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Cell Cycle Regulation in the Estrogen Receptor Beta (ESR2)-Overexpressing Hep3B Hepatocellular Carcinoma Cell Line

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Abstract

Epidemiological studies and experimental data have shown that the incidences of hepatocellular carcinoma in men are more frequent than in women. Evidence suggests that imbalance of hormones, including estrogen, androgen, prolactin, and growth hormone, modifies liver tumorigenesis. In this present study, we investigated how estrogen and estrogen receptor 2 (ESR2), regulates the cell cycle mechanism in Hep3B hepatocellular carcinoma cell line. Our results showed that ESR2 overexpression in the presence of 10^{-8} M 17-β-estradiol downregulated c-myc and cyclin D1 expression and simultaneously upregulated p27 expression. However, flow cytometry and MTT assays showed only minor G_1 phase arrest without affecting cell viability. Taken together, these observations indicate that ESR2 is required to lower tumorigenesis in males by altering cell cycle proteins in a ligand-dependent manner.

Key Words: c-myc, cyclin D1, ESR2, Hep3B cells

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancers in the world with approximately a million deaths annually (4, 23). Several cytotoxic agents have been tested in patients with advanced HCC; unfortunately, none displayed encouraging results and are often associated with unacceptable toxicity

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(2). Hormonal treatment plays an established role in several solid tumors. For example, estrogen replacement therapy exerts a protective role against colorectal cancer and in breast cancer (21, 24). Similarly, steroid hormone 17-β-estradiol (E_2) is a critical regulator of growth, differentiation and function in a wide range of target tissues, particularly in liver cancer in which the protective role of E_2 was well documented (19, 25). The main biological functions of estrogens are mediated through two distinct intracellular receptors ESR1 and ESR2 (12). ESR1 and 2 modulate gene expression by interacting with promoter response elements or other transcription factors in a ligand-dependent or -independent manner (10, 15). Foley *et al*. (2000) showed that malignant transformation in colon is related with decreased ESR2 protein expression (6). Similarly, progressive loss of *ESR2* expression during the process of carcinogenesis has also been documented in prostate (29), ovarian, and breast cancers (1, 9). All these evidences suggest that loss of ESR2 constitutes to cancer development.

The role of estrogens and their receptors in controlling cell cycle progression has drawn wide attention (3). Estrogen independently regulates the expression of cell-cycle genes like c-myc, cyclin D1, cyclin E and A, Cdc25A, $p45^{Skip2}$ and $p27^{Kip1}$ (7, 8). Similarly, ESR2 in T47D breast cancer xenografted tumors decreased tumor growth and angiogenesis (11). Breast cancer cell lines were extensively used to study the role of estrogen on cell cycle progression. However, only few studies were attempted in liver cancer cells to study this phenomenon. In this present study, we aimed to investigate whether ESR2 has any effect on the expression of cell cycle proteins such as c-myc, Cyclin D1 and p27. We showed that in overexpression of ESR2 downregulated c-myc and Cyclin D1 expression and upregulated p27 expression in a ligand-dependent manner.

Materials and Methods

Cell Culture and Transfection

Hep3B cell line was purchased from ATCC and maintained in MEM media (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, UT, USA), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 1% antibiotic-antimycotic (Invitrogen Corp, Carlsbad CA, USA) in 5% CO_2 humidified air at 37°C. For experiments on DNA fragmentation, RNA extraction, flow cytometry, and Western blotting, the cells were transferred to 6-cm culture dishes and allowed to grow to 60-70% confluency.

Hep3B cells were transfected with a plasmid carrying the *ESR2* gene using Lipofectamine (Invitrogen) according to the manufacturer's guidelines. After 6 h of transfection, cells were replaced with MEM supplemented 10% CharcoalDextran (CD) -FBS (Sigma) for 12 h and then with MEM medium containing 1% FBS and antibiotics for 6 h. Before treatment, cells were starved in MEM without phenol red with 1% antibiotics for 6 h and then replaced with phenol red-free MEM medium containing 1% FBS and vehicle or E_2 (Sigma) for different periods of time. All transfection experiments were repeated thrice with consistent results.

Construction of Plasmid

The human estrogen receptor β (ESR2) gene was generated by PCR from pRST7-ESR2 plasmid, and the PCR product was inserted into BamHI and SalI sites in the pTRE2-hygromycin B vector (Promega Corp., Madison NJ, USA). The insert was verified by DNA sequencing.

Flow Cytometry

Apoptosis was determined by Annexin V-FITC and propidium iodide staining. After removal of the cell aggregates by filtering through Falcon filter top tubes, the cellular DNA content was determined by fluorescence-activated cell sorting (FACS) analysis using a FACS Calibur flow cytometer (BD PharMingen, San Diegao, CA, USA). Data were analyzed using the CellQuest (BD PharMingen) and ModFit (Verity, Topsham, Topsham, ME, USA) programs.

MTT

Hep3B cells (1×10^4 cells per well) were seeded in a 24-well plate and then transfected with pcDNA3 or ESR2, or treated with E_2, or transfected with pcDNA3/ESR2 for desired time points. After treatment, the medium was replaced by the MTT (Sigma) solution (0.5 mg/ml) and incubated in 5% CO_2 at 37°C for 4 h. MTT solution was replaced by isopropanol to dissolve the blue formazan crystals, and absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Western Blotting

Western blotting was performed as described previously (5) with slight modifications. Cells were lysed at each time point with lysis buffer (50 mM Tris base, pH 7.4), 0.5 M NaCl, 1 M ethylenediamine – mercaptoethanol (BME), 1% NP-40, 10% glycerol, IGEPAL CA-630 (Sigma) and a protease inhibitor

Table 1. Sequence of PCR primers for *ESR2*, *c-myc*, *cyclin D1*, *p27* and *pHe7* promoter

Gene	Primer sequence (**5'→3'**)	bp	Temp.
ESR2	5'-ATGGATATAAAAAACTCACCA -3' 5'- TCACTGAGACTGTGGGTTCTG-3'	1,592	59°C
c-myc	5'-GCCCCTCAACGTTAGCTTCA -3' 5'- TTCCAGATATCCTCGCTGGG-3'	149	54°C
cyclin D1	5'-AACTACCTGGACCGCTTCCT -3' 5'-CCACTTGAGCTTGTTCACCA -3'	203	58°C
p27	5'-GGGGCTCGTCTTTTCGGGGTGTTT -3' 5'-GAGCGGGAGGGCGGAGAGGAG -3'	194	65°C
pHe7	5'- CTTCGAAAGGCAAGGAGGAA -3' 5'- ACGACCCTTAACATCTCGGT -3'	246	55°C

cocktail (Roche, Mannheim, Germany). Proteins were analyzed and separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with the following antibodies; ESR2, c-myc, p27, cyclin D1 and α-tubulin (Santa Cruz, CA, USA). The blots were incubated with a peroxidase-conjugated secondary antibody for 1 h. Bands were monitored using a Western blot chemiluminescence reagent (Santa Cruz).

RT-PCR

Total RNA extraction was performed as described earlier (18) with slight modifications. Reverse transcription was performed at 37°C for 60 min in 55.5 μl DEPC-H_2O, 4 μg total RNA, 0.5 μl RNase inhibitor at 40 U/μl (Promega), 20 μl 5X RT buffer, 8 μl 2.5 mM dNTP, 10 μl oligodT at 5 μM/ml (Mission Biotech, Taipei, Taiwan, ROC) and 2 μl MMLV reverse transcriptase at 200 U/μl (Promega). The resulting cDNA was added to the PCR reaction mixture containing 9.5 μl DEPC water, 2.5 μl 10X PCR buffer (MD Bio, Taipei, Taiwan, ROC), 2.5 μl 10 mM dNTP (Promega), 2.5 μl of each primer (5 μM) and 0.5 μl Taq DNA polymerase (2 U/μl) (MD Bio), 4 μl 2.5 mM dNTP mixtures. The cDNA was amplified by PCR with the primers listed on the Table 1. To verify quality and quantity of cDNA by PCR, the ribosomal gene pHE7 was used as an internal control. The PCR products were analyzed by electrophoresis on agarose gels containing ethidium bromide.

Statistical Analysis

All experiments were repeated at least three times using independent culture preparations. Values are shown as mean ± SEM. Student's *t*-test was used to calculate the statistical significance of the experimental results for two groups; $P < 0.05$ was considered significant.

Results

Efficiency of Transiently Transfected ESR2 in Hep3B Cancer Cells

Hep3B cells were treated with E_2 or transfected with pcDNA3 for 48 h or cells were transfected with ESR2 for 24 h and then treated with E_2 or ethanol for an additional 24 h and then analyzed for ESR2, c-myc, p27 and cyclin D1 mRNA and protein expression levels. As shown in Fig. 1, A and B, ESR2 and ESR2 plus E_2 treatment showed increased ESR2 and p27 levels and decreased c-myc and cyclin D1 levels. The data suggest that ESR2 overexpression could control the proliferation of Hep3B cells in a ligand-dependent fashion.

Effects of ESR2 on Cell Cycle Distribution

To study the effect of ESR2 on the different phases of cell cycle, Hep3B cells with E_2, or transfected with pcDNA3 for 48 h, or cells were transfected with ESR2 for 24 h and then treated with E_2 or ethanol for an additional 24 h and then analyzed with a FACS. The percentage of Hep3B cells at the G_1 phase accumulation was prominently increased in ESR2 and ESR2 plus E_2 treated group when compared to the control or E_2 treatment (Fig. 2). The results indicated that ESR2 could arrest Hep3B cell cycle at the G_1 phase.

ESR2 Inhibits Growth of Hep3B Cells in a Dose-Dependent Manner

The effects of ESR2 on cell proliferation were next examined. ESR2 and ESR2 plus E_2 were found to inhibit Hep3B cell growth in a time-dependent manner. Compared with the 48 h exposure group, 72 h exposure showed a greater alteration in the cell growth; however this decrease was not significant.

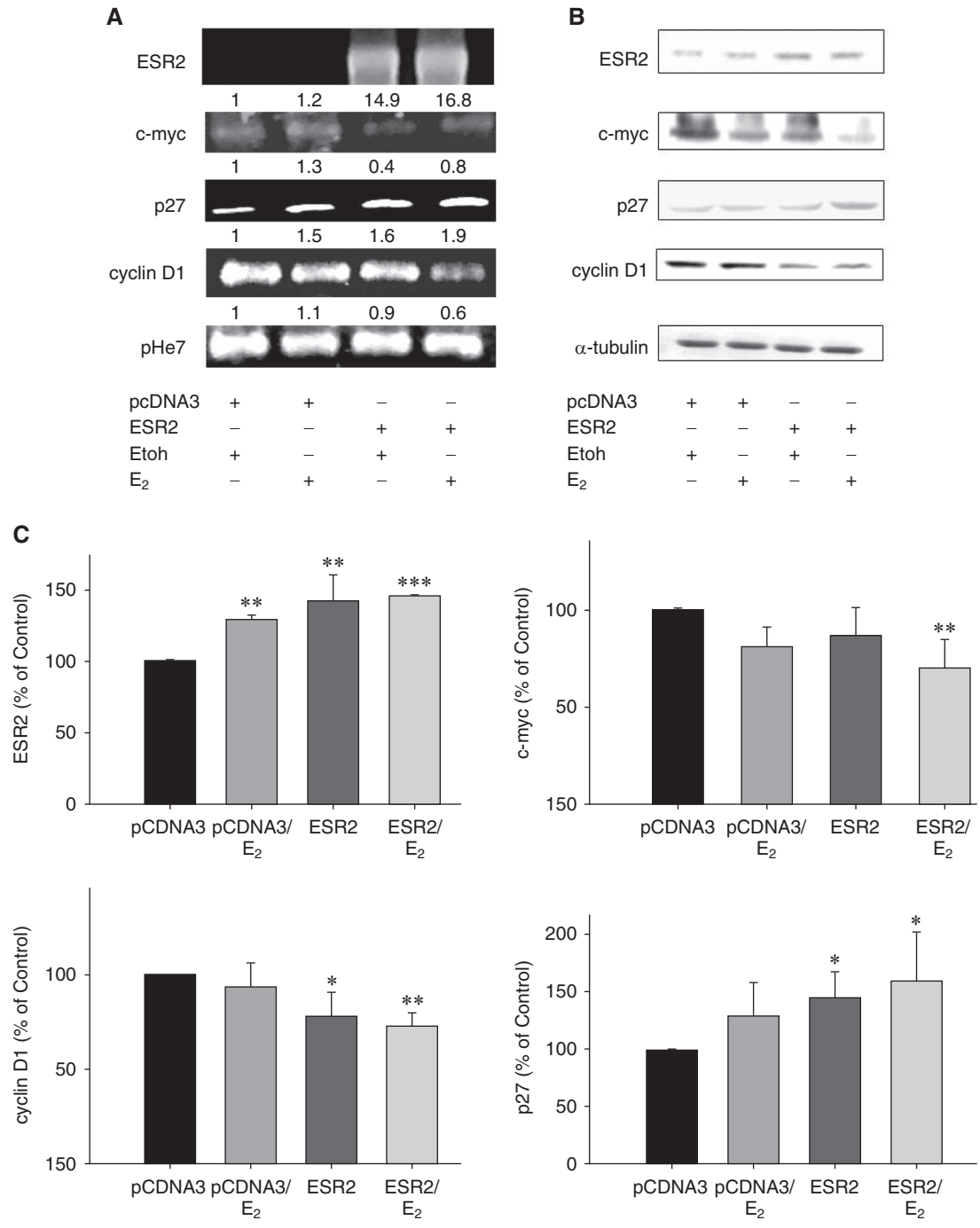

Fig. 1. Role of ESR2 in regulating cell cycle proteins. Hep3B cells were transfected with pcDNA3 for 24 h and then treated with E_2 or Etoh ethanol for 24 h (lanes 1 and 2); in a second group, cells were overexpressed with ESR2 and then treated with E_2 or ethanol for 24 h (lanes 3 and 4). A. RT- PCR; B. Western blot was used to detect ESR2, c-myc, p27 and cyclin D1 expression. C. Quantification of the protein bands. pHe7 and α-tubulin was used as loading controls, respectively. Data are shown as mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Discussion

Epidemiological reports have pointed out that despite of etiologies, male subjects have a higher incidence of HCC than the female (30). Women show significantly lower incidences of HCC than men, and a similar trend has also been observed in female rodents compared to males during chemically-induced carcinogenesis; ovariectomized females also showed higher levels of liver tumors than normal females (13, 27). Several evidences suggest that sex hormones and their receptors may play an important role in liver tumor-

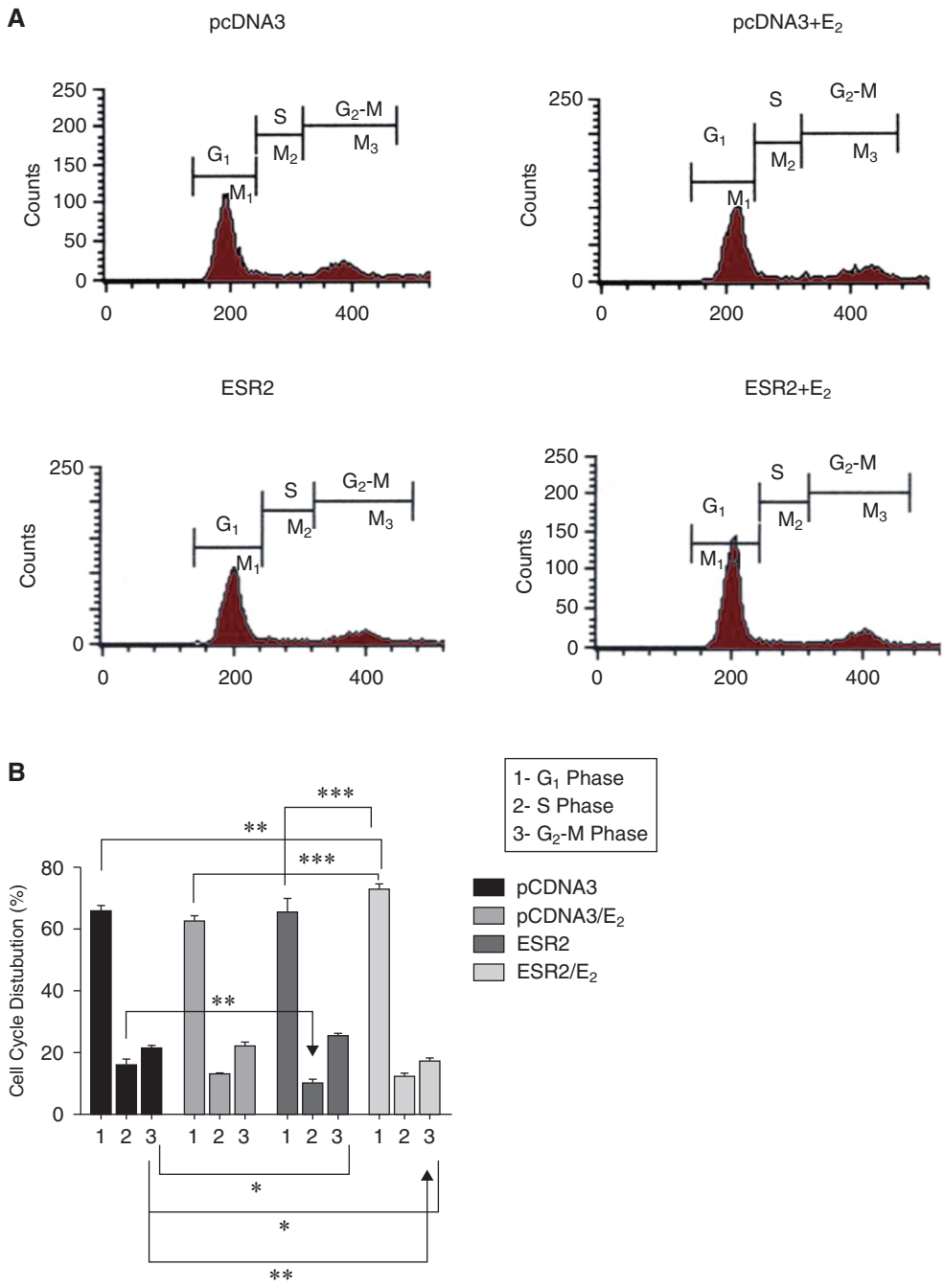

Fig. 2. Overexpression of ESR2 controls cell cycle. Hep3B cells were overexpressed with ESR2 or pcDNA3 for 24 h and then treated with E_2 or ethanol (Etoh) for 24 h. Cells were collected and fixed with Etoh, stained with propidium iodide and analyzed by flow cytometry. The data on each sample represent the percentage of cells in the Sub-G_1, G_1, S and G_2/M phases of the cell cycle, respectively. B. Representative column graph showing the percentage of cells (± SD) in each cell cycle. All the experiments were repeated three times. Data shown are means ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

genesis (28). For example, male mice exposed with estrogen showed fewer liver tumors than control males treated with diethylnitrosamine (DEN) (20, 25). In our recent study, we also found that over expression of ESR1 and ESR2 decreased PPARγ expression and further inhibited proliferation of Hep3B cells in a ligand-dependent manner (17). A potential role for ESR2 in liver carcinogenesis has been suggested by Iavarone *et al*. (2003), and decreased expression of ESR2 was found in patients with chronic hepatitis or cirrhosis and those with HCC (13). However, the specific role of ESR2 in HCC remains unclear and thus using hormonal therapy for HCC treatment remains contradictory (14). Therefore, in this present study, we analyzed

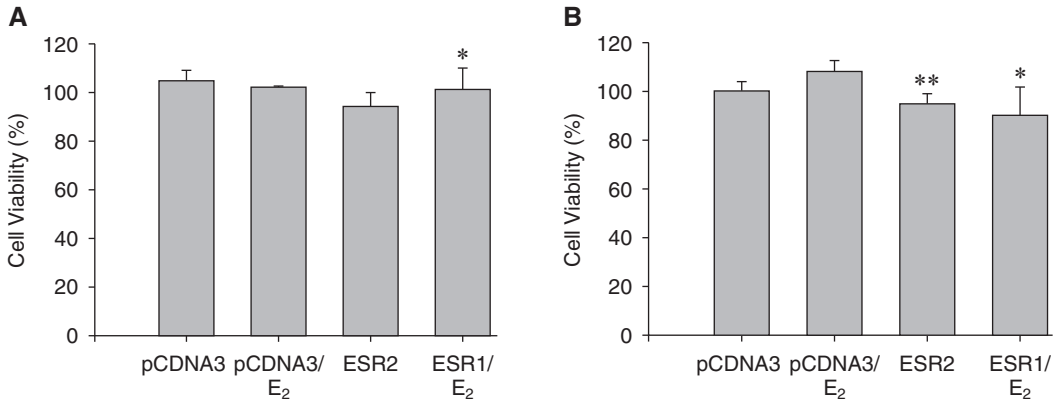

Fig. 3. ESR2 inhibits Hep3B cell viability in a time-dependent manner. A. The cells were overexpressed with ESR2 for 24 h and then treated with E_2 for an additional 24 h. B. The cells were overexpressed with ESR2 for 48 h and then treated with E_2 for an additional 24 h. The surviving cells were determined and presented as percentages of the untreated cells which were used as a control; the index of the control group was 100%. Data shown are means ± SD. *, $P < 0.05$; **, $P < 0.01$.

the functional role of ESR2 in Hep3B cell proliferation.

As suggested in different cellular models, ESR2 transaction appears to be fully functional with E_2 stimulation (16, 22). This assumption was not supported by the results obtained on the effects of E_2 on cell proliferation in ESR2-transfected Hep3B cells. The control mechanism of tumor promotion is by altering cell cycle regulators and by subsequent deregulation of the cell cycle. In T47D breast cancer cells, ESR2 overexpression decreased both cyclin E and A in a ligand-dependent manner (26). Similarly, in MCF-7 breast cancer cells, ESR2 inhibited cell proliferation and tumor formation by causing G_2 cell cycle arrest by inhibiting cyclin A and by decreasing p21CIP1, $p27^{KIP1}$ and c-myc expression at the transcription level (22). Similarly, in our present study, ESR2 overexpression decreased c-myc, cyclin D1 and increased p27 expression levels in a ligand-dependent manner.

In conclusion, ESR2 plays an anti-proliferative role in liver cancer cells in a ligand-dependent fashion in regulating cell cycle proteins and further causes G1 phase arrest. Further investigation using estrogen receptors in liver cancer is necessary to elucidate what other possible pathways are altered by overexpressing ESR2.

Acknowledgments

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