行政院國家科學委員會專題研究計畫　成果報告

典型鈣黏素(cadherin) 和 catenin 在卵巢漿液性囊腺癌與正常卵巢表面上皮的表現差異

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計畫主持人：陳澤昭

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中文摘要

卵巢黏液性囊腺瘤(ovarian mucinous cystadenoma)是常見的良性卵巢腫瘤，主要源生自卵巢表面上皮細胞，它可以長成巨大的囊腫。卵巢表面上皮細胞如何分化成卵巢黏液性囊腺瘤細胞，其真正機轉到目前為止還不清楚。鈣黏蛋白(cadherin)是一種存在於細胞膜上的細胞黏着分子(cell adhesion molecule)，其功能為細胞與細胞間的接合，以及在胚胎發育中主導不同組織及器官的形成，同時它與細胞的分化及腫瘤病變也有密切的關係。性腺賀爾蒙藉由其接受體來調控賀爾蒙依賴組織的生長。本研究利用 RT-PCR 和 DNA 定序，來找出所有存在於卵巢黏液性囊腺瘤的典型鈣黏蛋白。然後，再利用半定量 RT-PCR 來比較卵巢黏液性囊腺瘤與正常卵巢表面上皮細胞之間鈣黏蛋白 mRNA 的表現差異。另外也用半定量 RT-PCR 來比較性腺賀爾蒙接受體在卵巢黏液性囊腺瘤與正常卵巢表面上皮細胞之間的不同表現。我們確認卵巢黏液性囊腺瘤中有六種鈣黏蛋白，分別是 E-cadherin 、 P-cadherin 、 N-cadherin 、 R-cadherin 、 cadherin-6 和 cadherin-11。比較這六種鈣黏蛋白在卵巢黏液性囊腺瘤與正常卵巢表面上皮細胞的表現差異時，發現卵巢黏液性囊腺瘤中 E-cadherin 及 P-cadherin mRNA 的表現較正常卵巢表面上皮細胞明顯增加，而 N-cadherin、R-cadherin 及 cadherin-6 則明顯減少，但是 cadherin-11 則無差異。至於性腺賀爾蒙接受體的表現，無論是動情素接受體-α、動情素接受體-β、黃體素接受體或雄性素接受體在卵巢黏液性囊腺瘤中都比正常卵巢表面上皮細胞明顯增加。因此我們認

為，這六種鈣黏蛋白的消長可能是導致正常卵巢表面上皮細胞變成卵巢黏液性囊腺瘤的原因之一。也就是 E-cadherin 和 P-cadherin 的增加及 R-cadherin、N-cadherin 與 cadherin-6 減少，應該與卵巢黏液性囊腺瘤的形成有關。至於動情素接受體-α、動情素接受體-β、黃體素接受體或雄性素接受體的增加可能是卵巢黏液性囊腺瘤快速長大的主要因素之一。

ABSTRACT

Ovarian mucinous cystadenoma is a benign tumor originated from ovarian surface epithelium. It may grow to large dimensions quickly. However, the pathogenesis of this tumor remains unknown. The classical cadherins are cell adhesion molecules that regulate the morphogenesis of different tissues and steroid hormones regulate hormone-dependent cells through their receptors. In thess studies, six cadherins subtypes including E-cadherin, P-cadherin, N-cadherin, R-cadherin, cadherin-6 and cadherin-11 were identified in ovarian mucinous cystadenomas. In addition, we compared the messenger RNA levels of these cadherin subtypes in mucinous cystadenomas and normal ovarian surface epithelium by using semiquantitative RT-PCR. We found that the mRNA levels of E-cadherin and P-cadherin were significantly increased and N-cadherin, R-cadherin and cadherin-6 were significantly decreased in mucinous cystadenoma. The mRNA levels of cadherin-11 were similar between mucinous cystadenoma and normal ovarian surface epithelium. Furthermore, we also demonstrated that the mRNA levels of sex steroid receptors including estrogen receptor-α, estrogen receptor-β, progesterone receptor and androgen receptor were significant higher in mucinous cystadenomas than in ovarian surface epithelium. We speculate that the development of ovarian mucinous cystadenomas is mediated at least in part by up-regulation of the E-cadherin and

P-cadherin and down-regulation of the N-cadherin, R-cadherin and cadherin-6. The fast growth characteristic of this tumor is mediated at least in part by the over-expression of estrogen receptor-α, estrogen receptor-β, progesterone receptor and androgen receptor.

KEY WORDS

Ovarian mucinous cystadenoma, cadherin, steroid receptor, ovarian surface epithelium.

INTRODUCTION

Ovarian mucinous cystadenomas are the second common ovarian epithelial tumors. Previous studies have implicated that these tumors are originated from ovarian surface epithelial cells (OSE). However, the pathogenesis is not fully understood. 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). 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 (Takeichi, 1991, 1995; Potter et al., 1999). Ovarian cyst adenomas may grow up to a huge size in a short time, the mechanisms remain unknown. Gonadal steroids regulate the growth of hormone-dependent cells through their receptors. To date, little is reported regarding the steroid receptors in the mucinous cystadenomas.

In order to gain a better insight into the role(s) of the cadherins in the development of mucinous cystadenomas, we have performed a comprehensive examination of the cadherin subtypes present in these tumors 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 regulated in mucinous cystadenoma and normal OSE. We used the same strategies to compare steroid receptors including estrogen receptor-α (ER-α), estrogen receptor-β (ER-β), progesterone receptor (PR), and androgen receptor (AR).

MATERIALS AND METHODS

Tissues

Pathologically proved tissue samples of mucinous cystadenomas were obtained from patients (n=4; age range: 25-42 years) undergoing surgery for ovarian cysts. Tissue samples were used for determining cadherin subtypes using RT-PCR with degenerate primers for classical cadherins and all four samples were used for semiquantitative RT-PCR. Normal OSE were obtained from women (n=4, age range: 42-47 years) undergoing surgery for uterine myomas. 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.

Generation of first strand cDNA

Total RNA was extracted from the mucinous cystadenoma tissue samples and

OSE 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 mucinous cystadenomas

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

normal OSE and mucinous cystadenomas 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 mucinous cystadenomas and normal OSE

Semiquantitative PCR was performed to determine whether levels of the mRNA transcripts encoding these six cadherin subtypes are differentially regulated in mucinous cystadenomas and normal OSE. The published sequences of the primers for amplification of cadherins 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 subtype or GAPDH, an aliquot of first strand cDNA generated from either OSE and mucinous cystadenomas 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 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 35 and 25, 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 subtype in the mucinous cystadenomas or OSE 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 subtype to corresponding GAPDH for at least 3 independent experiments. Statistical differences between mucinous cystadenomas and OSE were assessed by the Kruskal-Wallis test. Differences were considered significant for $p \leq 0.05$.

RESULTS

Identification of the classical cadherins presented in mucinous cystadenomas

Six cadherin subtypes were detected in the total RNA extracts prepared from mucinous cystadenomas. Similarly, six cadherin subtypes were present in OSE.

Comparison of the cadherin mRNA levels present in mucinous cystadenomas and OSE

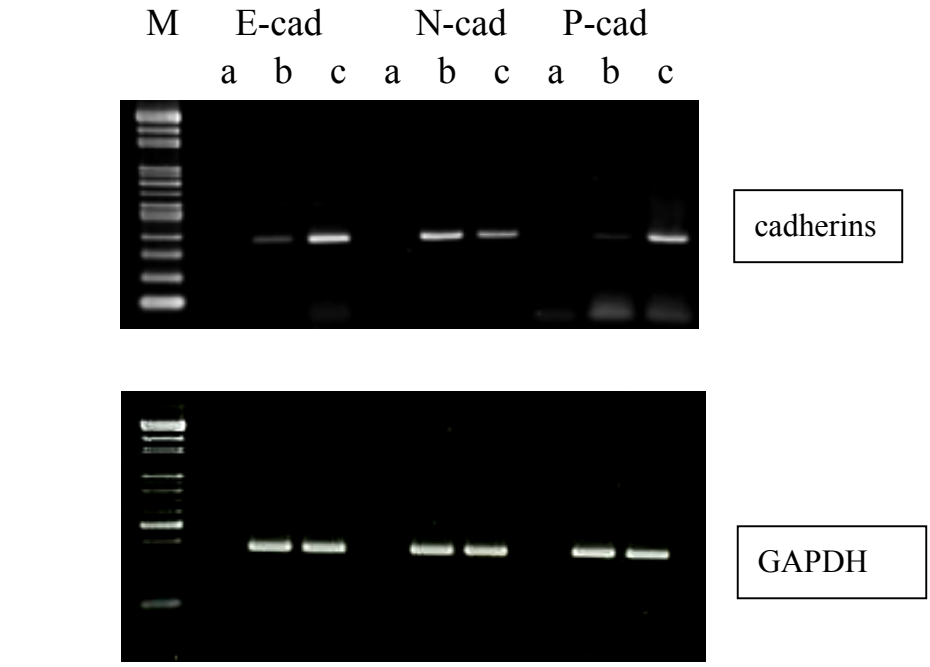
The mRNA levels of E-cad and P-cad were significantly increased and N-cad, R-cad and cad-6 were significantly decreased in mucinous cystadenoma. The mRNA levels of cad-11 were similar between mucinous cystadenoma and normal OSE. Furthermore, we also demonstrated that the mRNA levels of sex steroid receptors including ER-α, ER-β, PR and AR were significant higher in mucinous cystadenomas than in OSE.

Summary

Multiple cadherin subtypes were detected in the mucinous cystadenomas and normal OSE. These six subtypes were the same as previous reports. We speculate that the development of ovarian mucinous cystadenomas is mediated at least in part by up-regulation of the E-cadherin and P-cadherin and down-regulation of the N-cadherin, R-cadherin and cadherin-6. The fast growth characteristic of this tumor

is mediated at least in part by the over-expression of estrogen receptor-α, estrogen receptor-β, progesterone receptor and androgen receptor.

Fig. 1 The comparison of E-cad、N-cad 及 P-cad mRNAlevels in ovarian mucinous cystadenomas and OSE



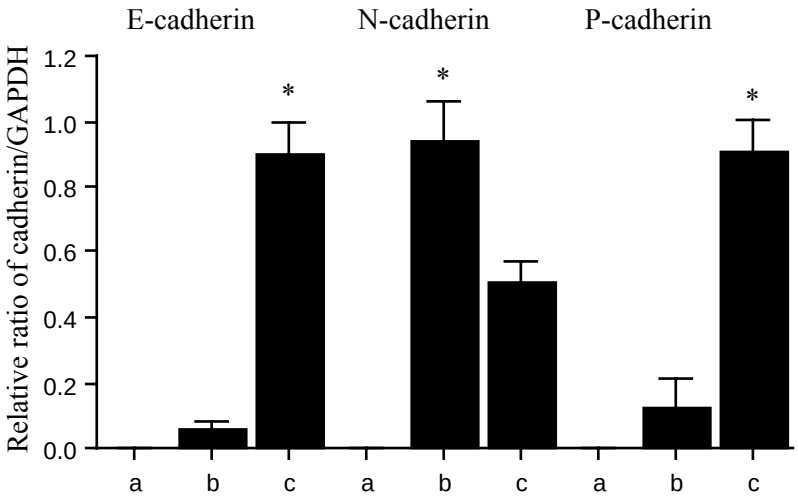
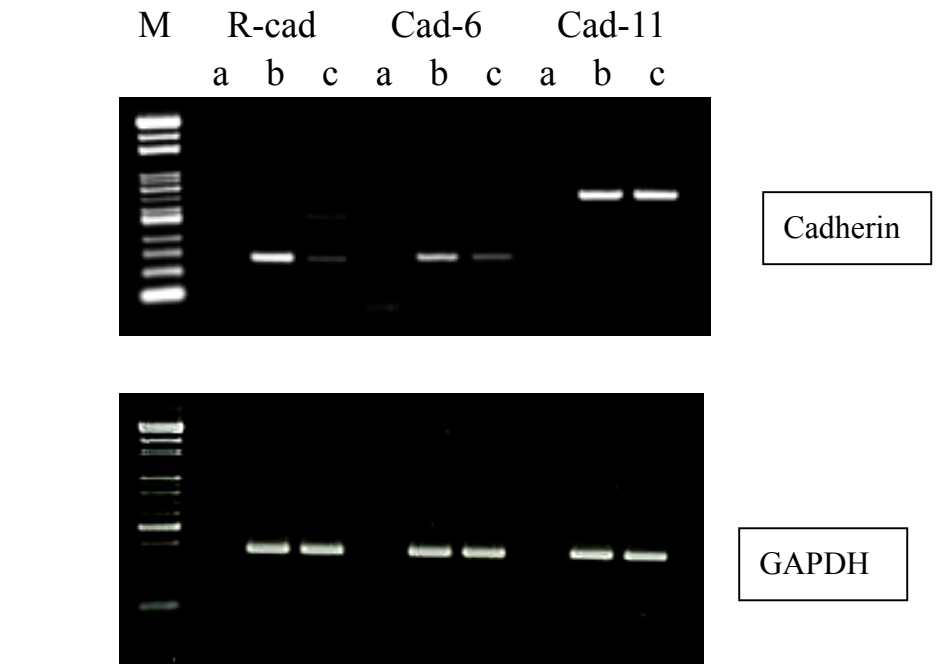


Fig 2. The comparison of R-cad, cad-6 and cad-11 mRNA levels in ovarian mucinous cystadenomas and OSE



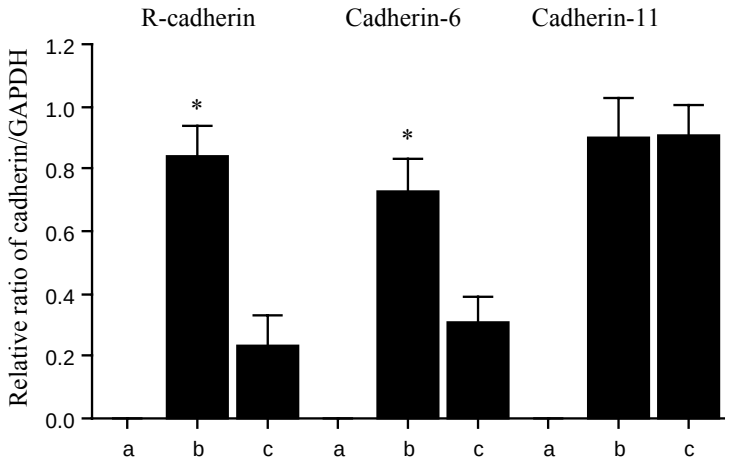


Fig. 3 The comparison of ER-α、ER-β and PR mRNA levels in ovarian mucinous cystadenomas and OSE

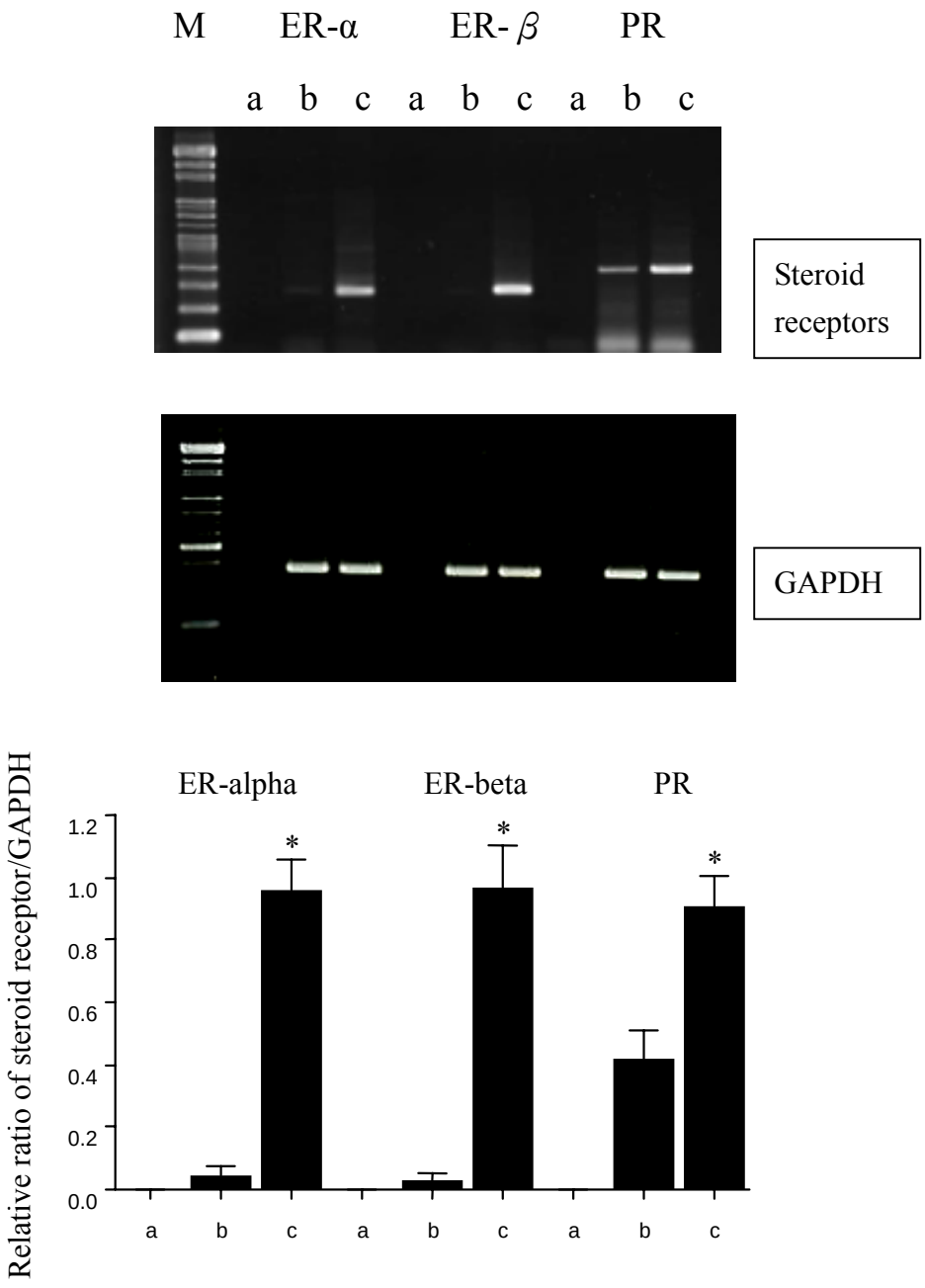
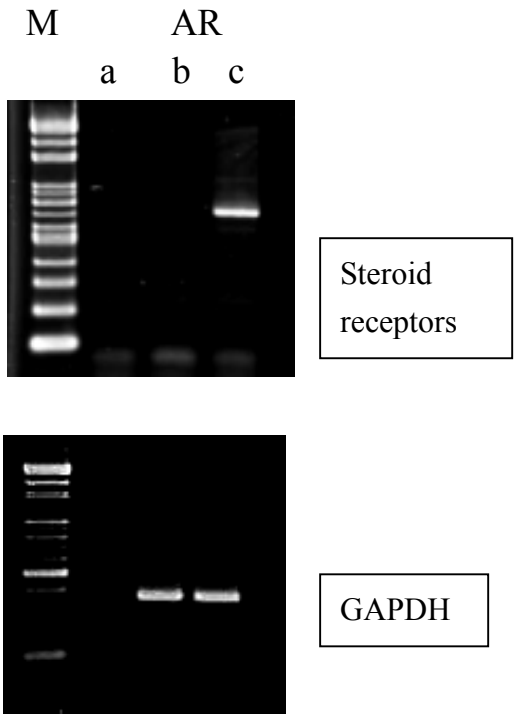


Fig. 4 The comparison of AR mRNAlevels in ovarian mucinous cystadenomas and OSE



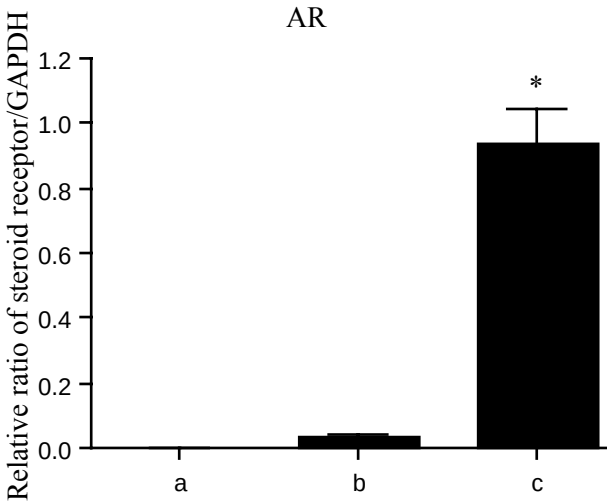


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Table 1: Primers for RT-PCR

Gene	Primer	Sequence	Size
Cadherin-degenerate	5’	5’-GAATTCACNGCNCCNCCNTAYGA*	180 bp
	3’	5’-GAATTCTCNGCNARYTTYTTRAA*	
E-Cadherin	5’	5’-TCCATTTCTTGGTCTACGCC	361 bp
	3’	5’-CACCTTCAGCCAACCTGTTT	
P-cadherin	5’	5’-GACCAACGAGGCCCCTTTTGTGCTG	357 bp
	3’	5’-GTGGTGGGAGGGCTTCCATTGTCCA	
N-cadherin	5’	5’-GTGCCATTAGCCAAGGGAATTCAGC	373 bp
	3’	5’-GCGTTCCTGTTCCACTCATAGGAGG	
Cadherin-6	5’	5’-TTCTTGCTGCTCTTTTGGGT	275 bp
	3’	5’-CCTGCTCCATCTCCTGAAAG	
Cadherin-9	5’	5’-CAAAACCTGGGCAGTTGATT	416 bp
	3’	5’-CCTCTTCAATGCAGCAAACA	
Cadherin-11	5’	5’-ACCAGATGTCTGTGTCAGA	742 bp
	3’	5’-GTCATCCTTGTCATCTGCA	
GAPDH	5’	5’-CCAGCCGAGCCACATCGCTC	359 bp
	3’	5’-ATGAGCCCCAGCCTTCTCCAT	

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