Differential Expression of *NUDT9* at Different Phases of the Menstrual Cycle and in Different Components of Normal and Neoplastic Human Endometrium

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SUMMARY

Objective: The human endometrium manifests different and distinct morphologies and physiologies during the different phases of the menstrual cycle. We aimed to determine which candidate genes demonstrate differential expression patterns in the endometrium during different phases of the menstrual cycle.

Materials and Methods: Using differential display reverse transcription polymerase chain reaction to compare day 5 and day 18 human glandular endometrium obtained by laser capture microdissection, we identified a specific gene, *NUDT9* (nucleoside diphosphate-linked moiety X motif 9). NUDT9 is known to function as a highly specific adenosine diphosphate ribose pyrophosphatase and has been mapped to chromosome 4q22.1. It gives rise to two alternatively spliced messenger RNAs, NUDT9α and NUDT9β, encoding a member of the Nudix hydrolase family. In this study, we purified NUDT9 protein and produced an antibody, which we then used for immunohistochemical studies.

Results: Using this anti-NUDT9 antibody, we successfully demonstrated that NUDT9 protein was differentially expressed in endometrial glandular cells at different phases of the menstrual cycle. NUDT9 was also found to be expressed more prominently in the epithelial glandular component than in the stromal component of human endometrial carcinomas.

Conclusion: We suggest that *NUDT9* may be involved in the regulation of the menstrual cycle and may be related to the proliferation of glandular cells in the human endometrium. [*Taiwan J Obstet Gynecol* 2009;48(2):96–107]

Key Words: ADP-ribose pyrophosphatase, endometrial carcinoma, endometrium, Nudix, NUDT9

Introduction

The endometrium of primates, including humans, is a complex heterogeneous structure comprising two major compartments, the glandular cells and the stromal cells, which respond differently to the same hormonal milieu [1]. Human endometrium undergoes significant changes during the menstrual cycle, from the early proliferative phase, through the late proliferative phase, ovulatory phase, early secretory phase, midsecretory phase and late secretory phase, to menstruation. The regulatory mechanisms involve hormonal, immunologic and genetic factors [2–6]. Differential display of eukaryotic messenger RNA (mRNA) using polymerase chain reaction (PCR) was devised in 1992 [7] and

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was used, combined with laser capture microdissection (LCM), by Torres et al in 2002 to study the endometrium of rhesus monkeys, and in human invasive endometrial carcinoma [1,8]. Our laboratory implemented a research project to identify genes that are differentially expressed in the proliferative endometrium, compared with the secretory endometrium. Various gene sequences were obtained, including *NUDT9* (nucleoside diphosphate-linked moiety X motif 9), which codes for an enzyme with pyrophosphatase activity using adenosine diphosphate ribose (ADPR) as its substrate [9–11]. It is an ADPR phosphatase that hydrolyzes ADPR to adenosine monophosphate and ribose-5′-phosphate. The *NUDT9* gene has two transcripts, NUDT9α (the dominant form, which contains the *N*-terminus leader sequence to the mitochondria) and NUDT9β (the aberrantly spliced form). The protein belongs to a superfamily of Nudix hydroxylases that catabolize potentially toxic compound within the cells [12]. It shares 39% sequence homology with the *C*-terminus cytoplasmic domain of the ADPR-gated calcium channel TRPM2 (transient receptor potassium channel 2) and is involved in the regulation of TRPM2 [13,14]. NUDT9 protein is expressed in the heart, skeletal muscles, liver, kidneys, and pancreas. However, its functions and expression are not yet fully understood [15]. In this study, we used immunohistochemistry to evaluate the expression profiles of NUDT9 in the endometrium during the proliferative and secretory phases, and in endometrial carcinoma.

Materials and Methods

Sample collection

Endometrial tissue fragments were obtained from surgical specimens of uteri removed because of benign uterine myoma. The patients, aged 30–50 years old (median, 42 years), had regular menstrual cycles without menometrorrhagia (indications for hysterectomy were pain or cancer risk). All patients had given birth to at least one child. In addition, for all patients, the pathology of the endometrium revealed no atypia, polyps, or cancer. Day 5 (proliferative phase, confirmed by pathologists) endometrium from six patients and day 18 (secretory phase, confirmed by pathologists) endometrium from four matched, controlled patients were obtained and stored at −80°C in Tissue-Tek OCT (Sakura Finetek Inc., Torrance, CA, USA) embedding compound prior to further processing. Paraffin sections of endometrioid-type endometrial carcinoma ($n = 12$), proliferative-phase endometrium ($n = 16$), and secretory-phase endometrium ($n = 14$) were also retrieved from the tissue bank for study.

LCM and total mRNA extraction

Cryostat sections were cut at −20°C at a thickness of 3–5 μm, placed on untreated plain glass slides, and immediately fixed in 75% ethanol prior to LCM. Slides were stained with hematoxylin and eosin using the following sequential solutions (10–30 seconds each): hematoxylin, distilled water, bluing reagent, 70% ethanol, 95% ethanol, and eosin Y. All solutions, including the stains, were supplemented with 0.5 U/μL of ribonuclease inhibitor (Promega, Madison, WI, USA). Slides were then dehydrated with two washings in 95% ethanol (10 seconds each) and two washings in 100% ethanol (10 seconds each). They were then washed twice in xylene (5 minutes each) and dried in a vacuum desiccator for 20 minutes prior to LCM. Glandular epithelial cells were harvested using a PixCell II LCM system (Arcturus Engineering, Mountain View, CA, USA), according to a protocol modified from Torres et al [1]. Tissue was collected on CapSure TF-100 caps (Arcturus Engineering) containing transfer film from two or three sections for each sample. Tissue samples from the same phase were pooled for subsequent analysis. RNA was extracted using a PicoPure RNA isolation kit and ExtracSure assembly in an alignment tray (Arcturus Engineering), following the manufacturer's recommendations. Briefly, the cap containing laser-captured cells was aligned in the ExtracSure assembly and filled with 10 μL of extraction buffer. After centrifugation to collect cell extract in a microcentrifuge tube, 10 μL of 70% ethanol was added to the cell extract and loaded onto a preconditioned RNA purification column. The cell extracts were then purified on the RNA purification column. The RNA was eluted in 11 μL of nuclease-free water and stored at −80°C [16].

Differential display reverse transcription PCR (DDRT-PCR)

Three micrograms of RNA and 3 μL H-T11A primer (2 μM) were mixed and the total volume was made up to 19.85 μL by adding diethyl pyrocarbonate-treated water. The solution was heated at 70°C for 10 minutes and then bathed in ice for 1 minute, followed by mixing with 6 μL of 5X buffer, 3.4 μL of 250 μM dNTP, and 0.75 μL of Maloney murine leukemia virus reverse transcriptase (200 U/μL) to give a total volume of 30 μL. The solution was incubated at 37°C for 60 minutes, then at 75°C for 5 minutes (to inactivate the reverse transcriptase). One microliter of complementary DNA (cDNA; produced by the above steps) was then used for PCR reaction with the following reagents: 25 μM dNTP, H-T11A primer (2 μM), random primers (HAP13–HAP24, 1 μL each, 2 μM; GenHunter Co., Nashville, TN, USA), 1 μL of 10X buffer, 0.1 μL of 1.25 U DNA

polymerase (Takara Bio Inc., Japan), 0.1 μL of α-^{35}S-deoxyadenosine triphosphate (temperature needed to be restored in oven before use), and 5 μL of water (to ensure PCR quality). The conditions for PCR were: 95°C for 5 minutes, then 40 cycles at 95°C for 30 seconds, 40°C for 2 minutes, 72°C for 30 seconds, then 72°C for 7 minutes. Gel electrophoresis was performed using 6% polyacrylamide DNA sequencing gels. The results were analyzed with PhosphorImager (Amersham Biosciences, Uppsala, Sweden). Reverse transcription PCR products that were differentially expressed in day 5 and day 18 endometrium were recovered, reamplified with PCR, inserted into pGEM-T Easy Vector (Promega) and sent for sequencing, according to methods modified from Bauer et al [17].

Sequence comparisons

Sequences obtained from DDRT-PCR were matched with known sequences in the GenBank (National Center for Biotechnology Information, USA) databases to determine if they were already known or were novel sequences.

Total mRNA extraction and preparation of NUDT9 cDNA by reverse transcription PCR

Tissues in RNA lysis buffer (Micro-to-Midi Total RNA Purification System, Invitrogen, Carlsbad, CA, USA) were disrupted using Kontes pestles (Fisher Scientific, Pittsburgh, PA, USA). Total mRNA was extracted, following the manufacturer's instructions: tissue lysate was centrifuged (15,000*g*, 3 minutes) and the cleared lysate was mixed with 700 μL of 70% ethanol. The reaction mixture was washed over an RNeasy mini column (Qiagen, Hilden, Germany), eluted using 50 μL of nuclease-free water, and stored at –80°C. Three micrograms of RNA and 3 μL of H-T11A primer (2 μM) were mixed and the total volume was made up to 19.85 μL by adding diethyl pyrocarbonate-treated water. The solution was heated to 70°C for 10 minutes and then bathed in ice for 1 minute, followed by mixing with 6 μL of 5X buffer, 3.4 μL of 250 μM dNTP, and 0.75 μL of Maloney murine leukemia virus reverse transcriptase (200 U/μL) to give a total volume of 30 μL. The solution was incubated at 37°C for 60 minutes, then at 75°C for 5 minutes (to inactivate the reverse transcriptase). Five microliters of cDNA (produced by the above steps) was added with 2 μL of forward primer with a *BamH*I site (5′-CAC TTG GGA TCC TCT GGT TCT AAT GGT TCC-3′), 2 μL reverse primer with a *Hind*III site (5′-CAC TTG AAG CTT TCC ACT GCT ATC CCT TTT-3′), 2 μL dNTP (25 μM), 0.5 μL DNA polymerase (TaKaRa, 1.25 U), 6.5 μL water (for PCR quality), and 2 μL 10X buffer. PCR conditions were 95°C for 5 minutes, then 35 cycles at 95°C for 45 seconds, 50°C for 45 seconds, 72°C for 1 minute, then 72°C for 10 minutes. A gene product anticipated to be *NUDT9* was recovered and PCR was repeated. The final product was analyzed in an ethidium bromide-containing 1% agarose gel and purified using a Viogene gel extraction kit, following the manufacturer's instructions.

Ligation

An insert sequence of *NUDT9* was recovered from the 1% agarose gel embedded with the PCR product from the previous section treated with *Bam*HI and *Hind*III, using a QIAquick gel extraction kit (Qiagen). pET32a plasmids were also treated with *Bam*HI and *Hin*dIII and acted as the vector DNA. The insert DNA was mixed with the vector DNA at a ratio of 3:1 and then admixed with T4 DNA ligase, 10X buffer, and double distilled water at 16°C for 16 hours, as instructed by the manufacturer (Promega).

Preparation of competent cells

Escherichia coli JM109 cells are endonuclease-deficient (*endA*$^-$) and recombination-deficient (*recA*$^-$), and were selected for our experiments. Two microliters of JM109 cell cultures were incubated overnight and transferred to 50-mL cultures for further incubation at 37°C, until the absorbance at 600 nm was 0.6–0.8. The *E. coli* cultures were centrifuged at 3,000 rpm (Beckman rotor JA-14; Beckman Coulter, Fullerton, CA, USA) for 15 minutes, and the supernatant was removed using aseptic techniques. The pellet was mixed in ice with 45 mL of sterile titration buffer (100 mM $CaCl_2$ buffer) for 60 minutes. Further centrifugation was performed at 3,000 rpm for 15 minutes. The supernatant was again removed. The pellet was admixed with 1 mL of titration buffer. Glycerol was added to a final concentration of 40%. Pellets were stored at –80°C.

Transformation

Ten microliters of the pET32a/NUDT9 ligation product was well mixed into 50 μL of competent cells and placed on ice for 30 minutes, followed by heat shock in a water bath at 42°C for 2 minutes 45 seconds, followed by immediate placing on ice for 5 minutes. The cells were cultured in 800 μL of Luria-Bertani medium for 2 hours. Two hundred-microliter cultures were inoculated on plates containing ampicillin (100 μg/mL) for further culture at 37°C for 16–18 hours. Positive clones were subsequently screened.

Preparation of NUDT9 protein extracts

Three milliliters of *E. coli* strain BL21 (DE3) cells containing pET32a/NUDT9 were inoculated at 1:100 into

400 mL of LB medium containing ampicillin (100 mg/mL). When the absorbance at 600 nm reached 0.8–1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the remaining cultures to 1 mmol/L of final concentration, and expression induction (250 rpm, 37°C) was continued for a further 4 hours. The cultures were centrifuged at 10,000 rpm (Beckman rotors JA-14 and JA-17) for 10 minutes each. Cultures were suspended in lysis buffer (20 mM Tris-HCl, 1 mM phenylmethanesulphonylfluoride, 0.3 mM NaCl, 10 mM imidazole) and then processed using an SLM Aminco French pressure cell press (Model FA-030; SLM Aminco, Urbana, IL, USA) 6–8 times. The broken cells were then centrifuged at 10,000 rpm (Beckman rotor JA-17) for 10 minutes. The supernatant was further centrifuged at 56,000 rpm (Beckman rotor Ti90) for 1 hour. The pellets were discarded and the supernatant recovered. NUDT9 fusion protein was now available for further analysis.

Purification of fusion protein by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography

Ni-NTA resin was pre-balanced with buffer (20 mM Tris-HCl, 200 mM NaCl, 10 mM imidazole). Four hundred milliliters of protein extracts was mixed with 1 mL of Ni-NTA resin (Qiagen) and purified through the Ni-NTA column (10 cm long and 1 cm in diameter) using wash buffer (20 mM Tris-HCl, 300 mM NaCl, 25 mM imidazole) and elution buffer (20 mM Tris-HCl, 300 mM NaCl, 500 mM imidazole), according to the manufacturer's instructions. Every 1 mL of eluted fluid was collected in a tube and a total of 9 mL was obtained. Tubes were marked as E1–E9. The resulting samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Quantification of NUDT9 protein

Concentration of NUDT9 protein extracts was determined after standardization with bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA) stained with Bradford dye. Briefly, 0, 4, 8, 12, 16, 20 or 24 μL of BSA (0.5 mg/mL) was added to 800 μL of ddH_2O. The absorbance at 595 nm was determined and a standard curve for BSA was generated. The concentrations of the NUDT9 protein extracts were then determined according to the BSA curve.

Preparation of rabbit anti-NUDT9 polyclonal antibody

Purified NUDT9 protein was used as antigen at a concentration of 1 mg/mL and mixed with Freund's complete adjuvant (Sigma-Aldrich) at a ratio of 1:1 (v/v), following the manufacturer's instructions. Then, 300–500 μL was injected subcutaneously into rabbits at four sites. The booster antigen was made by mixing purified NUDT9 fusion protein (0.5 mg/mL) with Freund's incomplete adjuvant (Sigma-Aldrich) at a ratio of 1:1 (v/v). Booster injections were given every other week using the same dosage and same method of administration as the initial injection.

Western blotting

Western blotting of cloned pET32a/NUDT9 was performed according to the local standard protocol in our laboratory, using 10% SDS-PAGE, with polyvinylidene-fluoride as the transfer membrane. Rabbit anti-NUDT9 antibody was used as the first antibody, with horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody (modified from Burnette et al [18] and following the instructions of the manufacturers, PerkinElmer and Sigma-Aldrich). The SuperSignal West Pico chemiluminescent substrate kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used, and detection was performed using a LAS-1000 luminescent image analyzer (Fujifilm, Tokyo, Japan). In addition, NUDT9 protein expression in various cell lines, including A549 (lung carcinoma cell), CH27 (human lung squamous carcinoma cell), H460 (human lung non-small cell carcinoma cell), Hep3B (human hepatoma cell line), Huh7 (human hepatoma cell line), HUVEC (human umbilical vein endothelial cells), OK (renal epithelial cell line), RK3E (rat kidney epithelial cells), THP1 (human monocytic cell line) and RVSMC (rat vascular smooth muscle cell), was also analyzed by Western blotting, using similar procedures and protocols to those listed above.

Immunohistochemical staining of NUDT9 in proliferative-phase endometrium, secretory-phase endometrium, and endometrial carcinoma

Immunostaining was performed using the streptavidin-biotin-peroxidase method [19,20]. Briefly, 5–6-μm thick sections were cut from each paraffin block and placed onto slides. The specimens were then deparaffinized in xylene and rehydrated in graded alcohols and distilled water. The endogenous peroxidase was consumed by immersing the sections in 3% hydrogen peroxide in absolute methanol for 20 minutes. Pressurized steam heat was applied to the slides while in a 10 mM citrate buffer solution (containing 0.1% nonylphenoxypolyethoxyethanol), pH 6.0, for 30 minutes. Nonspecific secondary antibody binding was blocked by incubation in 5% normal goat serum for 30 minutes. Affinity-purified rabbit anti-NUDT9 antibody, at a dilution of 1:100, was then applied for 90 minutes. The primary antibody was washed off with phosphate-buffered saline (PBS). The slides were then incubated with biotinylated

Table 1. Genes with differential expression profiles in the human endometrium at different phases of the menstrual cycle (day 5–18)

Gene locus	Protein	Chromosome locus	Size of sequences obtained (bp)	Homology with human loci (%)
SPG4	Spastin	2p21–22	798 (sequence 1) 846 (sequence 2)	98 (sequence 1) 98 (sequence 2)
NUDT10	NUDT10	Xp11.23	643	98
TMOD3	Tropomodulin 3	15q21.1–21.2	1,393	99
PLSCR4	Phospholipid scramblase 4	3q24	505	93
NUDT9	NUDT9	4q22.1	664	96

goat anti-rabbit IgG antibody (1:30), followed by streptavidin-peroxidase (1:30) for 20 minutes each. The chromogen 3,3′-diaminobenzidine tetrahydrochloride was then applied and reacted with the sections for 10 minutes. After washing in PBS, the sections were lightly counterstained with hematoxylin, washed in xylene, and coverslips were applied.

Immunofluorescence by confocal microscopy

Rabbit anti-NUDT9 antibody was mixed with pET32a to inhibit nonspecific binding prior to the experiment. The rabbit anti-NUDT9 antibody was then titrated to a titer of 1:100 and was incubated with the slides coated with A549 (lung carcinoma cell line), Huh7 (human hepatoma cell line) or HUVEC (human umbilical vein endothelial cells) cells. The three cell lines were provided by one of the coauthors (L.Y.C.). The slides were prepared at 37°C for 60 minutes, then washed with 1X PBS for 10 minutes at least three times. Titrated fluorescein isothiocyanate-conjugated secondary antibody at a titer of 1:200 was then incubated with the slides at 37°C for 30 minutes in darkness. The slides were washed with 1X PBS for 10 minutes, at least three times. The slides were mounted in a mixture of PBS and glycerol (1:1, v/v) and were observed under a laser confocal microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Nonparametric tests were chosen because of the small sample size and an inability to assume that the data followed a normal distribution. Fisher's exact test was used to compare the intensity of staining between secretory-phase endometrium and proliferative-phase endometrium. The Mann-Whitney test was used to compare the degree of staining of epithelial cells in endometrial cancers with that of the stromal cells in endometrial cancers. A p value < 0.05 was considered to be statistically significant.

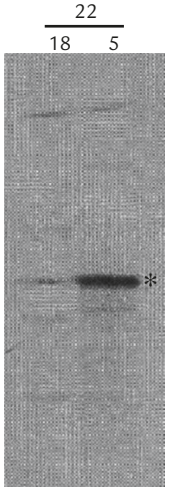

Figure 1. Levels of expression of NUDT9 in day 5 and day 18 endometrium were found to be markedly different, using differential display reverse transcription polymerase chain reaction (PCR). PCR of complementary DNA generated by reverse transcription of total messenger RNA extracted from day 5 (lane 5) and day 18 (lane 18) endometrium revealed a much higher expression of this PCR product in day 5 endometrium (*) than in day 18 endometrium.

Results

Sequences found by DDRT-PCR

DDRT-PCR revealed 26 sequences that showed marked differences in expression between day 5 and day 18 endometrium. Twelve out of the 26 sequences were later found to be the results of contamination by mitochondrial DNA. We were unable to find matches in the GenBank database for five of the 26 sequences, and three of the 26 sequences were reported cDNA sequences with unknown functions. The remaining six sequences were matched to well-documented gene sequences (Table 1). We concentrated on one of these six sequences that was much more strongly expressed in day 5 endometrium than in day 18 endometrium (Figure 1). The sequence of the PCR product was

determined to be: 5′-TGCATCCAACGCGTTGGGAG-CTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAA-TTCACTAGTGATTAAGCTTACCAGGTGAGATAATGG-ATAATCTTATGCTAGAAGCTGGAGATGATGCTGGAA-AAGTGAAATGGGTGGACATCAATGATAAACTGAAG-CTTTATGCCAGTCACTCTCAATTCATCAAACTTGTG-GCTGAGAAACGAGATGCACACTGGAGCGAGGACT-CTGAAGCTGACTGCCATGCGTTGTAGCTGATGGTC-TCCGTGTAAGCCAAAGGCCCACAGAGGAGCATACT-GAAAAGAAGGCAGTATCACAGAATTTATACTATA-AAAAGGGCAGGGTAGGCCACTTGGCCTATTTACTT-TCAAAACAATTTGCATTTAGAGTGNTTCGCATCAGA-ATAACATGAGTAAGATGAACTGGAACACAAAAT-TTTCAGCTCTTTGGTCAAAAGGAATTTAAGTAAT-CATATTTTGGATGGATTCGAATTAAGCATGGCTTAA-AATAATTTAAACCACTATGCTCTTTGAGATCATTA-TCAGATAAAGATAAATCTTGATCAGCTTAAAAAAA-AAAGCTATCGATTCCGCGCGCATGCGCGACATGCA-CGTCGGGCCATCGCCTNNGGAGCGATTCCATTACT-GGCCGCGTTTCACC-3′. The sequence was found to correspond to human *NUDT9* by a GenBank search, with a homology of 96% by Blast2 comparison. The other five sequences included two from spastin (*SPG4*), one from scramblase 4, one from tropomodulin 3, and one sequence from NUDT10 (Table 1).

Purification of NUDT9 fusion protein

Primers designed for PCR amplification of the endometrial tissue extracts needed to avoid the hydrophobic amino acids and also needed to contain suitable recognition sites for the restriction enzymes *Bam*HI and *Hin*dIII (Figures 2A and 2B). The size of the PCR product was 400 bp (Figure 2C). Induction of expression in *E. coli* carrying the pET32a/NUDT9 by IPTG revealed a 33-kDa protein (Figure 3) which was compatible with the size expected (14-kDa NUDT9 plus 19-kDa thioredoxin of pET32a). The extracts were then purified by Ni-NTA affinity chromatography and blotted by SDS-PAGE.

Titer in rabbit sera

The titer of the rabbit anti-NUDT9 rose to 1:27,000 at 5 weeks after immunization, but was weaker at 1:81,000 at 7 weeks, and remained at 1:81,000 at 9 weeks after immunization (Figure 4). Large amounts of anti-sera were collected at 9 weeks after immunization for future

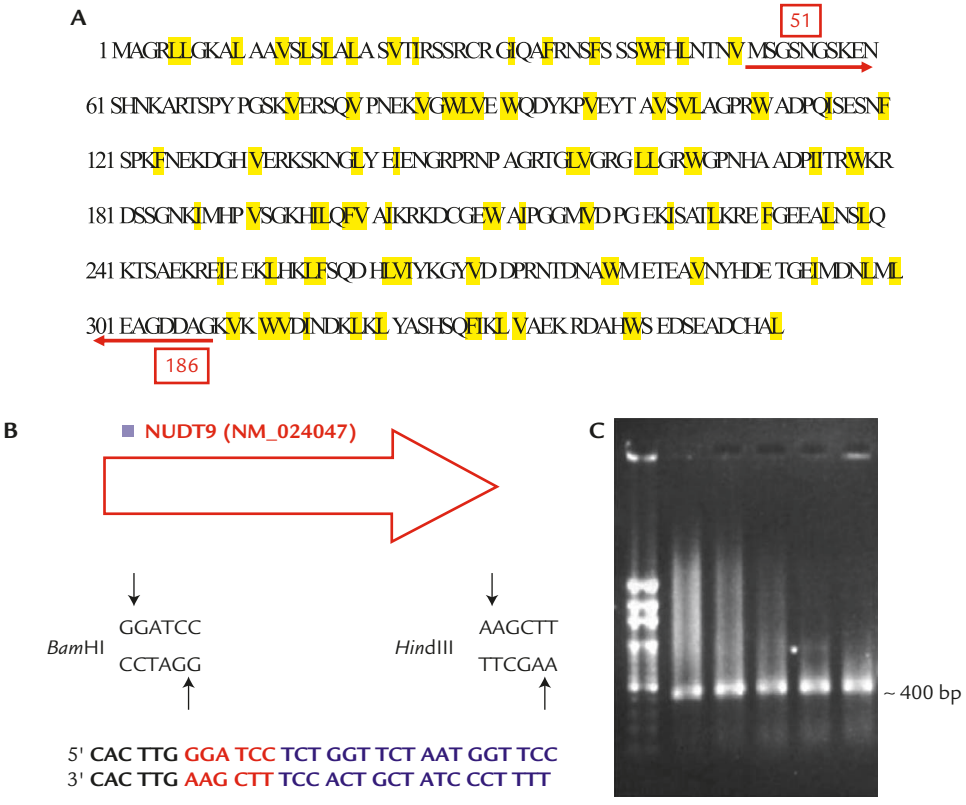

Figure 2. (A) Amino acid sequence of NUDT9. The hydrophobic amino acids are marked in yellow. The cloned segment of the NUDT9 peptide included the amino acids between 51 (5′-NH_2) and 186 (3′-COOH). (B) Diagram of the *NUDT9* gene and the design of primers, including a *Bam*HI recognition site (GGATTC) in the 5′ end and a *Hin*dIII recognition site in the 3′ end (AAGCTT). (C) The polymerase chain reaction product of the segment of the *NUDT9* gene being amplified and cloned thereafter. Each lane denotes a sample of endometrium extracts. A 400-bp DNA fragment was noted in each lane, a size compatible with the predicted size of the cloned segment.

use. To confirm the antigen–antibody relationship, the anti-sera were used to neutralize the NUDT9 fusion protein. Western blotting showed disappearance of the 33-kDa signal, indicating that the anti-sera did contain the specific rabbit polyclonal antibody for the NUDT9 fusion protein (data not shown).

Immunohistochemical study of NUDT9 in human endometrium

We found a marked increase in the expression of NUDT9 in the proliferative-phase endometrium compared with the secretory-phase endometrium (Figure 5). Fourteen out of 16 proliferative-phase endometrial samples were categorized as ≥ 2+, compared with only three out of the 14 secretory-phase endometrium samples ($p < 0.001$ using Fisher's exact test if categorized as a 2×2 table; see Tables 2 and 3). The expression of NUDT9 protein was also much more prominent in glandular carcinoma cells than in noncancerous stromal cells in human endometrial carcinoma (Figure 6). Table 4 shows the percentages of positively-stained cells in both the glandular and stromal samples. Two hundred glandular and 200 stromal cells were counted in each slide (total of 12 slides and 4,800 cells counted). A significantly higher percentage of NUDT9-positive staining was noted in the glandular cells, compared with the stromal cells ($p < 0.05$).

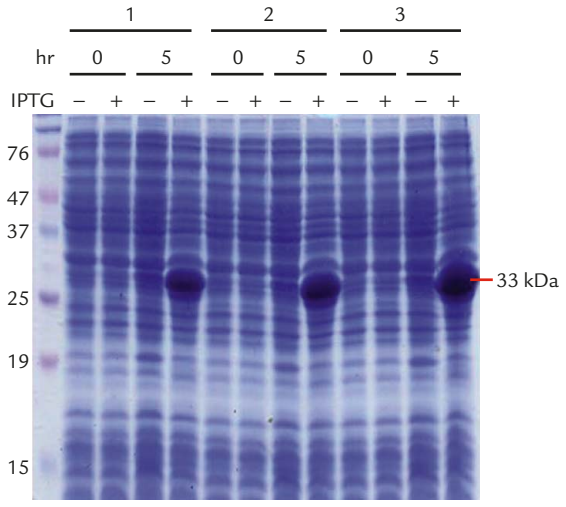

Figure 3. Western blotting of the NUDT9 fusion protein, showing the results of the three batches of samples (samples 1–3) stained with Coomassie blue to highlight the NUDT9 fusion protein. Addition (+) or non-addition (–) of the inducer, isopropyl-β-D-thiogalactopyranoside (IPTG), at 0 and 5 hours of culture revealed a 33-kDa product in the pET32a cloning system cultured for 5 hours after IPTG induction.

Immunofluorescence study of NUDT9 in three cell lines

Western blotting of NUDT9 expression in many noncancerous and cancerous cell lines revealed that all the cell lines studied expressed NUDT9 (Figure 7).

Immunofluorescence analysis using a fluorescein isothiocyanate-conjugated secondary antibody recognizing the rabbit anti-NUDT9 antibody was performed in A549 (lung carcinoma cell line), Huh7 (human hepatoma cell line), and HUVEC (human umbilical vein endothelial cells) cell lines, with the aid of confocal microscopy. NUDT9 protein was expressed both in the nucleus and in the cytoplasm (Figure 8). The nuclear expression was much more prominent than the cytoplasmic expression in HUVEC cells.

Discussion

Human endometrium undergoes cyclic changes marked by repeated proliferation and breakdown during the nongestational reproductive period in females. When

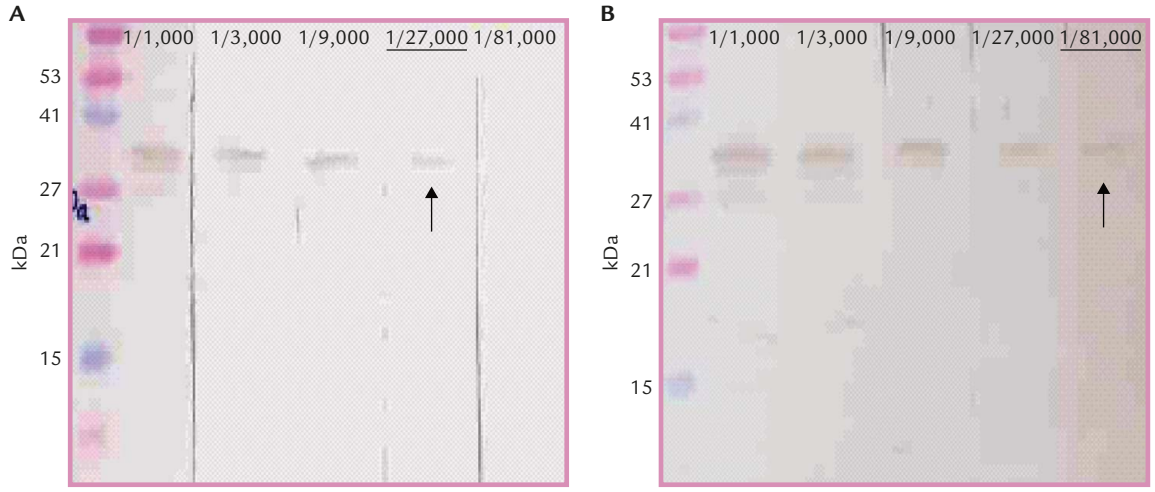

Figure 4. Titers of rabbit anti-sera at different time points. (A) At the first sampling (5 weeks after immunization), the titer was 1:27,000. (B) At the second sampling (7–9 weeks after immunization), the titer was weaker at 1:81,000.

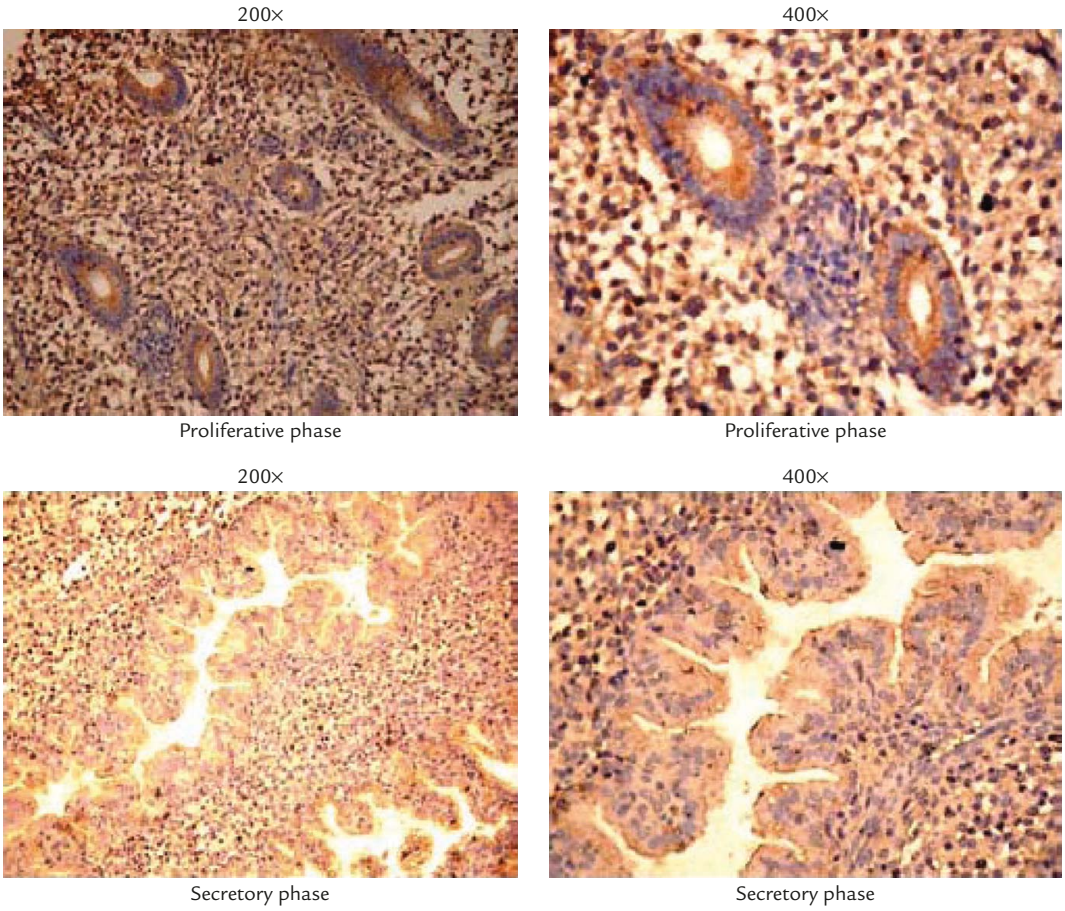

Figure 5. Immunohistochemical staining of proliferative- and secretory-phase endometrium. NUDT9 expression was higher in the proliferative-phase endometrium than in the secretory-phase endometrium.

Table 2. Distribution of different immunohistochemistry (IHC) intensities after staining with anti-NUDT9

	IHC category				
	0	1+	2+	3+	Total
Secretory-phase endometrium	6	5	3	0	14
Proliferative-phase endometrium	0	2	8	6	16

Table 3. Distribution of different immunohistochemistry (IHC) intensities after staining with anti-NUDT9 (further categorization)*

	IHC category	
	0 or 1+	2+ or 3+
Secretory-phase endometrium	11	3
Proliferative-phase endometrium	2	14

*$p < 0.001$ by Fisher's exact test.

necessary, it can be transformed into an environment suitable for implantation and maintenance of pregnancy, but the mechanisms involved are still not completely understood. A better understanding of the human endometrium may lead to breakthroughs in tackling problems such as implantation failure, early pregnancy loss, the safety of hormone replacement therapy, and even tumorigenesis/carcinogenesis. We, therefore, aimed to identify any genes with differential expression during the menstrual cycle. Of the potentially available methods including cDNA microarray, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and DDRT-PCR, we chose DDRT-PCR because it is cheaper, easier to perform, and less equipment-dependent [7,17,21]. LCM was used to ensure that the cells used for the extraction of mRNA were endometrial glandular cells, rather than a mixture of glandular and stromal cells. This is a critical step when trying to elucidate trivial differences in gene expression patterns [16]. No differences in expression of the usual markers of cell division and proliferation, such as Ki-67 and proliferative cell nuclear antigen [22,23], were found between day 5 and day 18 endometrium by DDRT-PCR in our study. However, we did find several genes that were differentially expressed in day 5 and day 18 human endometrium, including the human

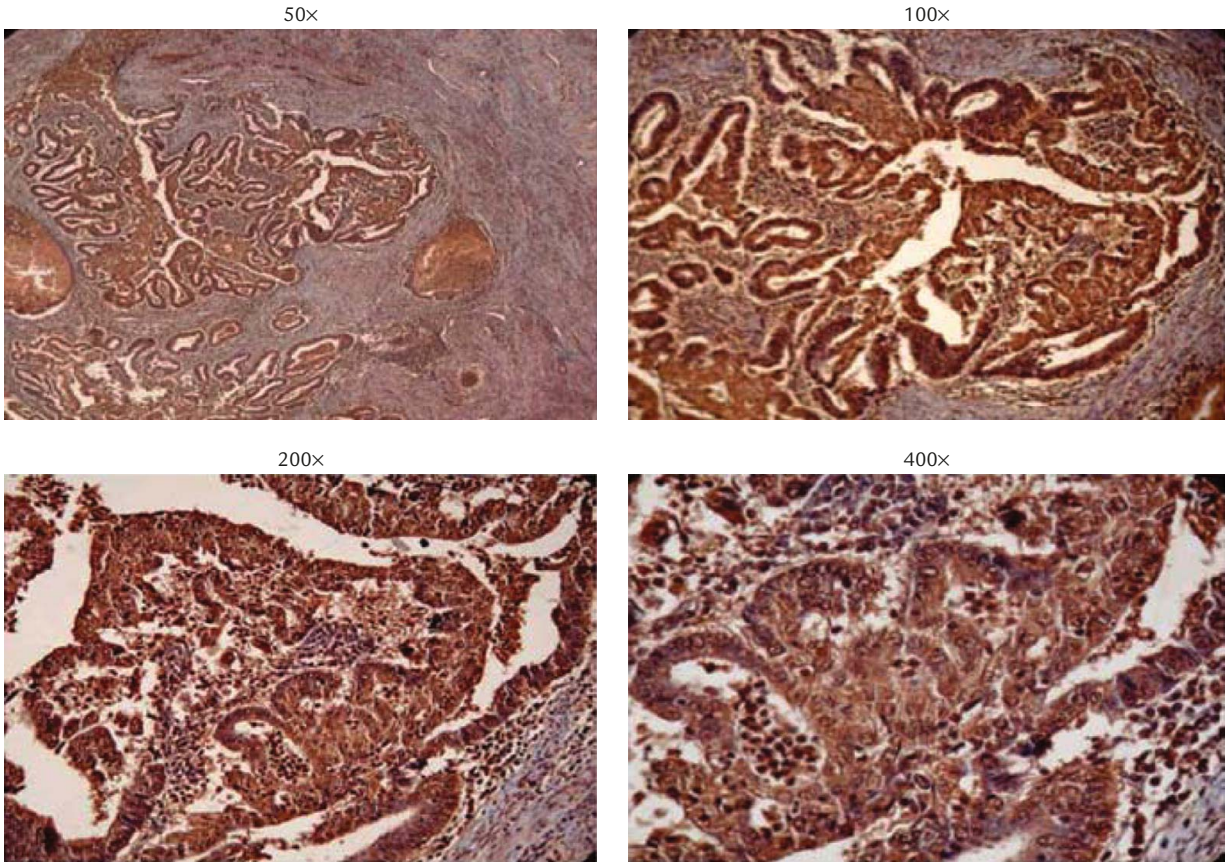

Figure 6. Immunohistochemical staining of sections of human endometrial carcinoma revealed higher NUDT9 expression in the glandular cells than in the stromal cells. Strong staining of NUDT9 protein was noted in the nuclei of the tumor cells.

Table 4. Distribution of NUDT9-positive cells and their relative abundances in the glandular and stromal compartments of human endometrial carcinoma specimens

Relative abundance	Distribution of NUDT9-positive cells (%)						
	0	1–5	6–25	26–50	51–75	76–100	Total
Glandular	0	0	0	1	3	8	12
Stromal	1	4	7	0	0	0	12

NUDT9. The presence of polymorphisms or sequencing errors hidden in the sequences could account for the fact that the homology between the sequences obtained in this study and the reported human gene sequences were only 93–99%, rather than 100%.

Human *NUDT9* maps to chromosome 4q22.1 and has two alternatively spliced mRNA transcripts: NUDT9α and NUDT9β. The protein is a member of the Nudix hydroxylase family (similar to yeast YSA1 and mouse Nudt5) and is able to hydrolyze ADPR to adenosine monophosphate and ribose-5-phosphate [11,24]. ADPR is a product of nicotinamide adenine dinucleotide hydrolysis and a breakdown product of the calcium-released second messenger cyclic ADPR [9,14].

The NUDT9α product is a full-length protein with a mitochondrial leader sequence and is expressed in the heart, liver, skeletal muscles, kidneys, and pancreas. The NUDT9β protein, however, lacks the *N*-terminal mitochondrial sequence and an intron, when compared with the NUDT9α protein [15]. Free ADPR is regarded as a toxic substance within the cell, but may be involved in the regulation of TRPM2, a transient calcium-permeable cation channel. TRPM2 is gated by oxidative and nitrosative stress. Its NUDT9 homology domain is critical for its activation by ADPR [12]. A previous study revealed that splicing variants of TRPM2, lacking some 34-amino acid residues, were insensitive to ADPR. Besides, if nicotinamide adenine dinucleotide hydrolysis was inhibited, or specific enzymes cleaved ADPR, the gating of TRPM2 was also suppressed. Such findings indicate an intimate relationship between NUDT9 and TRPM2 [13,14].

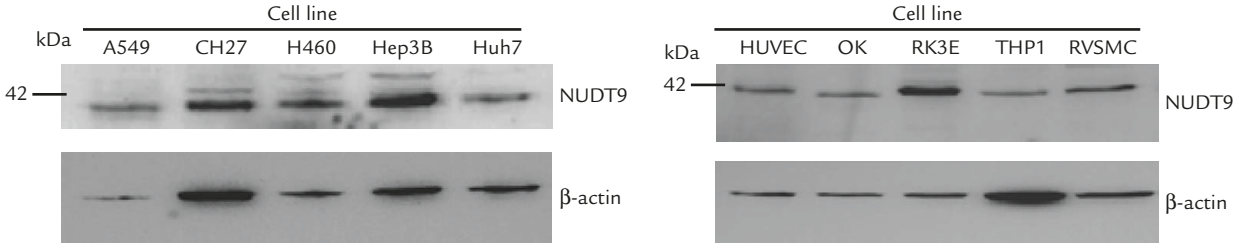

Figure 7. Expression of NUDT9 by Western blotting in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the cell lines: A549 (lung carcinoma cell), CH27 (human lung squamous carcinoma cell), H460 (human lung non-small cell carcinoma cell), Hep3B (human hepatoma cell line), Huh7 (human hepatoma cell line), HUVEC (human umbilical vein endothelial cells), OK (renal epithelial cell line), RK3E (rat kidney epithelial cells), THP1 (human monocytic cell line), and RVSMC (rat vascular smooth muscle cells).

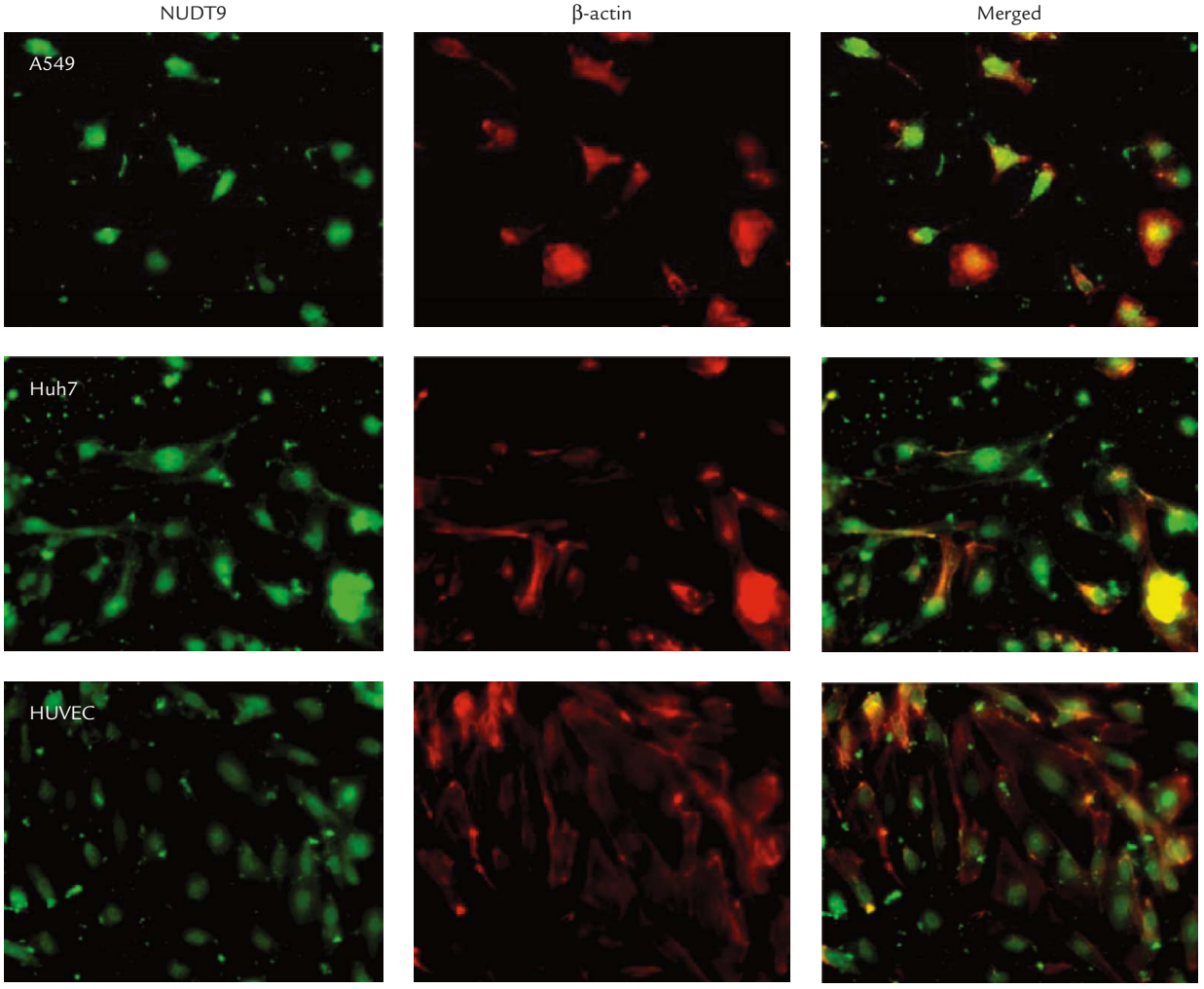

Figure 8. Immunofluorescence analysis of NUDT9 in three cell lines (A549, Huh7, HUVEC). β-Actin was used as a control; the images of NUDT9 and β-actin staining are merged. Marked nuclear expression of NUDT9 was demonstrated in the HUVEC cells. All three cell lines showed positive staining for NUDT9 in both the nucleus and in the cytoplasm.

NUDT9α is an evolutionarily conserved mitochondrial ADPR pyrophosphatase and is thus specific to mitochondria and functions as a monomer [10]. It has been speculated that NUDT9 might regulate the intracellular concentration of ADPR and help energy production. NUDT9 has also been used as a marker for mitochondria [24]. However, there have been only a few studies of NUDT9 and its functional relevance [13,14,24].

We used the amino acid sequence from 51–186 of the NUDT9 protein (Figure 2A). This segment shows 100% sequence identity between the two alternatively spliced mRNAs of NUDT9. Our anti-NUDT9 antibody

should, therefore, be able to recognize both NUDT9α and NUDT9β. NUDT9 can degrade the toxic substance ADPR in cells; thus, it is possible that an increased level of NUDT9 expression may prevent apoptosis or cell death, thus contributing to cell proliferation and even carcinogenesis. This could partly explain why the proliferative-phase endometrium expressed more NUDT9 than the secretory-phase endometrium, and also why glandular cancer cells had a higher level of NUDT9 expression than the adjacent stromal, noncancerous cells. NUDT9α, the major form of NUDT9 transcript, is thought to be localized within or associated with mitochondria, which are in turn considered to be involved in coordinating cell death or apoptosis. Increased NUDT9 expression could thus be a by-product of increased numbers of mitochondria in cancer cells or cells with a high proliferative index. Alternatively, NUDT9 could itself contribute to cell proliferation and carcinogenesis, independent of its association with mitochondria. Further studies are needed to clarify this relationship, as well as its relationship with proteins and enzymes involved in cell cycle regulation, such as cyclin D1, cyclin E, p53, p21, and hormones such as estrogen, progesterone and their receptors. Notably, all the cell lines studied in this report, whether cancerous or noncancerous in origin, had detectable (if variable) levels of NUDT9 expression (Figure 7). Nuclear expression of NUDT9 was noted in A549, Huh7 and HUVEC cells (Figure 8), implying that its distribution was not restricted to the mitochondria. The alternatively spliced NUDT9β, or other products of NUDT9 degradation, may account for the nuclear expression noted in our study. Because NUDT9 is expressed in many cell lines, in both the nucleus as well as the cytoplasm, its detection in other gynecologic cancers (as for the Haymaker p38.5 protein in the carcinogenesis of gynecologic cancers), or even in cancers of other origins, may help to clarify its real role in carcinogenesis [20,25]. Previous studies of NUDT9 have mostly focused on its biochemical functions and structural relevance. Our study thus appears to be the first to identify NUDT9 as a potential biomarker for human endometrium and endometrial carcinogenesis.

In summary, we identified *NUDT9* as a gene that is differentially expressed during different phases of menstruation in the human endometrium. This was confirmed by immunohistochemistry that showed NUDT9 to be more highly expressed in proliferative-phase endometrium than in the more quiescent, secretory-phase endometrium. We also revealed that NUDT9 is widely expressed at the protein level in actively proliferating cells, and is expressed in both the nucleus and cytoplasm. Finally, we demonstrated that glandular epithelial cells expressed more NUDT9 than stromal cells in human endometrial carcinomas. Thus, NUDT9 is involved in and can be a biomarker of proliferation of human glandular endometrial cells. Based on these findings, we suggest that this molecule could be associated with tumorigenesis in the human endometrium.

Acknowledgments

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