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典型鈣黏素和 catenins 在子宮內膜癌及正常子宮內膜的不同表現：鈣黏素和 catenins 在子宮內膜癌形成扮演的角色

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COMPARISON OF CADHERIN AND CATENIN SUBTYPES PRESENT IN THE POOR-DIFFERENTIATED ENDOMETRIAL ADENOCARCINOMA AND NORMAL ENDOMETRIUM*

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Running Title: Cadherins in endometrial adenocarcinoma

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ABSTRACT

Background

The incidence of endometrial cancer is increasing possibly due to the increasing of obesity women, Tamoxifen and hormone therapy users in Taiwan. Previous studies have demonstrated that endometrial cancers are associated with sex hormones, especially estrogen. However, the mechanisms by which sex steroids mediate the formation of endometrial cancers remain to be elucidated.

Cadherins are a family of integral membrane glycoproteins that mediate cell-cell interactions in a homophilic manner. Cadherins have been shown to be involved in not only cell-cell adhesion bot also cell differentiation, tissue formation, tumorigenesis and cancer invasion. Catenins are intracellular proteins that link cadherins to the cytoskeleton to form a functional complex and promote the functions of cadherins. Previous studies have showed that catenin expressions are associated with various cancers. To date, only a few reports have studied this topic and all focused on E-cadherin. In view of these observations, it is valuable to study the differential expressions of cadherins and catenins other than E-cadherin between endometrial cancers and normal endometria.

Methods

Six specimens of grade-3 endometrial adenocarcinoma were obtained from patients who were undergoing staging operations. The cadherin subtypes presented in endometrial cancers were identified using RT-PCR with degenerate primers of classical cadherins and DNA sequencing. After the identification of the cadherin subtypes, we collected six specimens of normal endometrium of early secretory phase from patients undergoing hysterectomy for uterine myomas and compared the differential expressions of these cadherins and catenins with cancer specimens using semiquantitative RT-PCR for the mRNA levels.

Results

We demonstrated six subtypes of classical cadherin in grade-3 endometrial cancers; they are E-cadherin, P-cadherin, N–cadherin, cadherin-6, cadherin-9 and cadherin-11. Among these cadherins, the mRNA levels of E-cadherin were significantly decreased and P-cadherin levels were increased in the endometrial cancers than normal endometria. There were no significant differences of the mRNA levels of α-catenin, β-catenin and γ-catenin between cancer lesions and normal endometria.

Conclusion

As cadherins are major morphoregulators, we speculated that tumorigenesis of poor-

differentiated endometrial adenocarcinoma is mediated at least in part by the down-regulation of E-cadherin and the up-regulation of the P-cadherin.

Key words: cadherins, cell adhesion molecules, P-cadherin, peritoneum, endometriosis, reverse transcription-polymerase chain reaction

INTRODUCTION

The cadherins are a gene superfamily of integral membrane glycoproteins that mediate calcium-dependent cell adhesion in a homophilic manner (Takeichi, 1991, 1995; Potter et al., 1999; Nollet et al., 2000). To date, more than 20 cadherin subtypes have been identified (Nollet e al., 2000). These cell adhesion molecules (CAMs) have been shown to play pivotal roles in a diverse array of biological and pathological processes that includes tissue morphogenesis, embryogenesis and cancer metastasis (Takeichi, 1991, 1995; Potter et al., 1999).

To date, six cadherin subtypes have been detected in human endometrium in vivo and in vitro: E-cadherin (E-cad), P-cadherin (P-cad), cadherin-6 (cad-6), cadherin-9 (cad-9), and cadherin-11 (cad-11) (Chen et al, 2002). The spatiotemporal expression of these cadherin subtypes in the endometrium during the menstrual cycle suggests that these CAMs play distinct roles in the formation and organization of this dynamic tissue (MacCalman et al., 1998; Getsios et al., 1998). However, the role(s) of the cadherins in the development of endometrial cancer remains poorly understood.

In order to gain a better insight into the role(s) of the cadherins in the endometrial cancers, we have performed a comprehensive examination of the cadherin subtypes present in grade-3

endometrial adenocarcinoma using a reverse transcription-polymerase chain reaction (RT-PCR) strategy previously described by Suzuki et al. (1990). Semiquantitative RT-PCR was then performed to determine whether the levels of the mRNA transcripts encoding these cadherin subtypes were differentially expressed in endometrial cancers and normal endometria. We have determined that the same repertoire of cadherin subtypes is present in endometrial cancer and normal endometrium. However, P-cad mRNA levels were found to be significantly greater and E-cad levels were decreased in the endometrial cancer lesions. In view of these observations, it is tempting to speculate that P-cad plays a key role in the development of endometrial cancer..

MATERIALS AND METHODS

Tissues

Endometrial adenocarcinoma samples were obtained from patients (n=6; age range: 45-68 years) undergoing staging operations for endometrial cancers. First three samples were used for determining cadherin subtypes using RT-PCR with degenerate primers for classical cadherins and all six samples were used for semiquantitative RT-PCR. Normal endometrium (n=6) were obtained from women undergoing surgery for uterine myomas during early

secretory phase. These women had not received hormonal treatments for at least three months prior to the time of surgery. The research was approved by The Committee of Ethical Review of Research Involving Human Subjects, China Medical College, Taichung, Taiwan. All of the women provided informed written consent.

Samples were collected either snap-frozen for mRNA study or fixed in 4% formaldehyde for histological study. Paraffin sections, prepared from the fixed endometrial tissues samples, were then stained and subjected to histological evaluation.

Generation of first strand cDNA

Total RNA was extracted from the adenocarcinoma or normal endometrium by using Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD) according to a protocol supplied by the manufacturer. The concentration and purity of the total RNA extracts was quantified by spectrophotometry (A 260/280). The integrity of the total RNA extracts was also determined by gel electrophoresis followed by ethidium bromide staining.

First strand cDNA was generated from aliquots (1 μg) of these total RNA extracts using a RT reaction mixture that contained Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units Promega, Madison, WI), random primers (0.5 μg), 20mM Tris-HCl,

pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 1 mM deoxynucleotides, to a final volume of 25 μl. This RT reaction mixture was incubated at 37°C for 60 min after which, the MMLV-RTase was heat-inactivated at 94°C for 5 min.

Identification of the cadherins present in normal peritoneum or endometritiotic lesions

PCR was performed using the method described by Suzuki et al. (1990). Briefly, oligonucleotides that correspond to the amino acid sequences of two regions conserved among the known cadherin subtypes were used as degenerate primers. The first strand cDNAs generated from total RNA extracted from endometrial cancers were used as templates in these PCR reactions.

The PCR products, approximately 180 bp in size, were gel-purified and subcloned into the PCR-II vector using a TA cloning Kit (Invitrogen, San Diego, CA). Approximately 30 clones per tissue sample were subjected to DNA sequence analysis using an automated DNA sequencer (Applied Biosystems) employing Taq DyeDeoxy sequencing reagents. The nucleotide sequence obtained for each clone was compared to the Genbank/EMBL databases using the "BLAST" computer program (NCBI, Bethesda, MD).

Semiquantitative PCR analysis of the cadherin mRNA levels present in eutopic endometrium or endometriotic lesions.

Semiquantitative PCR was performed to determine whether levels of the mRNA transcripts encoding these six cadherin subtypes are differentially expressed in endometrial cancers and normal endometrium. The published sequences of the primers for amplification of cadherins, catenins or the housekeeping gene, GAPDH, were listed in Table 1 (MacCalman et al., 1997; Pishvaian et al., 1999; Mialhe et al., 2000).

To determine the optimum number of cycles required for the amplification of each cadherin and catenin subtype or GAPDH, an aliquot of first strand cDNA generated from either endometrial cancer or normal endometrium was amplified with the respective primers using an increasing number of PCR cycles (20-40). To avoid primer dependent artifacts, the reaction mixtures were denatured at 95°C for 5 min prior to the addition of the Taq polymerase (2.5 U Promega). The subsequent cycling programs consisted of denaturation at 95°C for 30 sec, annealing at 60°C for E-, P-, and N-cad and GAPDH, at 55°C for cad-6, cad-9, and cad-11 for 30 sec and extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min.

A linear relationship between the band intensity of the PCR products and the number of amplification cycles performed was observed. Based upon these observations, the optimum number of cycles for the amplification of each cadherin subtype and GAPDH, was determined to be 30 and 20, respectively. A PCR reaction in which the first strand cDNA was omitted served as a negative control for these studies. To avoid technical error, each PCR experiment has been done three times.

The PCR products were separated on 2% agarose gels, stained with ethidium bromide, and photographed using Polariod film. The photographs were scanned and digitized using a Hewlett Packer Digital Scanner. The intensity of the bands specific for each cadherin subtype or GAPDH was quantified using the Phoretix Gel Analysis Software Version 3.01 (NonLinear Dynamics, UK). The relative mRNA levels of each cadherin or catenin subtype in the endometrial cancer or normal endometrium obtained from the same patient were normalized to the corresponding GAPDH levels.

Statistical analysis

The results of semiquantitative RT-PCR are presented as the ratio of the mean relative

absorbance of each cadherin or catenin subtype to corresponding GAPDH for at least 3 independent experiments. Statistical differences between endometrial cancer and normal endometrium were assessed by the Mann-Whitney U test. Differences were considered significant for $p \leq 0.05$.

RESULTS

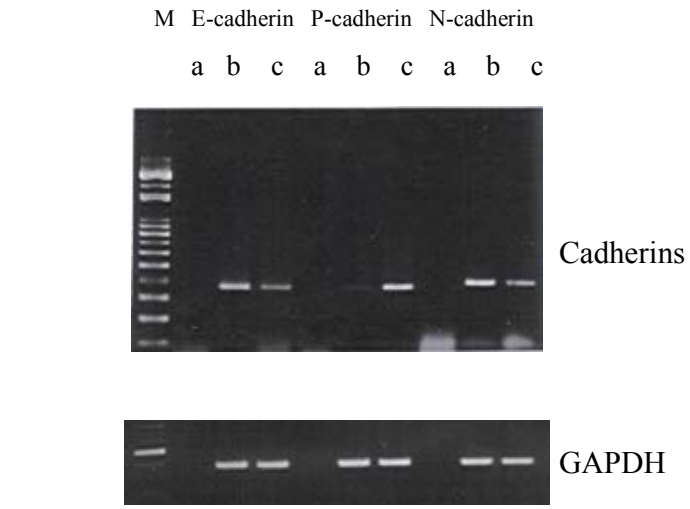
Table. Primers used for PCR in these studies

Gene	Primer	Sequence	Size
Cadherin-degenerate	5’	5’-GAATTCACNGCNCCNCCNTAYGA-3’*	180 bp
	3’	5’-GAATTCTCNGCNARYTTYTTRAA-3’*	
E-Cadherin	5’	5’-TCCATTTCTTGGTCTACGCC-3’	361 bp
	3’	5’-CACCTTCAGCCAACCTGTTT-3’	
P-cadherin	5’	5’-GACCAACGAGGCCCCTTTTGTGCTG-3’	357 bp
	3’	5’-GTGGTGGGAGGGCTTCCATTGTCCA-3’	
N-cadherin	5’	5’-GTGCCATTAGCCAAGGGAATTCAGC-3’	373 bp
	3’	5’-GCGTTCCTGTTCCACTCATAGGAGG-3’	
Cadherin-6	5’	5’-TTCTTGCTGCTCTTTTGGGT-3’	275 bp
	3’	5’-CCTGCTCCATCTCCTGAAAG-3’	
Cadherin-9	5’	5’-CAAAACCTGGGCAGTTGATT-3’	416 bp
	3’	5’-CCTCTTCAATGCAGCAAACA-3’	
Cadherin-11	5’	5’-ACCAGATGTCTGTGTCAGA-3’	742 bp
	3’	5’-GTCATCCTTGTCATCTGCA-3’	
α-catenin	5’	5’-CAGAGGGAGCATGACTTCGG-3’	290 bp
	3’	5’-CTACAGCAGCCACCAACTCT-3’	

β-catenin	5’	5’-AAGGTCTgGAGGAGCAGCTTC-3’	668 bp
	3’	5’-TGGACCATAACTGCAGCCTT-3’	
γ-catenin	5’	5’-ATGGAGGTGATGAACCTGATGG-3’	284 bp
	3’	5’-CCTGACACACCAGGGCACAT-3’	

* R = either A or G, Y = either C or T, N = either A, C, G, or T.

Fig. 1


M E-cadherin P-cadherin N-cadherin
a b c a b c a b c
Cadherins
GAPDH

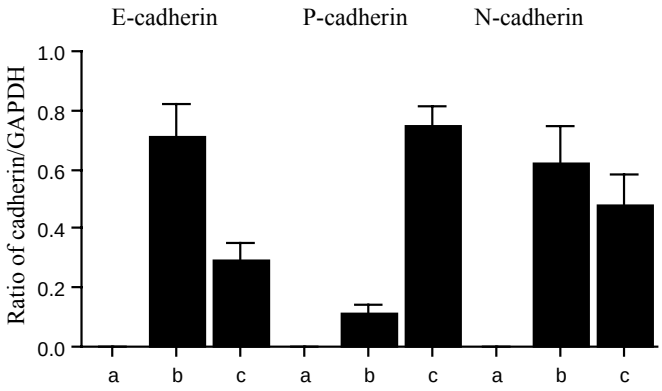

E-cadherin
P-cadherin
N-cadherin
Ratio of cadherin/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
a b c a b c a b c

Fig. 1. Comparison of the cadherin mRNA levels present in endometrial adenocarcinoma or normal endometrium obtained during early secretory phase of the menstrual cycle. Photographs of ethidium bromide-stained gels containing PCR products generated using primers specific for E-cad P-cad or N-cad at 361, 357 and 373 base pairs respectively. For each cadherin subtype, PCR products were obtained using template cDNAs generated from normal endometrium endometrium (lane b) or endometrial adenocarcinoma (lane c). A PCR reaction in which the first strand cDNA was omitted was performed as a negative control (lane a). Products amplified using GAPDH specific primers at 359 base pairs are also shown for each sample. DNA markers are presented on the left hand-side of each gel.

The absorbance values obtained for each cadherin subtype were normalized to the values obtained from the corresponding GAPDH. The results derived from this analysis as well as from two other independent experiments (photographs not shown) were standardized to the control. The values obtained from this study, as well as from 5 other studies are presented (mean $\pm$SEM; n=5) in the bar graphs (*$p < 0.05$).

Fig. 2

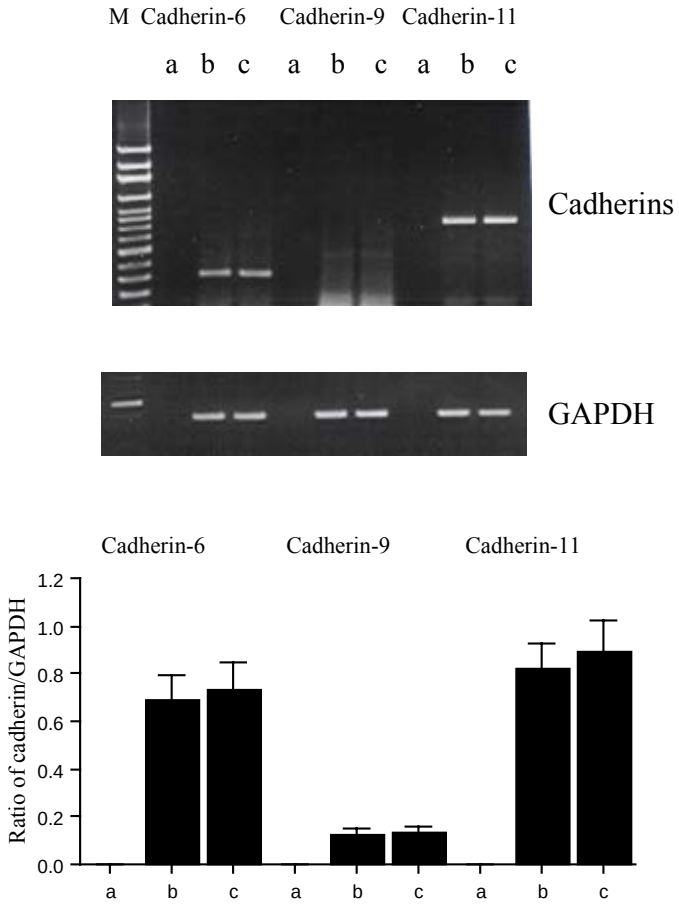
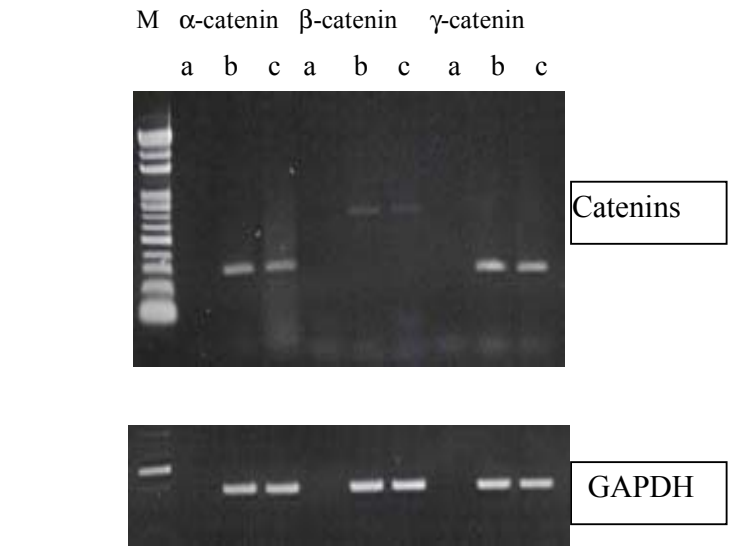

M Cadherin-6 Cadherin-9 Cadherin-11
a b c a b c a b c
Cadherins
GAPDH
Cadherin-6
Cadherin-9
Cadherin-11
Ratio of cadherin/GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a b c a b c a b c

Fig. 2. Comparison of the cadherin mRNA levels present in endometrial adenocarcinoma or normal endometrium obtained during early secretory phase of the menstrual cycle. Photographs of ethidium bromide-stained gels containing PCR products generated using primers for cad-6, cad-9 or cad-11 at 275, 416, and 742 base pairs respectively (Panel B). For each cadherin subtype, PCR products were obtained using template cDNAs generated from normal endometrium (lane b) or endometrial adenocarcinoma (lane c). A PCR reaction in which the first strand cDNA was omitted was performed as a negative control (lane a). Products amplified using GAPDH specific primers at 359 base pairs are also shown for each sample. DNA markers are presented on the left hand-side of each gel.

The absorbance values obtained for each cadherin subtype were normalized to the values obtained from the corresponding GAPDH. The results derived from this analysis as well as from two other independent experiments (photographs not shown) were standardized to the control. The values obtained from this study, as well as from 3 other studies are presented (mean $\pm$SEM; n=4) in the bar graphs (*$p < 0.05$).

Fig. 3


M α-catenin β-catenin γ-catenin
a b c a b c a b c
Catenins
GAPDH

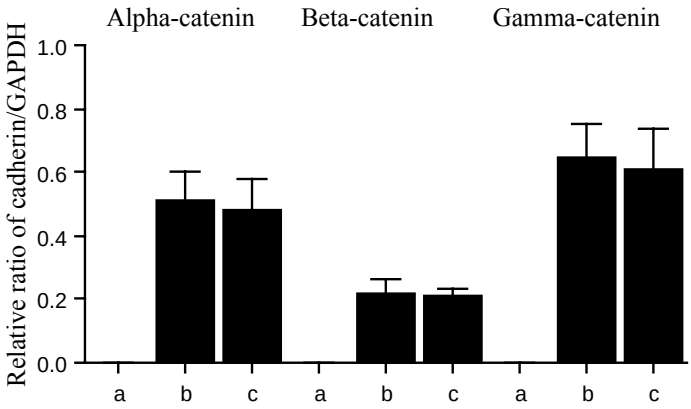

Alpha-catenin
Beta-catenin
Gamma-catenin
Relative ratio of cadherin/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
a b c a b c a b c

Fig. 3. Comparison of the catenin mRNA levels present in endometrial adenocarcinoma or normal endometrium obtained during early secretory phase of the menstrual cycle. Photographs of ethidium bromide-stained gels containing PCR products generated using primers for α-catenin, β-catenin, and γ-catenin (Panel B). For each catenin subtype, PCR products were obtained using template cDNAs generated from normal endometrium (lane b) or endometrial adenocarcinoma (lane c). A PCR reaction in which the first strand cDNA was omitted was performed as a negative control (lane a). Products amplified using GAPDH specific primers at 359 base pairs are also shown for each sample. DNA markers are presented on the left hand-side of each gel.

The absorbance values obtained for each catenin subtype were normalized to the values obtained from the corresponding GAPDH. The results derived from this analysis as well as from two other independent experiments (photographs not shown) were standardized to the control. The values obtained from this study, as well as from 3 other studies are presented (mean $\pm$SEM; n=4) in the bar graphs (*$p < 0.05$).

DISCUSSION

Multiple cadherin subtypes were detected in the endometrial cancers in aggrement with the cadherin subtypes previously identified in the glandular epithelial and stromal cells of the human endometrium or endometriosis in vivo and in vitro (MacCalman et al., 1997; Getsios et al., 1998, Chen et al 2002).

In general, the repertoire of cadherins present in normal endometrium and the levels of the mRNA transcripts encoding these CAMs were maintained in the endometrial cancer. However, there is a marked reduction in the expression of E-cad in the endometrial cancers. P-cad mRNA levels, in contrast to the other cadherin subtypes, were significantly higher in the endometrial cancers. To date, the role(s) of P-cad in the pathogenesis of endometrial cancers remain to be elucidated. Elevated levels of P-cad have also been associated with the progression of cancer. For example, P-cad becomes the predominant cadherin present in high-grade squamous intraepithelial lesions of uterine cervix (De Boer et al., 1999). Similarly, there appears to be an increase in P-cad expression and a concomitant decrease in E-cad levels during the progression of Barret's esophagus dysplasia to adenocarcinoma (Bailey et al., 1998). Collectively, these observations suggest that an increase in P-cad expression may

promote cellular invasion, a developmental process that is often associated with tumorogenesis (De Boer et al., 1999; Bailey et al., 1998) and endometriosis (van der Linden, 1996; Starzinski-Powitz et al., 1998; Scotti et al., 2000).

In summary, we have demonstrated that E-cad is increasing and P-cad is decreasing in endometrial cancer. Collectively, these studies suggest that P-cad may play a pivotal role in the development of endometrial cancer.

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