PHYSIOLOGY

Clinical Correlation of Cyclin D2 mRNA Expression in Human Luteinized Granulosa Cells

DOOSEOK CHOI,^{1,2,4} EUN YOUNG LEE,² SARAH YOON,³ SEONGSOO HWANG,²
BYUNG-KOO YOON,¹ and JE-HO LEE¹

Submitted: March 29, 2000

Accepted: June 11, 2000

Purpose: The aim was to evaluate whether the expression of cyclin D2 mRNA in human luteinized granulosa cells correlates with the clinical parameters of women undergoing in vitro fertilization–embryo transfer (IVF-ET) programs.

Methods: Using reverse transcriptase–polymerase chain reaction (RT-PCR) techniques, the cyclin D2 mRNA in human luteinized granulosa cells of large follicles was measured. Correlation analysis between cyclin D2 mRNA expression and clinical parameters [age; day3 follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2); E_2 and P4 at the day of human chorionic gonadotropin (hCG); total amount of gonadotropin and the number of days of gonadotropin administration; pregnancy outcome] were performed.

Result(s): Cyclin D2 mRNA expression showed negative correlation with patient's age ($R = -0.60$, $P < 0.005$) and total amount of gonadotropins ($R = -0.45$, $P < 0.03$), and also showed positive correlation with serum E_2 levels at the day of hCG administration ($R = 0.55$, $P < 0.02$). Other clinical parameters showed no statistical significance. Cyclin D2 mRNA expression of pregnant group was higher than that of nonpregnant group ($P < 0.03$).

Conclusions: Cyclin D2 mRNA expression in human luteinized granulosa cells positively correlates with clinical parameters of ovarian function and may be a novel marker for the health of human ovarian follicle.

KEY WORDS: Cyclin D2 mRNA; human luteinized granulosa cell; clinical parameters.

INTRODUCTION

One of the methods to estimate the ovarian function is to evaluate the ability of granulosa cells (GC) to proliferate and to synthesize estradiol (E_2) within the follicle. The number of granulosa cells that ultimately develop to a preovulatory follicle is determined by the balance between the rate of granulosa cell proliferation and that of cell death. Granulosa cell death via an apoptotic mechanism has been regarded as a negative regulator for follicle growth (1–3). On the contrary, as a positive regulator, the insulinlike growth factors (IGF-I and IGF-II) promote ovarian follicular cell mitosis and differentiated function and can prevent granulosa cell apoptosis (4,5). Recent studies provide support for the involvement of the cell cycle regulatory molecules in the control of granulosa cell proliferation in response to hormones during follicular development (6). Especially D-type cyclins (cyclin D1, D2, and D3) have been known as cell cycle activators in ovary. Among D-type cyclins, cyclin D2 is known as a rate-limiting positive cell cycle regulator that has a pivotal role in granulosa cell proliferation in mouse and rat ovary, whereas

¹ Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea.

² IVF Clinic, Samsung Medical Center, Seoul, Korea.

³ Clinical Research Center, Samsung Biomedical Research Institute, Seoul, Korea.

⁴ To whom Correspondence should be addressed at: Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwan-dong, Kangnam-ku, Seoul, Korea 135-710.

cyclin D1 and D3 are localized to theca cells (6). Also, a critical role for cyclin D2 in the hormone-dependent phase of follicular growth is illustrated by the ovarian follicles of mice null for cyclin D2 (7). But, in humans, only one study was reported regarding the cyclin D2 expression in granulosa cell tumor (7). So the expression pattern and role of cyclin D2 in human granulosa cells remain to be elucidated. In this study, we assessed the expression of cyclin D2 mRNA in human luteinized granulosa cells and tested whether or not cyclin D2 mRNA expression in human luteinized granulosa cells correlates with the clinical parameters of ovarian function in women undergoing in vitro fertilization–embryo transfer (IVF-ET) programs.

MATERIALS AND METHODS

Patients and Follicle Aspiration

Human luteinized granulosa cells were obtained from 20 women participating in an IVF program between January 1999 and April 1999 at Samsung Medical Center, Seoul, Korea. Eleven with infertility of unknown origin, four were associated with male factor infertility, two had a tubal factor, two had endometriosis, and one had an age factor (Table I). The

mean age of study group was 34.5 years (26–50 years old). This study was approved by Samsung Medical Center Ethical Committee, and written informed consents were obtained. All patients received a gonadotropin-releasing analogue (Synarel; G.D. Searle & Co., Chicago) and highly purified urinary follicle-stimulating hormone (FSH) (Metrodin HP; Serono, Aubonne, Switzerland) only or with human menopausal gonadotropin (hMG) (Pergonal; Serono, Randolph, MA). During the period of gonadotropin stimulation, follicle size was measured by transvaginal ultrasound (5.0 MHz, Kretz) until at least one follicle achieved a mean diameter of 18 mm. Transvaginal oocyte retrieval was performed 35 hr after hCG injection (10,000 IU) (Profassi; Serono, Aubonne, Switzerland). Follicular fluid from at least three large follicles (mean diameter $\geq$ 20 mm at the time of aspiration) was collected per patient. After isolation of oocyte–cumulus cells under a dissecting microscope, luteinized granulosa cell preparation was performed.

Granulosa cell Preparation

The follicular aspirates from large follicles were centrifuged for 10 min at 600 g and the cell pellet was resuspended with Ham's F-10 (Gibco-BRL, Grand

Table I. Profiles of Patients Who Underwent an IVF-ET Program

Patient No.	Age	LH/FSH/E ₂ ^a	E ₂ /P4 ^b	Amount of gonadotropins ^c	Number of days of gonadotropin	Cyclin D2 ^d	Pregnancy	Diagnosis ^e
1	36	6.1/3.9/26.7	5100/2.27	1800	6	0.57		Unexpl.
2	33	3.2/7.3/27	1841/1.77	2250	7	0.45		ES
3	30	3.8/6.8/12	669/0.83	1200	6	0.74	P	MF
4	35	2.2/6.0/35.3	847/1.1	2250	9	0.35		Unexpl.
5	38	4.5/9.5/17	203/0.68	4425	16	0.33		Unexpl.
6	50	3.3/5.7/58	274/0.55	3525	12	0.24		Age
7	34	0.4/6.8/10	1137/1.13	1500	8	0.34		Unexpl.
8	33	3.2/7.3/27	802/0.67	2025	7	0.51		ES
9	33	5.9/5.9/28	1964/1.75	1725	8	0.69	P	Unexpl.
10	26	3.1/6.5/31	3910/1.44	1350	9	0.63	P	MF
11	39	2.6/5.3/19	2177/1.73	2025	8	0.45		Unexpl.
12	28	9.4/6.1/22	6880/1.68	1200	8	0.56	P	Unexpl.
13	32	4.0/6.4/14	1978/1.39	1350	6	0.73	P	TF
14	41	2.0/7.2/32	1130/1.01	3450	17	0.46		MF
15	31	5.8/14.0/14.9	172/1.3	1800	6	0.29		TF
16	41	4.9/8.8/16.7	519/0.54	3225	11	0.45		Unexpl.
17	41	4.9/8.8/16.7	125/0.68	3750	11	0.40		Unexpl.
18	31	4.4/5.7/33	979/0.5	2025	9	0.65		Unexpl.
19	27	5.5/3.0/25	1397/1.02	1200	8	0.60		Unexpl.
20	30	4.1/6.0/26	2740/1.09	1500	10	0.65		MF

^a LH, FSH, and E₂ levels on menstrual cycle day 3. LH and FSH (mIU/ml); E₂ (pg/ml).

^b E₂ and P4 (ng/ml) levels at the day of hCG administration.

^c Total amount of gonadotropin administration (IU).

^d Cyclin D2 mRNA, arbitrary unit.

^e ES: Endometriosis; MF: Male factor; TF: Tubal factor.

Island, NY). The resuspended cells were layered in 3 ml 50% Percoll solution (Sigma, St. Louise, MO) and centrifuged for 5 min at 600 *g* to pellet the blood cells. Purified granulosa cells were aspirated from the interphase (1 to 2 ml) and washed twice with Ham's F-10 (8).

Hormone Assay

Serum levels of FSH, luteinizing hormone (LH), and E₂ on menstrual cycle day 3 were measured in a spontaneous menstrual cycle usually prior to the stimulated cycle. Serum levels of E₂ and P4 were determined at the day of hCG administration. Serum FSH, LH, and E₂ were measured with a commercially available radioimmunoassay kit (FSH-IRMA, LHsp-IRMA, E₂-RIA-CT, Biosource, Belgium), and serum P4 level was measured by a commercially available double-antibody radioimmunoassay kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). The sensitivities of the FSH, LH, E₂, and P4 assay were 0.1 mIU/ml, 0.20 mIU/ml, 4.8 pg/ml, and 0.02 ng/ml, respectively. The intra- and interassay coefficients of variation of FSH, LH, E₂, and P4 were <6%, <9%, <10%, and <15%, respectively.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from granulosa cells by Trizol reagent (Gibco-BRL) according to the manufacture's instructions. Total RNA (2 µg) was reversely transcribed in final 25-µl Tris buffer (50 mmole/liter; pH8.3) supplemented with KCl (75 mmole/liter), MgCl₂ (3 mmole/liter), DTT (5 mmole/liter), deoxynucleotide triphosphate (0.5 mmole/liter, each) (Gibco-BRL), oligo dT (1.5 µ mole/liter), and moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (200 U, Promega) at 42°C for 60 min. Polymerase chain reaction (PCR) amplification was performed in a final 50-µl reaction volume containing 2 µl of reverse transcribed cDNA, 0.4 µ mole/liter of each primer, 1U of Taq DNA polymerase (Promega, Madison, WI), 1x Taq DNA polymerase buffer, MgCl₂ (2.5 mmole/liter), and deoxynucleotide triphosphate (0.2 mmole/liter each). The PCR reaction was conducted in a Perkin Elmer DNA Thermal Cycler (version 2.3) with the following program: denaturing for 1 min at 95°C, annealing for 45 sec at 56°C (for cyclin D2) and at 60°C (for GAPDH), and elongation for 2 min at 74°C for a total of 30 cycles, except for the last cycle, for which

the elongation step was prolonged to 10 min at 74°C. The amount of template cDNA used in each reaction was normalized to the amount of GAPDH mRNA. Amplification of template cDNA was in the linear range for the number of PCR cycles and RT-PCR of GAPDH transcript was performed using the same amount of cDNA from each sample as a template. PCR products were visualized by electrophoresis on 1.5% (wt/vol) agarose gels.

Oligonucleotide Primers

Oligonucleotide PCR primers were selected with the assistance of a computer program (DNA STAR) designed to optimize GC content and melting temperature and to minimize hairpin and dimer formation. PCR primers were cyclin D2 for 5'-TACTTCAAGTGCGTGCAGAAAGGAC-3'; cyclin D2 rev 5'-TCCCACACTTCCAGTTGCGATCAT-3'; GAPDH for 5'-ACCACAGTCCATGCCATCAC-3'; GAPDH rev 5'-TCCACCACCTGTTGCTGTA-3', which resulted in product of 489 bp and 452 bp, respectively.

Densitometry

Gel image was scanned with a gel documentation system (Gel Doc 1000, Bio-Rad Hercules, CA) and relative densities were analyzed using a multi-analyst fingerprinting program (version 1.1). The relative densities of the bands were expressed as arbitrary absorbance units per area.

Statistical Analysis

The correlation between cyclin D2 mRNA expression expressed by arbitrary unit and clinical parameters was tested by Spearman's correlation analysis. Differences of cyclin D2 mRNA expression between pregnant and nonpregnant group were analyzed by Wilcoxon's rank sum test. Statistical significance was given when *P* value was less than 0.05.

RESULTS

Four hundred eighty-nine base pair cyclin D2 and 452 bp GAPDH RT-PCR products were obtained in human luteinized granulosa cells. After the correction of cyclin D2 mRNA by GAPDH mRNA, the cyclin D2 mRNA expression was expressed by arbitrary unit. While cyclin D2 mRNA expression in lu-

teinized granulosa cells showed negative correlation with age ($R = -0.60$, $P < 0.005$) and total amount of gonadotropins used ($R = -0.45$, $P < 0.03$), cyclin D2 mRNA expression showed positive correlation ($R = 0.55$, $P < 0.02$) with serum E_2 levels at the day of hCG administration (Fig. 1). Although menstrual cycle day 3 serum FSH levels and the number of days of gonadotropin administration showed negative correlation with cyclin D2 mRNA expression ($R = -0.41$ and $R = -0.37$), there were no statistical significance ($P = 0.08$ and $P = 0.11$) (Fig. 2). Other parameters (day 3 LH, E_2 ; P4 at the day of hCG) showed no correlation with cyclin D2 mRNA expression. Cyclin D2 mRNA expression of pregnant group was higher than that of nonpregnant group ($P < 0.003$) (Fig. 2).

DISCUSSION

The cyclin D2 is one of critical governors of the cell cycle clock apparatus during the G1 phase of the mammalian cell cycle. Entry into the S phase is positively regulated by cyclin D2 (9–11). The cyclin D2 binds with cyclin-dependent kinase (cdk) 4 and 6, which allows phosphorylation by cdk-activating kinase (CAK), thereby forming an active complex. The cyclin-cdk complex phosphorylates cellular substrates, leading to activation of the E2F family of transcription factors. These result in the activation of numerous genes involved in DNA synthesis and ultimately enable cells to begin the transition from G1 to S phase. The targeted deletion of cyclin D2 gene indicates that it is essential for normal ovarian function. In mice null for cyclin D2, granulosa cell proliferation is impaired, the ovarian follicles remain small, and ovulation fails to occur (7). This finding suggests that cyclin D2 is a rate-limiting activator of cell cycle progression and is essential for granulosa cell proliferation. Therefore, we postulated that cyclin D2 mRNA expression in granulosa cells reflects the follicle health especially in a dominant follicle that is destined to ovulate. In this study, we revealed that cyclin D2 mRNA is expressed in luteinized granulosa cells of all patients undergoing IVF-ET program for the first time. To our knowledge, there was only one study regarding the cyclin D2 expression in the human ovary in which ovarian granulosa cell tumors expressed very high levels of cyclin D2 mRNA compared with normal ovaries (7). In rat, cyclin D2 mRNA is known to be down-regulated within 4 hr after hCG injection in granulosa

cells of preovulatory follicles that are destined to ovulate (6). Though we did not analyze the specific preovulatory follicles, the cyclin D2 mRNA in rat ovarian granulosa cells treated with PMSG showed a different pattern of expression after hCG injection. The decrease of cyclin D2 mRNA after hCG injection was not profound compared to before hCG injection (maximum 16% decrease 8 hr after hCG injection), and the cyclin D2 mRNA recovered thereafter up to the level of before-hCG injection (data not shown). This finding suggests that there may be no significant down-regulation of cyclin D2 mRNA expression of human luteinized granulosa cells in controlled ovarian hyperstimulation cycle at 35 hr after hCG injection. In human, 35 hr after hCG injection cyclin D2 mRNA was still detected in large follicles (≥ 20 mm at the time of aspiration) that were considered as preovulatory follicles. Cyclin D2 mRNA expression showed negative correlation with age ($R = -0.60$, $P < 0.005$) and amount of gonadotropins used ($R = -0.45$, $P < 0.03$). Although there was no statistical significance, cyclin D2 mRNA expression also showed negative correlation with day 3 FSH ($R = -0.41$, $P = 0.08$) and the number of days of gonadotropin administration ($R = -0.37$, $P = 0.11$). Clinical parameters that showed negative correlation with cyclin D2 mRNA expression in this study are all known to be negatively associated with ovarian function. Also, cyclin D2 mRNA expression in the pregnant group was higher than that of the nonpregnant group ($P < 0.003$). These results indirectly suggest that cyclin D2 mRNA expression in human luteinized granulosa cells positively correlates with the clinical parameters of ovarian function.

In conclusion, analysis of cyclin D2 mRNA expression in human luteinized granulosa cells suggests that cyclin D2 mRNA expression in human luteinized granulosa cells positively correlate with the clinical parameters of ovarian function and may be a novel marker for the health of the human ovarian follicle.

ACKNOWLEDGMENTS

This work was supported by the Samsung grant, #SBRI C-99-005-1

Poster presented at the 11th World Congress on In Vitro Fertilization and Human Reproductive Genetics, 9–14 May, 1999, Sydney, Australia.

Fig. 1. Correlation analysis between cyclin D2 mRNA and (A) patients age, (B) total amount of gonadotropins, (C) E_2 level at the day of hCG, (D) menstrual cycle day 3 serum FSH level, and (E) number of day of gonadotropin administration.

Fig. 2. Comparison of cyclin D2 mRNA expression in human luteinized granulosa cells between pregnant and nonpregnant group ($P < 0.003$).

REFERENCES

1. Seifer DB, Gardiner AC, Ferreira KA: Apoptosis as a function of ovarian reserve in women undergoing in vitro fertilization. *Fertil Steril* 1996;66:593–598
2. Nakahara K, Saito H, Saito T: Incidence of apoptotic bodies in membrana granulosa of the patients participating in an in vitro fertilization program. *Fertil Steril* 1997;67:302–308
3. Nakahara K, Saito H, Saito T: The incidence of apoptotic bodies in membrana granulosa can predict prognosis of ova from patients participating in vitro fertilization programs. *Fertil Steril* 1997;68:312–317
4. Giudice LC: Insulin-like growth factors and ovarian follicular development. *Endocrinol Rev* 1992;13:641–669
5. Chun SY, Billig H, Tilly JL: Gonadotropin suppression of apoptosis in cultured preovulatory follicles: Mediatory role of endogenous insulin-like growth factor I. *Endocrinology* 1994;135:1845–1853
6. Robker RL, Richards JS: Hormone-induced proliferation and differentiation of granulosa cells: A coordinated balance of the cell cyclin regulators cyclin D2 and p27^{KIP1}. *Mol Endocrinol* 1998;12:924–940
7. Sicinski P, Donaher JL, Geng Y: Cyclin D2 is an FSH-respective gene involved in gonadal cell proliferation and oncogenesis. *Nature* 1996;384:470–474
8. Dirnfeld M, Goldman S, Yael G: A simplified coculture system with luteinized granulosa cells improves embryo quality and implantation rates: A controlled study. *Fertil Steril* 1997; 67:120–122
9. Sherr CJ: Cancer cell cycles. *Science* 1996;274:1672–1677
10. Inaba T, Matsushim H, Valentine M: Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. *Genomics* 1992;13:565–574
11. Xiong Y, Menninger J, Beach D: Molecular cloning and chromosomal mapping of CCND genes encoding human D-type cyclins. *Genomics* 1992;13:575–584