

Increased expression of calpain 6 during the progression of uterine cervical neoplasia: Immunohistochemical analysis

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Abstract. Calpain 6 (Capn6) is one of the calcium-dependent intracellular nonlysosomal proteases and is known for tissue-specific expression, primarily in the placenta. We investigated whether the expression of Capn6 is associated with the progression of uterine cervical neoplasia. We analyzed 81 formalin-fixed paraffin-embedded cervical tissues that included six normal cervical epithelium, 20 low-grade squamous intraepithelial lesions (LSIL), 17 high-grade squamous intraepithelial lesions (HSIL), 20 invasive squamous cell carcinomas (ISCC) without lymph node (LN) metastasis and 18 ISCCs with LN metastasis. The expression of Capn6 was determined by immunohistochemistry and was undetectable in normal cervical squamous epithelium. The expression of the protein gradually increased in accordance with the progression from normal to LSIL ($P=0.000$) and from LSIL to HSIL ($P=0.003$). Capn6 was detected in all cases of ISCC. However, there was no significant difference between HSIL and ISCC ($P=0.945$), ISCC with LN metastases and ISCC without LN metastases ($P=0.862$). The distribution of staining was diffuse. The cytoplasm and nucleus were stained evenly. These results suggest that Capn6 may play a significant role in the development of LSIL and may also function in the progression of LSIL to HSIL. However, its role in tumor development and metastases is not clear.

Introduction

Cervical cancer is the result of a multi-step process involving a sequential transition from normal epithelium to preneoplastic

cervical intraepithelial neoplasia (CIN) and then to invasive cervical cancer (1). Although it is well accepted that high-risk human papillomaviruses (HPVs) are associated with cervical cancer (1), HPV infection alone is insufficient for malignant transformation and specific cellular gene alterations are also required for the development of cancer. Identification of these genes has important implications for cervical cancer screening and treatment.

Calpains constitute a family of cytosolic calcium-dependent cysteine proteases. Several mammalian isoforms of calpain (Capn1-13) that are widely expressed in many tissues have been described (2). The classic calpain proteases consist of an isoform-specific large subunit and an invariant small subunit (3). The large 80 kDa subunit of Capn2 can be subdivided into four domains (D-I to D-IV) and the small 30 kDa subunit consists of two domains, D-V and D-VI. D-IV and D-VI have been proposed to confer calcium dependency on the activity of calpain (4). The physiological function of calpains is unclear; however, it has been postulated that calpains are involved in cell migration through alteration of the architecture of cell adhesion and cytoskeletal components and through involvement in intracellular signaling pathways (5).

Capn6 possesses most of the residues conserved in calpain family members; however, the C-terminal region of Capn6 lacks homology to the calmodulin-like domain (D-IV) present in other vertebrate calpains (6,7) and is referred to as Domain T (D-T). The D-T domain lacks any site conforming to the EF-hand (Ca^{2+} -binding) consensus sequence, suggesting that it may not bind Ca^{2+} . Moreover, in humans the amino acids that constitute the canonical active site of the predicted Capn6 protein, Cys, His and Asn, are modified to Lys, His, and Asn, making it unlikely that Capn6 is proteolytically active (7). Capn6 was recently shown to be a microtubule-stabilizing protein that is expressed in embryonic tissues and may be involved in the regulation of microtubule dynamics and cytoskeletal organization. In Capn6 transfected cells, cytokinesis was retarded and was often aborted to yield multinucleated cells (8).

Capn6 has much more tissue-specific expression in adults than the ubiquitously expressed Capn1 and Capn2. Although it is expressed in a number of embryonic tissues, it has been found primarily in the placenta in adults. In 1997, it was reported that *Capn6* mRNA was expressed only in the placenta out of 50 normal tissue RNAs tested (6). In 2003, the *Capn6*

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gene was found to be overexpressed in leiomyomas compared with normal myometrium by gene-expression analysis and was either not expressed or expressed at very low levels in 18 other tissue types (9). Few reports exist in the literature on *Capn6* expression in human cancer. Skubitz *et al* used microarray analysis to show that *Capn6* was expressed in two of four uterine leiomyosarcoma (LMS) samples and in one of the four non-uterine LMS samples (10). We previously reported expression of *Capn6* in endometrial stromal sarcomas, malignant mullerian mixed tumors and uterine LMS with no association to tumor stage and survival (11). As a continuation of our previous study, we further studied *Capn6* expression in several other gynecological malignant samples, including cervical cancer. To investigate whether an altered expression of *Capn6* is associated with neoplastic development and/or progression in the uterine cervix, we evaluated *Capn6* expression in biopsy samples corresponding to normal cervical epithelium, CIN lesion and invasive squamous cell carcinoma (ISCC). We present data showing that expression of *Capn6* increases in parallel with the progression of cervical neoplasia.

Materials and methods

Tissue samples. A total of 81 tissue specimens, including 20 low-grade squamous intraepithelial lesions (LSIL), 17 high-grade squamous intraepithelial lesions (HSIL), 20 ISCC without lymph node (LN) metastasis, 18 ISCC with LN metastasis and six normal specimens from the cervix were obtained from the Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine. All tissue specimens were obtained from conization procedures or radical hysterectomies of 81 independent patients from 1994 to 2004. Two gynecological pathologists reviewed all the cases in regard to the histological type, histological grading and LN status. Three-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded blocks and were applied to routine histopathology slides and immunohistochemistry slides. Representative tumor sections including adjacent non-tumor mucosa were analyzed by immunohistochemistry.

Immunohistochemical analysis. Formalin-fixed paraffin-embedded tissue sections were immunostained with anti-*Capn6* D-T (anti-DT) polyclonal antibody (1:50 dilution, Affinity BioReagents, Deerfield, IL) using a two-step EnVision technique. Paraffin sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Microwave antigen retrieval (2 x 15 min) was performed in citric acid (pH 6.0). Following overnight incubation at 4°C with the primary rabbit polyclonal antibodies, slides were incubated with an EnVision kit (Dako, Copenhagen, Denmark) for 30 min at room temperature. Specimens were then stained with diaminobenzidine and counterstained with Mayer hematoxylin. Normal myometrial tissue was used as a negative control and placental tissue as a positive control (Fig. 1). The analysis was performed in a semiquantitative manner: for each specimen an immunohistochemical score from 0 to 3 was determined based on the staining intensity. Positive controls were designated as moderate staining and scored as 2⁺. The staining intensity of

specimens was evaluated as follows: no staining as 0, weaker than the positive control as 1⁺, equivalent to the positive control as 2⁺ and stronger than the positive control as 3⁺. Slides were scored independently by two pathologists (Y.L.C. and G.H.A.) and a consensus score was obtained for each slide.

Statistical analysis. Statistical analyses were performed using SPSS for Windows version 11.0. The Jonckheere-Terpstra test was applied to correlate the expression of *Capn6* with progression from normal cervical tissue to ISCC with LN metastasis and the least significance test using ranks was applied for multiple comparisons between the five groups. A P-value of <0.05 was considered statistically significant.

Results

Clinical data. The median ages of patients with the different histological types were 44, 39, 38, 51 and 49 years for normal, LSIL, HSIL, ISCC without LN metastasis and ISCC with LN metastasis, respectively (Table I).

Immunohistochemistry. *Capn6* expression was undetectable in normal cervical squamous epithelium of all control samples; however, the basal layer of a normal endocervical gland exhibited nuclear and cytoplasmic *Capn6* staining (Fig. 1) and some of the fibroblasts and endothelial cells in the stroma of the cervix showed immunoreactivity against anti-DT. If the results were restricted to the squamous cell layers of the normal cervix, all control cases showed negative staining for *Capn6*. In contrast, *Capn6* was detected in all cases of ISCC regardless of lymph node metastasis (Table II, Figs. 1 and 2). *Capn6* staining was diffuse and the cytoplasm and nucleus appeared to stain similarly. For LSIL, expression of *Capn6* was also observed in 100% (20/20) of the cases. However, none of the LSIL slides stained strongly and 18 of the LSIL cases (90%) stained weakly (1⁺) with a similar pattern to ISCC (Table II). All HSIL cases (17/17) demonstrated expression of *Capn6* (Table II, Figs. 1 and 2); four cases (24%) stained strongly and eight cases (47%) stained weakly. When the four grades (normal, LSIL, HSIL and ISCC) were compared with respect to the intensity of staining, the expression of *Capn6* gradually increased in accordance with the progression from normal to LSIL to HSIL (normal vs. LSIL, P=0.000; LSIL vs. HSIL, P=0.003) (Table II, Figs. 1 and 2). There was a statistically significant difference between LSIL and ISCC (P=0.000), though not between HSIL and ISCC (P=0.945). In addition, there was no significant difference between ISCC with LN metastases and ISCC without LN metastases (P=0.862).

Discussion

This is the first report describing the expression of *Capn6* during the progression of human uterine cervical neoplasia. Although the physiological function of *Capn6* remains unknown, the results of this study indicate that *Capn6* may be involved in neoplastic progression in the uterine cervix. The current study demonstrated that *Capn6* was expressed in all uterine cervical neoplasias (LSIL, HSIL and ISCC) examined, in contrast to the negative expression in all normal

Characteristics	Normal (n=6)	LSIL (n=20)	HSIL (n=17)	ISCC, LN (-) (n=20)	ISCC, LN (+) (n=18)
Age (yr)					
Median	44	39	38	51	49
Range	36-53	26-56	28-47	28-72	29-71
Menopause	2/6 (33%)	1/20 (5%)	0/17 (0%)	11/20 (55%)	7/20 (35%)
Stage					
Ib1				6	6
Ib2				5	6
IIa				9	6

LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ISCC LN (-), invasive squamous cell carcinoma without lymph node metastasis; ISCC LN (+), invasive squamous cell carcinoma with lymph node metastasis.

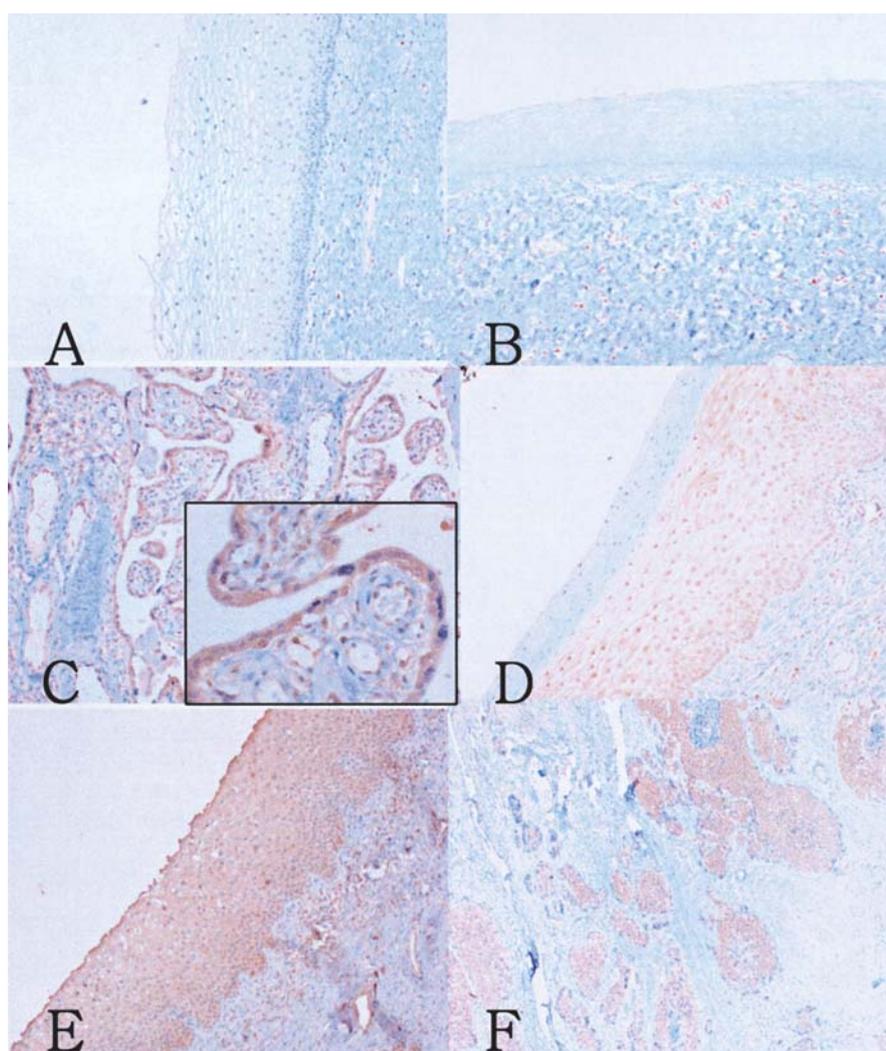


Figure 1. Immunohistochemical staining of anti-Capn6 D-T antibodies. (A,B) Normal cervical squamous epithelium showing no immunostaining. (C) Normal placenta (score 2, magnification, x100; inset, magnification, x400), positive staining. (D) Low-grade squamous intraepithelial lesion (LSIL) showing 1+ immunostaining. (E) High-grade squamous intraepithelial lesion (HSIL) showing 2+ immunostaining. (F) Invasive squamous cell carcinoma (ISCC) showing 2+ immunostaining.

Table II. The expression of calpain 6 in normal cervical squamous epithelium, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), invasive squamous cell carcinoma (ISCC) without lymph node (LN) metastasis and ISCC with LN metastasis.

Histological grade	Expression of calpain 6			
	0	1+	2+	3+
Normal ^a	6/6 (100%)	-	-	-
LSIL ^b	-	18/20 (90%)	2/20 (10%)	-
HSIL ^c	-	8/17 (47%)	5/17 (29%)	4/17 (24%)
ISCC, LN (-) ^d	-	9/20 (45%)	7/20 (35%)	4/20 (20%)
ISCC, LN (+) ^e	-	7/18 (39%)	8/18 (44%)	3/18 (17%)

^{a-b}($P=0.000$); ^{b-c}($P=0.003$); ^{c-d,e}($P=0.945$); ^{b-d,e}($P=0.000$); ^{d-e}($P=0.862$).

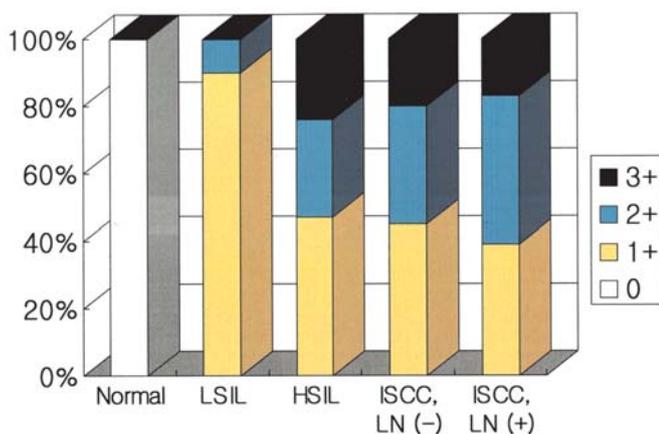


Figure 2. Percentage of scoring of immunohistochemical staining of calpain 6 in normal cervical squamous epithelium, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), invasive squamous cell carcinoma (ISCC) without lymph node (LN) metastasis and ISCC with LN metastasis ($P<0.01$).

cervical squamous cell epithelium samples (Table II, Figs. 1 and 2). The expression of Capn6 was observed from the early stages in the progression of cervical neoplasia and increased significantly with stage progression from LSIL to HSIL and ISCC (Table II, Figs. 1 and 2). Protein expression was evident in all cases of HSIL and ISCC and there was no significant difference in expression between these two stages. These results suggest that expression of Capn6 begins early during disease progression and is associated with disease progression of CIN rather than the malignant transformation of the cervical squamous cells. However, it should be noted that the cases of normal epithelium included in our study did not show the transformation zone and further studies with normal tissues containing a transformation zone are required.

It is well known that cases of LSIL are heterogeneous with respect to natural history and viral presence. While the majority of LSIL regress during long-term follow-up, between 8 and 25% progress to HSIL and occasionally to invasive cancer (12,13). Management of women with LSIL is currently limited by the inability to predict which patients are at risk of progression. The ability to predict the risk of progression using

markers that identify genetic variants associated with transformation or HPV genomes and proteins associated with transformation would greatly facilitate prevention and early intervention (14). In addition to HPV variants, candidate markers currently being studied include HPV, E6 and E7 messenger RNA, FHIT, p16, loss of heterozygosity at specific chromosomal loci and DNA ploidy (15-19). Although there is an expectation that one or more markers associated with a high-risk of progression will be identified, there is currently no such marker that can be clinically applied.

In the current study, we found that 90% of LSIL cases stained weakly with anti-DT antibody and none stained strongly. In contrast, 24% of the HSIL cases and 18% of the invasive cervical cancers stained strongly with the anti-DT antibody. Thus, it can be hypothesized that increased expression of Capn6 is one genetic alteration required for the progression from LSIL to HSIL and that the expression of Capn6 may be used as a biomarker to predict which patients with LSIL are at highest risk of disease progression. Our data also indicate that Capn6 expression is not associated with age or hormone status (Table I), although further evaluations with larger sample sizes are necessary. One clinical application would be to monitor Capn6 expression during a follow-up of patients with LSIL who are not receiving therapy to predict the risk of disease progression. Therefore, a prospective observational study to elucidate the role of Capn6 in the progression of LSIL patients is warranted. An understanding of the role of Capn6 in uterine cervical neoplasia will likely provide insight into cervical carcinogenesis and clues for identifying strategies for a better diagnosis, treatment and prevention of cervical cancer. Capn6 has recently been shown to be a microtubule-stabilizing protein expressed in embryonic tissues and may be involved in the regulation of microtubule dynamics and cytoskeletal organization (8). Cytokinesis was retarded in Capn6 transfected cells and was often aborted producing multinucleated cells. The altered regulation of microtubule dynamics and cytoskeletal organization by Capn6 may be associated with cervical neoplasia development and/or progression.

The classic calpains are abundant in the cytoplasm of the cell with a slight predominance around the nucleus (20). These



SPANDIDOS PUBLICATIONS can cleave many intracellular signaling and proteins and active calpains have been found predominately in the plasma membrane, indicating that membrane localization may regulate calpain activity (5). A previous study reported that Capn6 was mainly distributed in the perinuclear region and that the localization largely overlapped with thick microtubule bundles (8). Notably, our study revealed an even distribution of Capn6 between the cytoplasm and nucleus within the tumor cells. Further experiments using Western blot or RT-PCR analysis on cytoplasmic and nuclear fractions are required to confirm the nuclear expression of Capn6. At the present time, we are unable to exclude the possibility of non-specific binding of antibodies to the nucleus.

In conclusion, this study demonstrated that the expression of Capn6 increased during the progression of cervical neoplasia from the early stages of LSIL to ISCC, indicating that Capn6 might play an important role in the progression of uterine cervical neoplasia. Further studies are necessary in order to elucidate the role of Capn6 in tumor progression.

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