-PCR Assays

Sample	Type of Tumor	RCAS1/GAPDH ratio
1	Squamous cell carcinoma	0.36
2	Squamous cell carcinoma	1.03
3	Squamous cell carcinoma	0.59
4	Squamous cell carcinoma	0.4
5	Squamous cell carcinoma	0.78
6	Squamous cell carcinoma	1.04
7	Squamous cell carcinoma	0.58
8	Squamous cell carcinoma	1.00
9	Squamous cell carcinoma [a]	0.48
10	Squamous cell carcinoma [a]	1.29
11	Adenocarcinoma	1.85
12	Adenocarcinoma	1.29
13	Adenocarcinoma	0.99
14	Adenocarcinoma	1.73
15	Adenocarcinoma	1.47
16	Adenocarcinoma	1.9
17	Adenocarcinoma	0.31
18	Adenocarcinoma	7.45
19	Adenocarcinoma	1.73
20	Adenocarcinoma	1.1
21	Adenocarcinoma	0.91
22	Adenocarcinoma	1.00
23	Adenocarcinoma [a]	0.69
24	Adenocarcinoma [a]	1.62

a =Immunoreactivity staining of RCAS1 was negative

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