§3. Results

3.1 Identification of the recombined plasmid

The plasmid pSG-hERRα was excised and open to two linear fragments after digested by the *EcoR*I and *Bam*HI double restricted endonuclease system. The DNA fragments containing the hERRα full-length cDNA and pSG vector were separated by electrophoresis in 1% agarose gel (Figure 3.1). The agarose gel containing the full-length cDNA of hERRα (2221 bp) was excised carefully and melted in the QIA buffer. According to the protocol provided by the manufacturer, the full-length hERRα cDNA was extracted by the QIAquick gel extraction kit. Treated with the *EcoR*I and *Bam*HI double restricted endonuclease system, the pCN3-GFP vector was also open to a linear vector. The hERRα-GFP plasmid was constructed by inserting the full-length cDNA of hERRα into the linear pCN3-GFP vector.

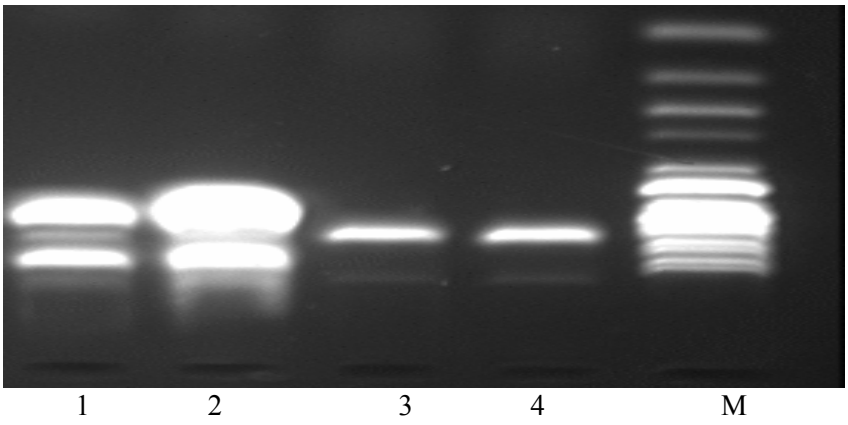


Figure 3.1: The elecrophoresis of linear plasmid DNA fragments. After digested by the *EcoR*I and *Bam*HI double restricted endonuclease system, the plasmid DNA fragments were separated by 10% agarose gel electrophoresis. Lane 1, 2 are the pSG-hERRα plasmid (UP, hERRα full-length cDNA, 2.2 Kbp; DOWN, pSG vector 3.3 Kbp), lane 3,4 are the linear pCN3-GFP plasmid vector. M, 5 Kbp marker (Invirtrogen, USA)

After construction, the hERRα-GFP reporter plasmid or pD-GADPH plasmid was transformed into the Top-10 one shot cells or QIAGEN EZ competent cells, respectively, and seeded in X-gal-ampicillin screening plate. The successful construction broke the *Lac Z* coding domain by inserting the target fragments, which results in the successfully transformed clone lost the ability to decompose X-gal and can not show a blue color (Figure 3.2 A and B). Thus, the negative clone will remain blue and the successfully

transformed cells, which became resistant to the treatment with ampicillin, could grow in the ampicillin selective culture medium. A single positive clone (white) was picked from the primary screening plate and injected into 250 μl SOC medium for culturing 4 h while shaking at 300 rpm and seeded again in a new screening plate. Cultured overnight (12 h), a pure positive-clone-plate was formed (Figure 3.2 C and D). A single positive clone was chosen from the pure positive-clone-plate and cloned in the LB-selective medium as protocol listed in chapter **2.3.3**.

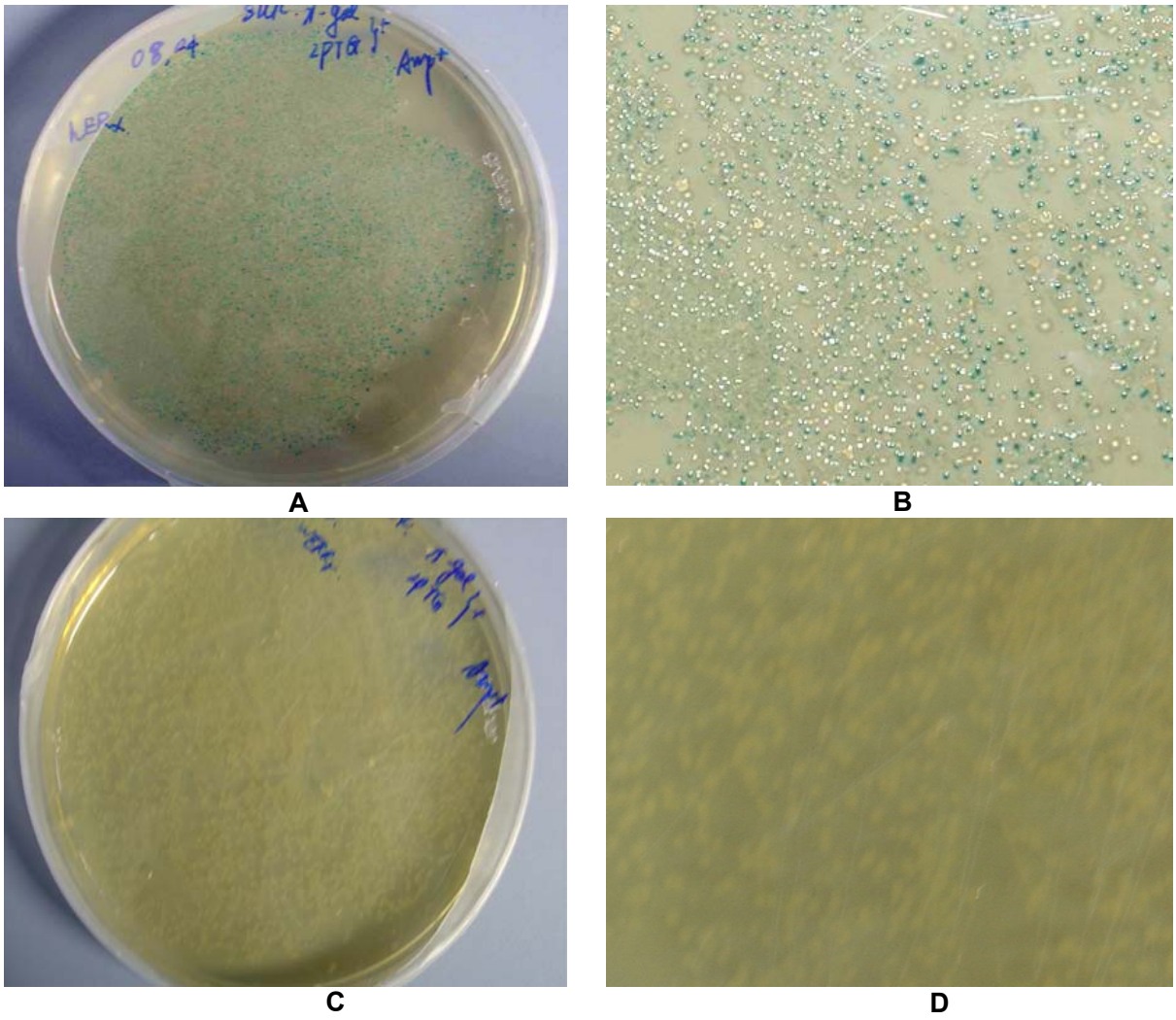


Figure 3.2: Purification of positive transformed monoclonal by blue/white screening (A-D). (A) and (B) After transformed, the bacteria were seeded in the primary screening plate. A single white clone (positive) from (B) was chosen and cultured in SOC medium for 4 h, and seeded in the second screening plated for overnight (C). A purified positive transformed clone was developed (picture C and D)

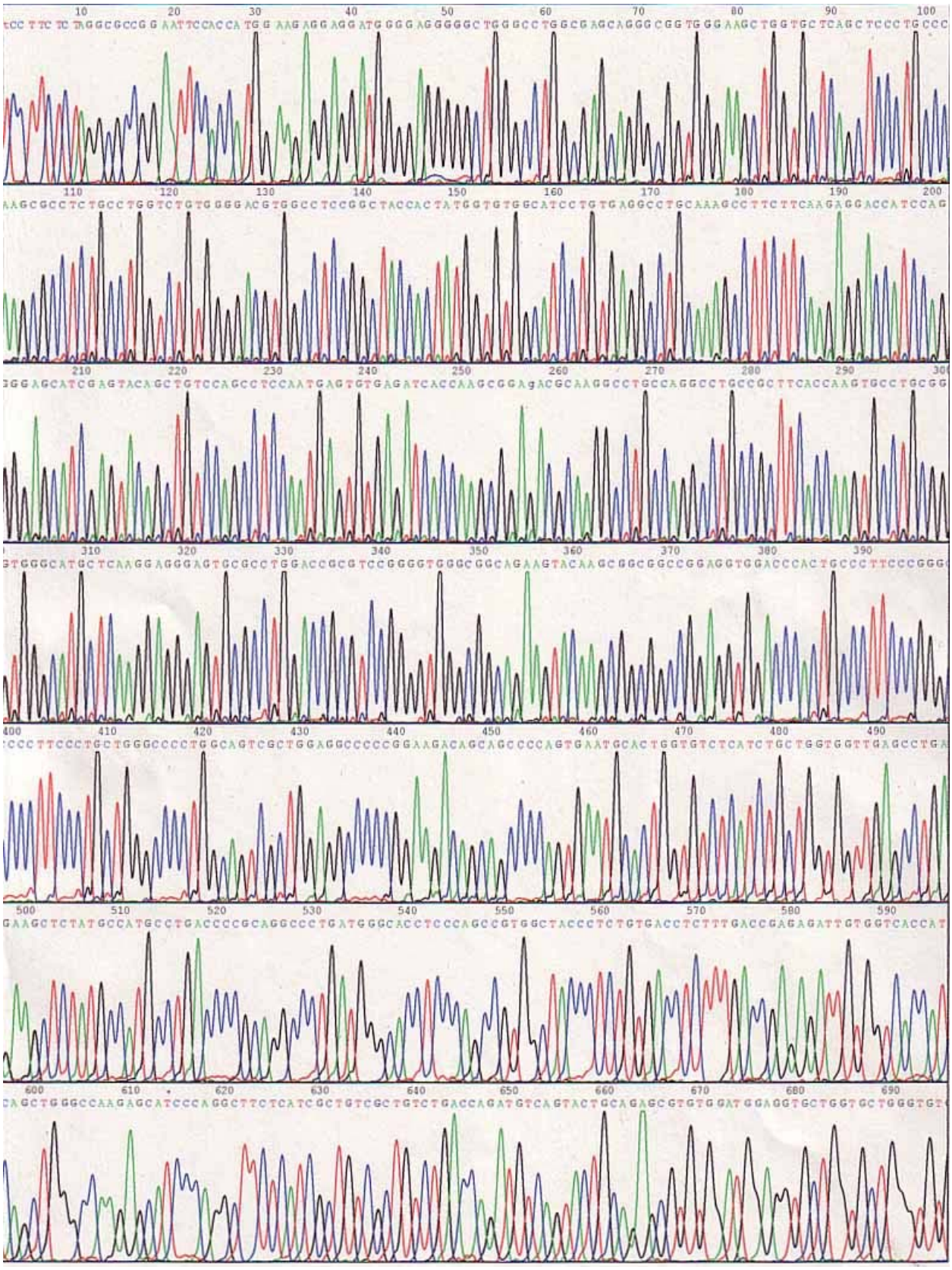


Figure 3.3: Sequence analysis of the recombined hERRα-GFP plasmid. The partial result form the sequenced examination was showed a 99.9% identify with the original hERRα full-length cDNA. The ligated reaction is correct.

The correction of ligation was examined by sequence analysis. The sequenced examination was performed by TIB mol lab. The Figure 3.3 gives the partial result of the inserted fragment sequence. Compared with the original sequence of hERRα full-length cDNA, the ligation is corrected. The recombinants were also used as templates for PCR reaction to check the ligated corrections (Figure 3.4).

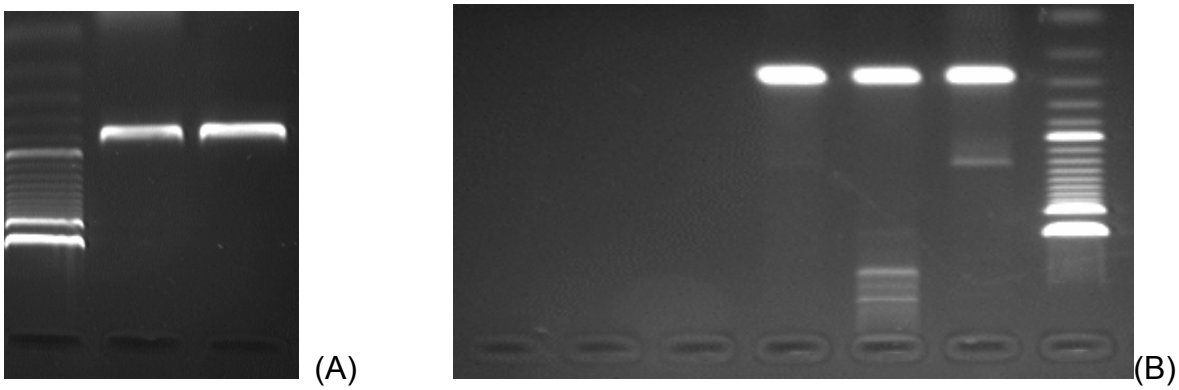


Figure 3.4: A normal PCR was also running by using the hERRα-GFP recombinant (A) or pD-GADPH recombinant (B) as template. (A) A normal PCR amplification from hERRα-GFP reporter plasmid, a 482 bp product was clearly seen in the Lane 1 and 2, (B) Lane 1-3 are unsuccessfully transformed clones and Lane 4-6 are successfully transformed clones. M, 100bp ladder (Invirtrogen, USA)

3.2 Expression of exogenous ERRs fusion protein

To analyze the subcellular expression of ERRs protein in the ovarian cancer cells, the plasmids of hERRα-GFP and HA-tag-hERRγ were transfected into the ovarian cancer cell lines OVCAR-3 and SKOVR-3. Examined by Confocal Scan and Western-Blot method, these exogenous fusion proteins can report the subcellular distribution of ERRs. In general, results from the Confocal Scan and Western-Blot demonstrate that both of the exogenous hERRα and hERRγ were chiefly expressed in the ovarian cancer cell nucleus.

3.2.1 Subcellular localization of hERRα-GFP fusion protein

After successful construction of the hERRα-GFP reporter plasmid, 1 μg hERRα-GFP reporter plasmid mixed with 6 μl FuGENE6 per chamber was transfected into the ovarian cancer cells SKOV-3 and OVCAR-3 cultured on the 4-chamber-sildes (Roche, Mannheim, Germany). Derived by the thymidine kinase promoter, the hERRα-GFP reporter plasmid can produce a fusion protein of the green fluorescent protein and hERRα protein. Excited by the 480 nm illuminations (FITC), the part of green fluorescent

protein can produce a high-level auto-fluorescent green signal (Figure 3.5 A, B). To more clearly distinguish the cellular nuclear and cytoplasm, the transfected cells were also stained by 1:1000 diluting DAPI. Excited by the 330 nm illuminations (UV), the cell nucleus stained with DAPI can give a blue colour (Figure 3.5 E-H). The hERRα-GFP fusion protein was chiefly observed in the nucleus of ovarian cancer cell OVCAR-3 judged by using confocal scanning microscopy (Figure 3.5 A, D). Compared to the high green signal expressed in the nucleus of ovarian cancer cell, almost no auto-fluorescent green signal could be observed in the cytoplasm (Figure 3.5 E, F). Serum-free DMEM medium was used as a negative control. In the control cells, which were cultured without the transfection, neither the cell nucleus nor cytoplasm could be observed the green signal (Figure 3.5 C, D, G, H).

3.2.2 Expression of exogenous HA-tag-hERRγ protein

3.2.2.1 Quantification of protein (BSA assay)

The protein concentration was determined by ELISA method (Pierce). A standard protein concentration-O.D. absorbance curve was set by the different O.D absorbance of the varied concentration of standard protein (Figure 3.6). According to the standard protein concentration-O.D. absorbance curve and the formula, the protein concentration of sample could be calculated out by relatively OD absorbance. In my studies, ovarian cancer cell lines ES-2, OVCAR-3 and SKOV-3 were plated at a density of 3×10^5 cells/well in 35-mm plates. After 24 h culture, in 17 μl serum-free DMEM medium, 0.5 μg or 1 μg pSG-HA-tag-hERRγ plasmid was mixed with 3 μl FuGENE6. The mixture was added into the cells cultured in the 35-mm plates. After transfection for 72 hours, nuclear protein and whole-cell protein were extracted and quantitative according to the protocol provided by the kits (Clontech, PaloAlto, CA, USA).

3.2.2.2 Western-Blot analysis of HA-tag-hERRγ

Some reports had shown the expression of ERRs protein by western-blot detection of the recombined fusion HA-tag-ERR protein in some *in vitro* transcription and translation systems [12,31]. In my studies, 10 μg nuclear protein, 50 μg cytoplasmic protein or 100 μg whole-cell protein per lane was loaded on an 8% polyacrylamide gel. After blotting, the HA-hERRγ recombined fusion protein could be detected by antibodies anti to HA-tag epitope in the nuclear protein extraction and total protein extraction of ovarian cancer cell lines SKOV-3, OVCAR-3 and ES-2 (Figure 3.7 A and B) but not in the cytoplasm protein extraction (Figure 3.7 C). A control group was also set up by treatment with serum-free

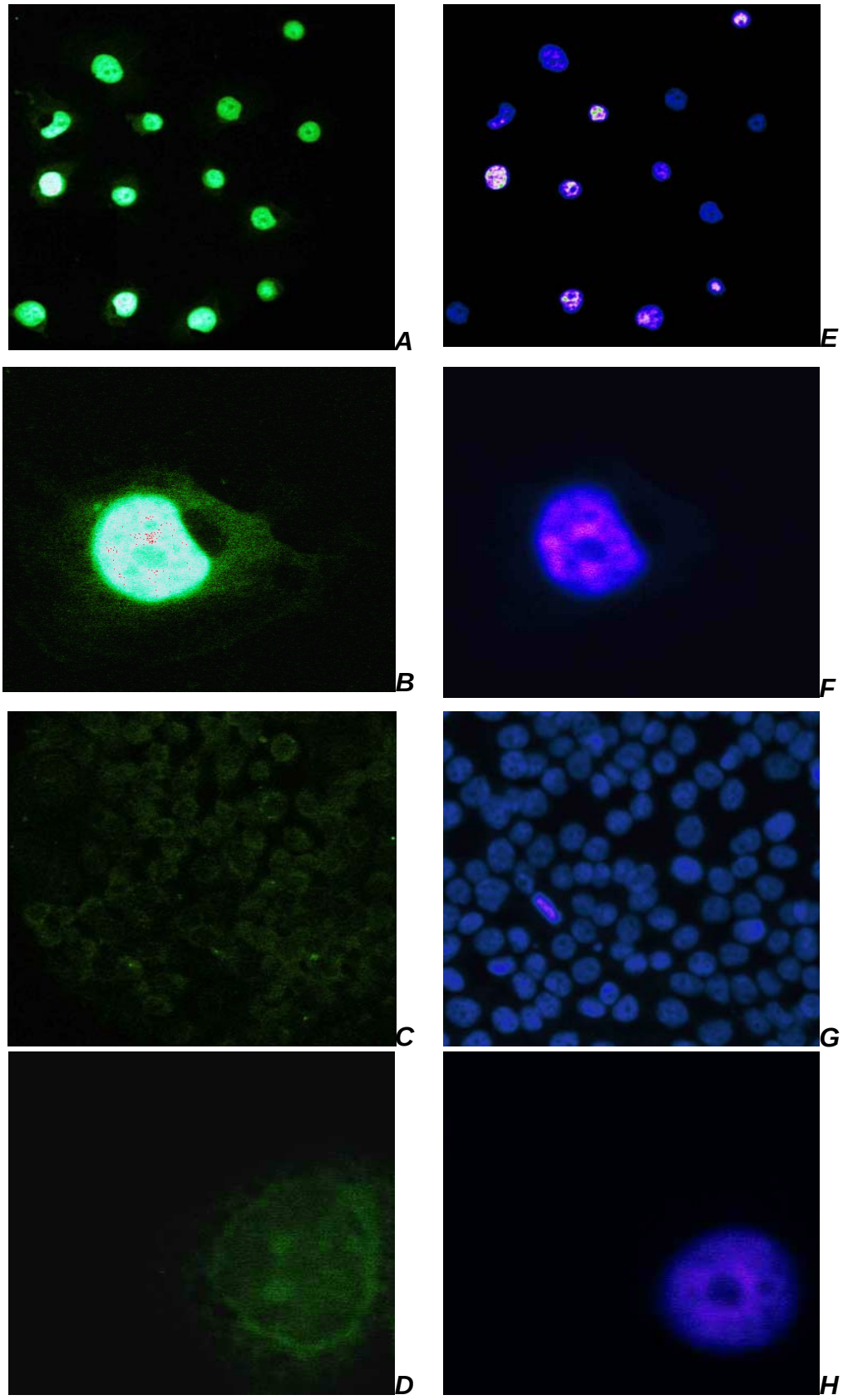


Figure 3.5: The double excited illumination confocal scan microscope. Excited by 480 nm or 380 nm illumination, a green or blue signal could be observed.

Table 3-1: OD absorbance of standard protein (BSA assay)

BSA (μg/ml)	OD	OD	MW
0	0	0	0.000
5	0	0	0.000
10	0.002	0.01	0.006
25	0.022	0.018	0.020
50	0.035	0.037	0.036
100	0.072	0.078	0.075
250	0.158	0.163	0.161
500	0.288	0.303	0.296

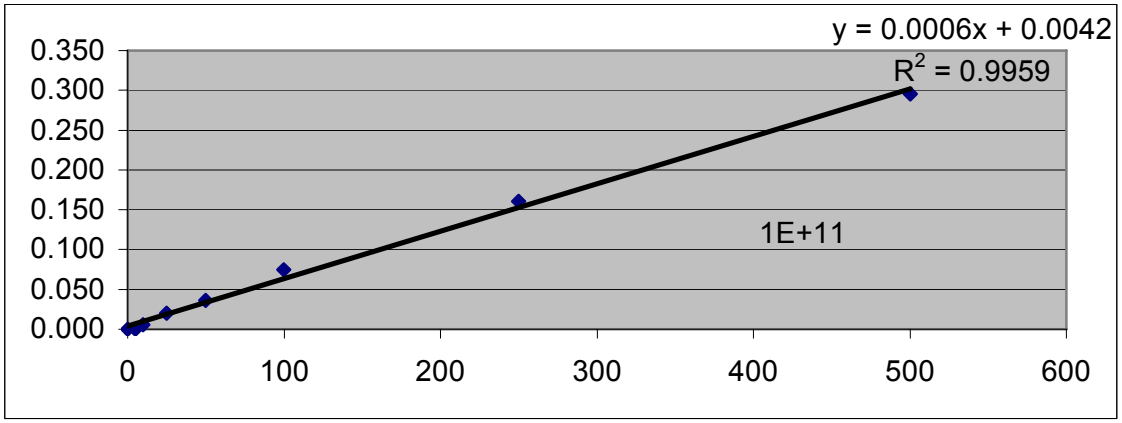


Figure 3.6 The protein concentration-OD absorbance standard curve

DMEM medium. In contrast to the transfected group, there was almost no visible HA-tag-hERRγ special band in the control group (Figure 3.7 B and C).

3.3 Expression of endogenous ERRs protein

An *in vivo* analysis of endogenous ERRs protein expression was also performed in the ovarian cancer cell line as soon as the commercial antibodies anti to hERRα, hERRβ, hERRγ were available [71]. Moreover, 33 samples of ovarian cancer and 12 samples of normal ovary were also included in the immunohistology analysis. The expression of endogenous protein was scored as immunoreativity. More than 500 cells were calculated for each sample and labeling index (LI) as follows: 0%-1% positive staining cells, index as (-); 2%-9% positive staining cells, index as (±); 10%-30% positive staining cells, index as (+); 31%-50% positive staining cells, index as (++); >50% positive staining cells index as (+++). According to some reports, the samples with more than 10% (≥10%) positive staining cells could be considered as positive-expression sample.

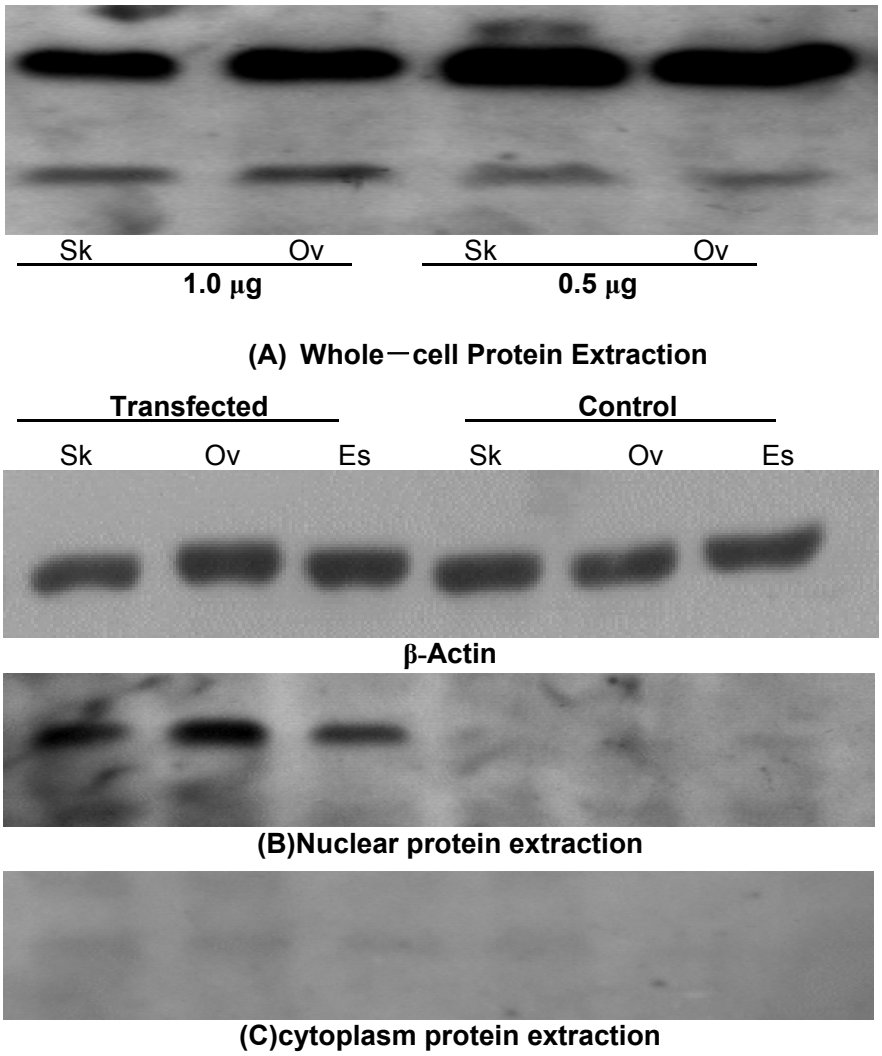
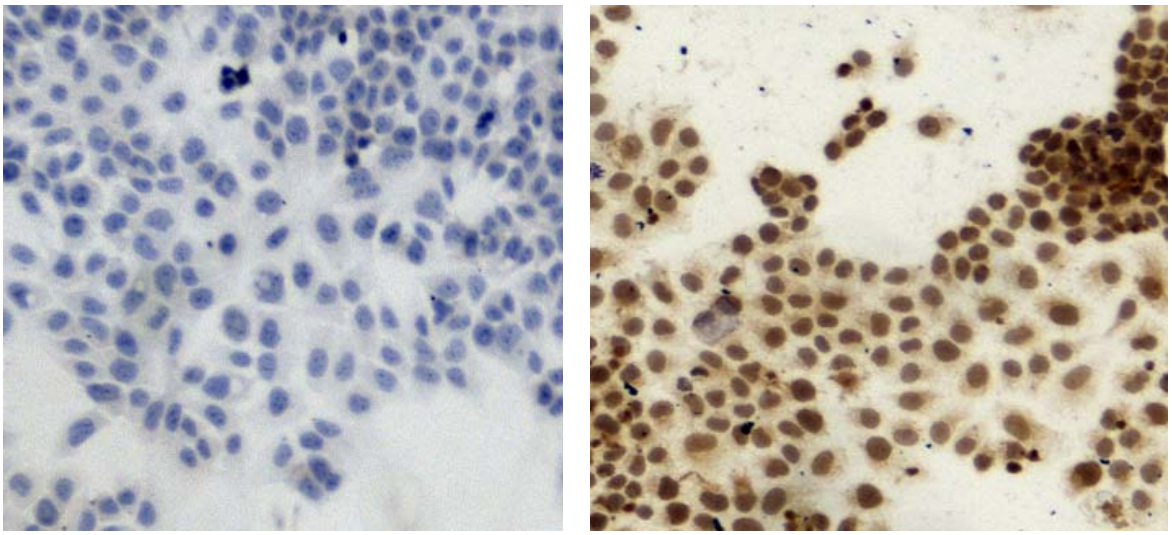


Figure 3.7: Expression of HA-tag-hERRγ protein (A) Whole-cell protein extraction: ovarian cancer cell line SKOV-3 (SK) and OVCAR-3 (OV) were transfected with 1.0 μg or 0.5 μg pSG5-HA-hERRγ plasmid. After 48 h incubation, the HA-tag-hERRγ fusion protein can be detected in the whole cell protein. (B) Nuclear protein extraction and (C) Cytoplasm protein extraction: transfected with 1.0μg pSG5-HA-hERRγ plasmid and incubated for 48 h, a special band was detected in the nuclear protein extraction from ovarian cancer cell lines ES-2 (ES) ,SKOV-3 (SK) and OVCAR-3 (OV). In contrast, there was almost no visible band of hERRγ fusion protein in the control group, which was only treated with DMEM serum-free medium. Moreover, no visible bands could be detected in the nuclear of cytoplasm extraction.

3.3.1 Expression of ERRs protein in the established ovarian cancer cell lines.

All of the five ovarian cancer cell lines were performed immunochemistry analysis of hERRα, hERRβ as well as hERRγ and scored as LI. Results from the immunochemistry

also showed the endogenous ERRs protein of cultured ovarian cancer cells are chiefly expressed in the cell nucleus (Figure 3.8), which is similar to the results from the exogenous ERRs protein analysis. All the cell lines are hERRα positive-expression, moreover, cells MDAH-2774, OVCAR-3, SKOV-3 are strong positive-expression of hERRα (LI>30%) and the immunoreactivity of hERRα in ovarian cell lines OAW-42 and ES-2 are 23% and 13%, respectively. Not as the high immunoreactivity could be detected in the ovarian cancer cell lines, the hERRβ protein showed limited immunoreactivity in these five cell lines. Only cell lines OVCAR-3 and SKOV-3 were observed with an increasing immunoreactivity of hERRβ-1, and SKOV-3 showed a positive immunoreactivity of hERRβ-2 (LI=17%). The average immunoreactivity of hERRγ was 23% in these cell lines. Among them, OVCAR-3 has a very strong positive-expression of hERRγ (+++) and the cell lines SKOV-3 and MDAH-2774 had mildly positive-expression of hERRγ (+~++).



A ***Objective×10, lens×20***

B ***Objective×10, lens×20***

Figure 3.8: Immunochemistry result of hERRα in ovarian cancer cell line OVCAR-3 (A) cells without treated with anti-hERRα antibodies was used as negative control (B) positive-staining of hERRα

3.3.2 Expression of the ERRs protein in the *in vivo* ovarian cancer tissues

The mean value of ERRα immunoreactivity in the 33 ovarian cancer cancers and the 12 normal ovaries examined was 22.4% (0-87%). The immunoreactivity and positive-expression rate of ERRs in the ovarian cancer samples and normal ovarian tissues were summarized in the Table 3-1 and 3-2. The mean immunoreactivity of

hERRα (32.3%, rang from 0%-87%) in the ovarian cancers was significantly higher than in the normal ovaries (9.2%, range from 0%-26%). The mean immunoreactivity of hERRβ or hERRγ between the ovarian cancer group and the normal ovaries group has no significance difference. The hERRα positive-expression (defined as the positive staining cells>10%) was detected in 19 of 33 ovarian cancer (57%) and 2 of 12 normal ovaries (16%). Compared to the normal ovarian tissues, ovarian cancer showed a higher hERRα positive-expression rate (p=0.02).

Table: 3-2: Immunoreactivity of ERRs in ovarian cancers and normal ovaries

	Ovarian cancer Mean(range)	Normal ovaries Mean(rang)	*P**
hERRα **	32.3(0%-87%)	9.2(0%-26%)	0.048
hERRβ **	10.3(0%-21%)	6.8(0%-9%)	0.662
hERRγ **	22.7(0%-58%)	20.9(0%-63%)	0.245

*Chi-square test; ** by using immunochemistry, ERRα, ERRβ and ERRγ can not be distinguished with their isoforms.

Table 3-3: Positive-expression rate of ERRs in ovarian cancers and normal ovaries

	Ovarian cancer N=33(100%)	Normal ovaries N=12(100%)	*P**
hERRα **			
Positive	19(57.6%)	2(16.7%)	0.020
Negative	14(43.4%)	10(83.3%)	
hERRβ**			
Positive	3(9.1%)	0(0%)	0.543
Negative	30(90.9%)	12(100%)	
ERRγ**			
Positive	16(48.5%)	4(33.3%)	0.045
Negative	17(51.5%)	8(66.7)	

*Chi-square test; ** by using immunochemistry, ERRα, ERRβ and ERRγ can not be distinguished with their isoforms.

The mean immunoreactivity of hERRβ (10.3% range from 0%-21%) in the ovarian cancers was similar to the mean immunoreactivity of the normal ovaries (6.8%, range from 0%-9%). Only 3 ovarian cancer samples were observed with hERRβ positive expression. We did not detect any positive expression of hERRβ protein in the normal ovaries. The positive-expression rate of hERRβ (including the hERRβ1 and hERRβ2)

was 9.1% in the ovarian cancer and 0% in the normal ovarian tissues. There are no different positive-expression rate and immunoreactivity of hERRβ between the ovarian cancers and normal ovarian tissues (p=0.543 and p=0.662). hERRγ positive-expression was observed in 16 of 33 ovarian cancer (49%) and 4 of 12 normal ovaries (33%). Similar to hERRα, the positive-expression of hERRγ was also significantly increased in ovarian cancer patients (p=0.045). However, the difference of mean immunoreactivity between the ovarian cancers (22.7%) and the normal ovaries (20.9%) was not observed to be significant (p=0.245).

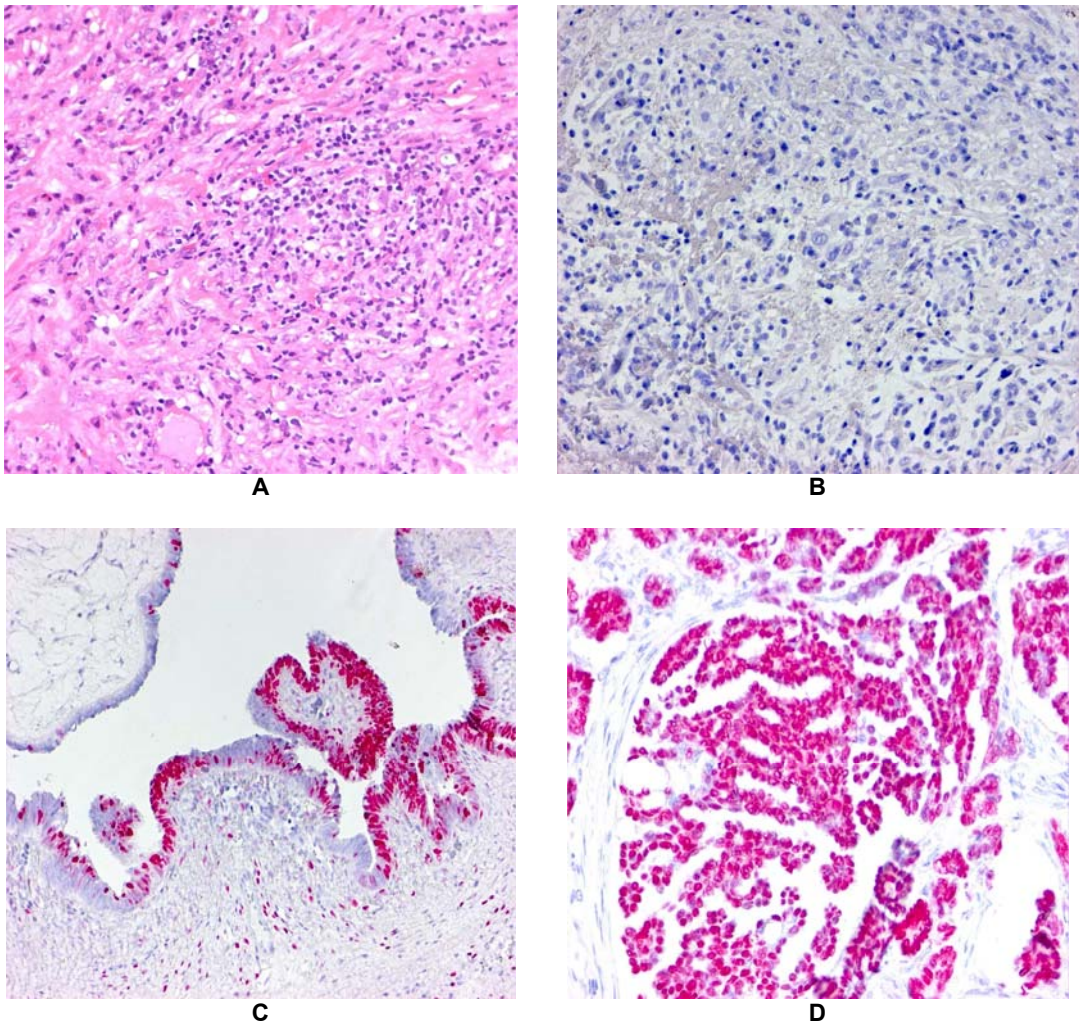


Figure 3.9: The *in vivo* expression of hERRα in the ovarian cancer samples. (A) HE staining of the ovarian cancer section, (B) One of the hERRα negative-expression samples. (C) One of the hERRα positive-expression and label index (LI) as (++); (D) one sample of very strong positive-expression of hERRα and label index as (+++).

The immunochemistry staining of hERRα expression can be seen in the Figure 3.9. The

same staining method was also performed in the detection of hERRβ and hERRγ expression (the image did not show here). Compared with the stroma, the expression of ERRs are chiefly located in the ovarian epithelium, which is the major tissue aroused ovarian cancer.

3.4 Quantification analysis on the ERRs mRNA levels

The *in vivo* mRNA levels of human ERRα, ERRβ, ERRγ as well as ERα and ERβ in ovarian cancer tissues were analyzed by LightCycler Quantitative RT-PCR (Figure 3.10). A series of increasing-concentration plasmids (serial diluted in TE buffer, from 10^7 copies/μl to 10 copies/μl) containing the target genes was performed amplification during every analysis to set up the standard curves of the fluorescence intensity plotted against the logarithm of the plasmid concentration (Figure 3.10 A). According to the protocol provided by the manufacturer, the accuracy of experiments was confirmed by using a controlled standard curve with an error rate less than 0.2 (Figure 3.10, B and C), and each amplification was repeated 3 times. The quantitative concentration of the target genes was analyzed by the software (Figure 3.10).

A housekeeping gene, GADPH, was also amplified to control the mRNA integrity.

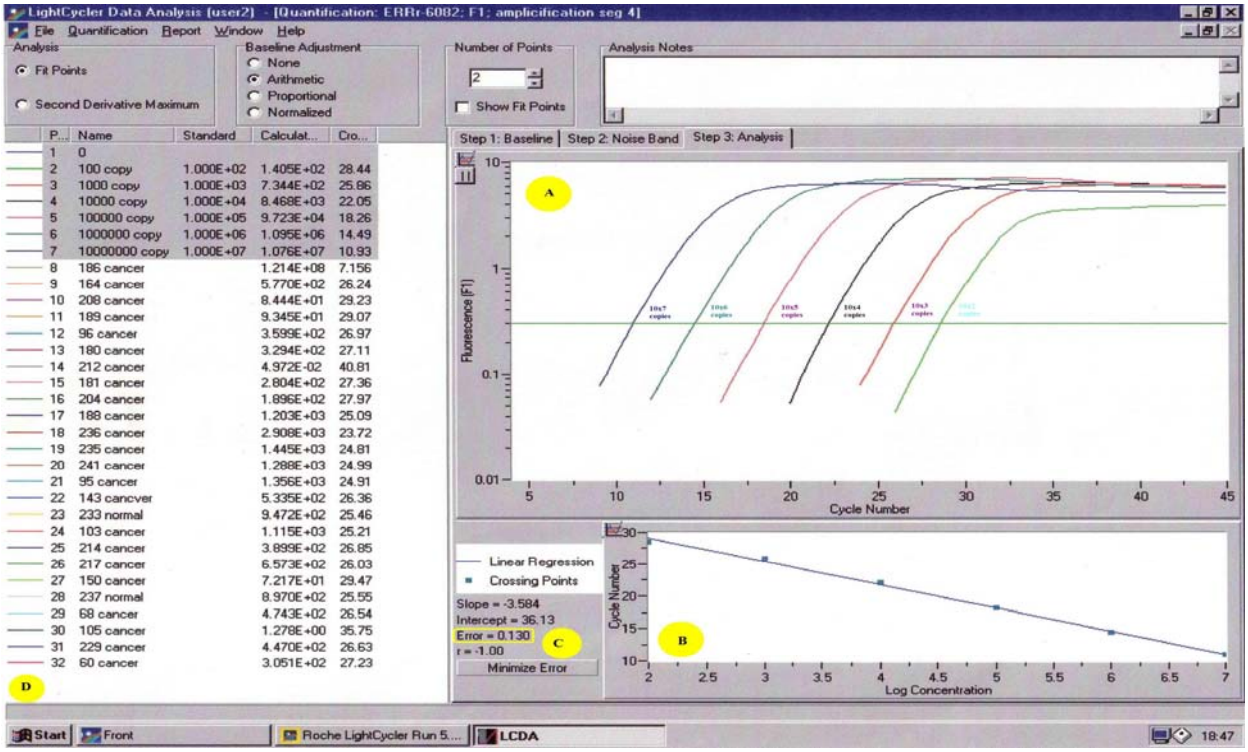


Figure 3.10, The result of LightCycler Quantitative PCR analysis. (A) Amplification of control plasmid contain the target genes from 10copies/μl to 10^7 copies/μl (B) The standard curve of target gene concentrations (copies/μl) and fluorescence intensity plotted against the logarithm. (C) The error control of the standard curve should be less than 0.2. (D) result of the sample.

Furthermore, after the quantitative analysis of two-step reverse transcriptional PCR, the amplified product was also running on a 2% agarose gel and examined under a UV light. Stained by the SYBR GREEN I, the amplifications of DNA can be seen under the UV light (Figure 3-11).

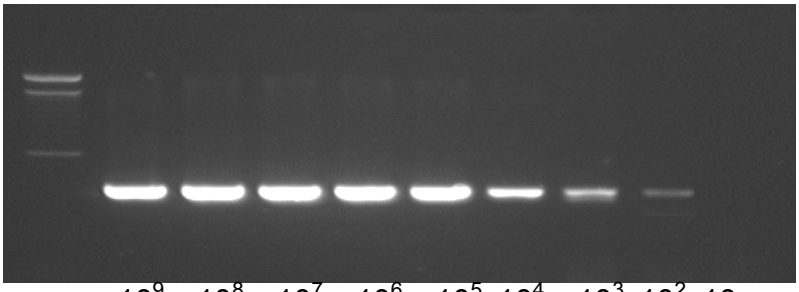


Figure 3-11: After LightCycler PCR: the amplifications of standard plasmids containing the target genes were also running on 2 % agarose gel. The result confirmed the accuracy of LightCycler Q-PCR.

3.4.1 ERRs mRNA levels in ovarian cancer cell lines

There are at least two major isoforms of ERRα, human ERRα (full length cDNA 2421 bp) and human ERRα-1 (full length cDNA 2221 bp) [33,40,47,58]. I use a special primer set to amplify a 482 bp fragment on A/B domain of ERRα-1, which can be found both in the ERRα and ERRα-1. These products were observed in all 5 ovarian cancer cell lines and name as hERRα in this study. In contrast to the high expression of hERRα, expression of hERRβ-1 and its isform hERRβ-2 seemed minimal in the ovarian cancer cell lines. hERRβ-1 was detected in ovarian cancer cell lines OVCAR-3 and SKOV-3, hERRβ-2 could only be detected in cell lines SKOV-3. By applying ERRγ special primer set, an amplified product of 395 bp fragment was detected in ovarian cancer cell lines MDAH-2774, OVCAR-3 and SKOV-3. The results of LightCycler Q-PCR were also analyzed by running on a 2% agarose gel (Figure 3-12, A-H). The quantitative mRNA levels in these established ovarian cancer cell lines were listed in the Table 3-4.

In general, the Q-PCR results of mRNA levels and the immunoreactivity of the protein expression had a closed correlation (r=0.768, p=0.041). The mRNA concentrations of different ovarian cancer cell lines were listed in the Table 3-4. In this study, the concentrations of amplifications transcripted from total RNA is higher than 100 copies/ng RNA could be seen a visible band in a 2% agarose gel staining by the SYBR Green I. The sample's mRNA concentration, which is higher than 2×10^2 copies/ng RNA was defined as mRNA positive-expression.

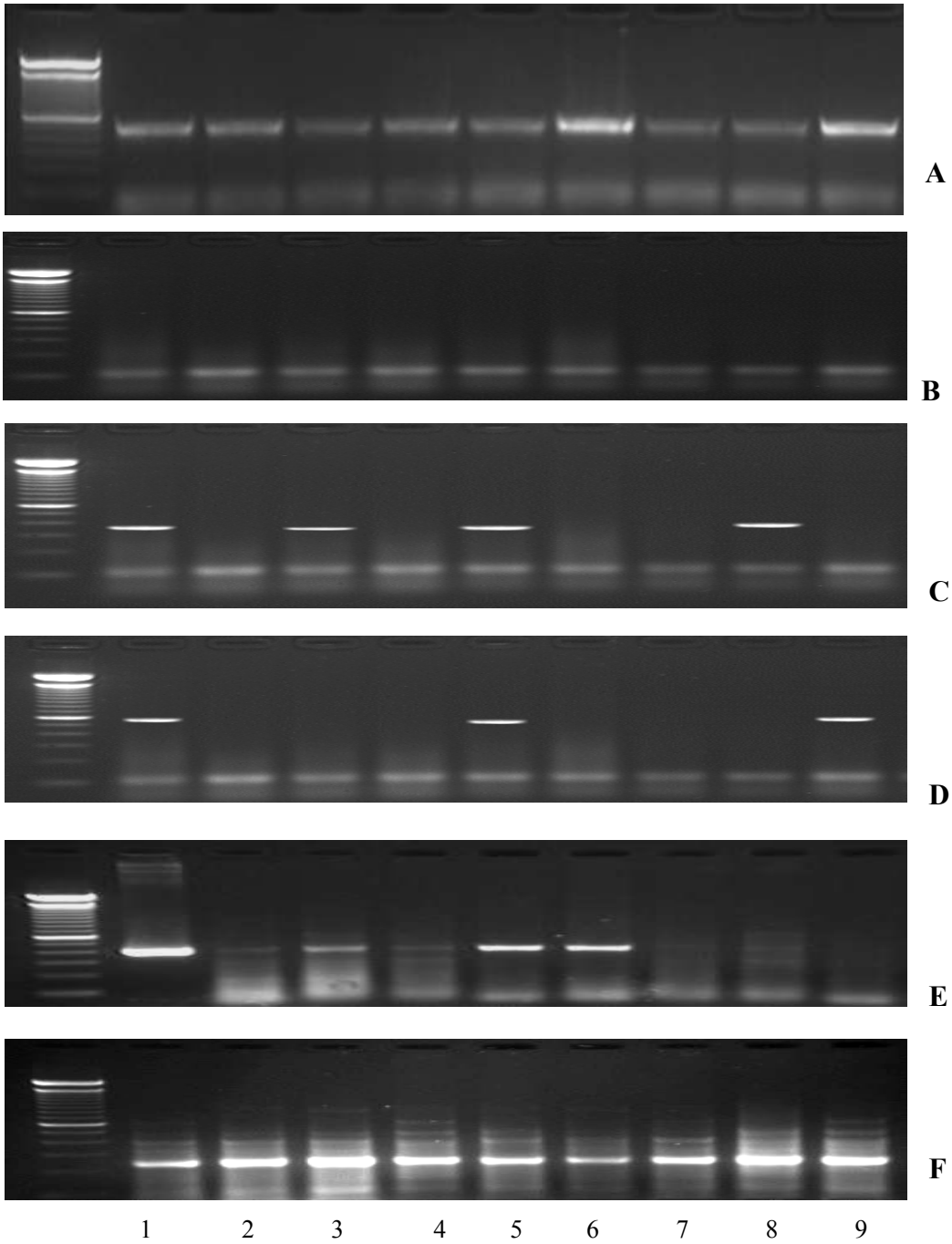


Figure 3-12: Lane 1 represent for the positive controls (plasmids containing the target sequence) lane 2-6 stand for ovarian cancer cell lines ES-2, OVCAR-3, OAW-42, SKOV-3, MDAH-2774, 7-9 represent for the ovarian cancer samples. M represent marker (100bp ladder). Picture (A) ERRα, (B) ERRα-1, (C) ERRβ-1, (D) ERRβ-2, (E) ERRγ, (F) GADPH.

Table 3-4: The mRNA level of ERRs and ERs in ovarian cancer cell lines

Cell lines	hERα 115bp	hERβ 105 bp	hERRα* 100bp	hERRβ* 340bp	hERRγ 395 bp
ES-2	(-) 178.00	(+) 392.00	(+) 488.00	(-) 98.00	(-) 132.00
Mdah-2774	(+) 216.00	(-) 196.00	(+) 3040.00	(-) 166.00	(+) 1626.00
SKOV-3	(+) 3998.00	(+) 2526.00	(+) 9968.00	(+) 872.00	(+) 2394.00
OVCAR-3	(+) 12240.00	(+) 18960.0	(+) 3002.00	(+) 298.00	(+) 10118.00
OAW-42	(-) 88.00	(-) 94.00	(+) 944.00	(-) 102.00	(-) 124.00
MCF-7	(+) 32260.00	(+)8890.00	(+) 284.20	(-)188.00	(+)1208.00

*hERRα and hERRβ-1 primer sets were used to stand for hERRα and hERRβ

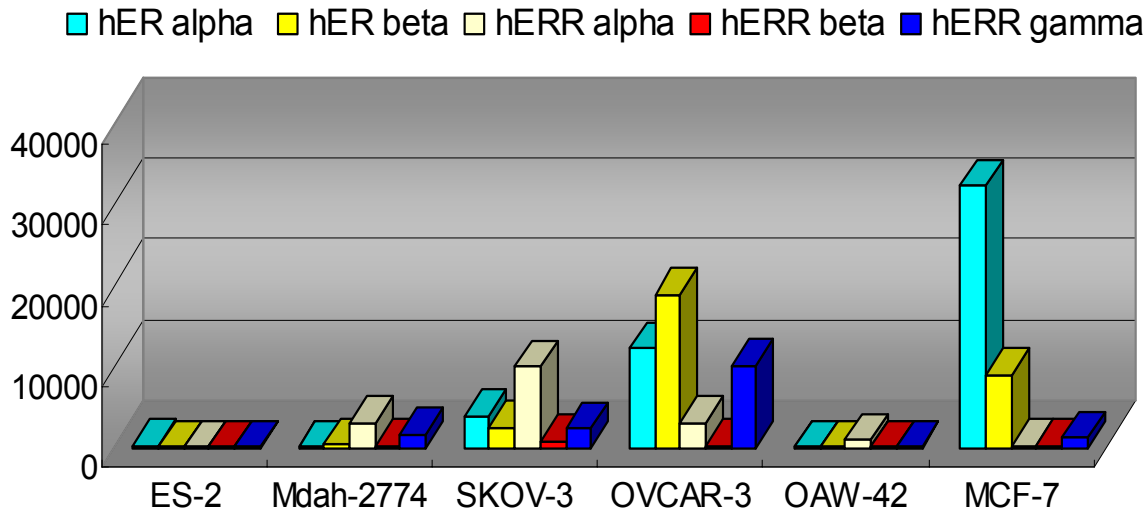


Figure 3.13: Quantitative RT-PCR results of ER family and ERR family members mRNA levels in ovarian cancer cell lines.

3.4.2 The ERRs mRNA levels in the ovarian cancer

To conveniently compare the mRNA expression levels of ERRα, ERRβ, ERRγ, ERα and ERβ, the hERRα-1 special primers set and hERRβ-1 premiers set were used in the Q-PCR and stand for the hERRα and hERRβ. Because some reports had pointed out that the hERRα-1 and hERRβ-1 are the major isoforms expressed in the human [40,44,47]. In this research, the results demonstrate that ERRα and ERRγ mRNA expressed broadly in the ovarian cancer cell lines and the primary ovarian cancers, however, ERRβ expressed poorly in the ovarian cancers. The sample size in this study was modest: 33 ovarian cancers and 5 established ovarian cancer cell lines. Hence,

some important results or relationship in ERRs should be remained discussing by a large-scale research.

Table 3-5: mRNA level of ERRs and ERs in ovarian tissues

Genes	Ovarian cancer (N=33)				Normal ovaries (N=12)				P*
	Min	Max	Mean	Median	Min	Max	Mean	Median	
hERRα	68.0	14432.0	2456.2	1184.0	7.6	4514.0	666.8	188.0	<0.05
hERRβ	2.0	1816.0	209.4	55.8	15.2	741.8	189.3	55.8	>0.05
hERRγ	60.0	12140.0	1796.6	974.8	102.2	7064.0	1493.8	730.8	>0.05
hERα	47.6	22308.8	4224.0	2850.0	56.8	18266.0	2554.0	1194.0	<0.05
hERβ	32.8	9748.0	1556.0	704.8	66.6	5182.2	15840	958.0	>0.05

**t* test were performed

Using the Q-PCR analysis, the mRNA expression of ERRs and ERs could be detected in almost all the samples (from 2.0 copies/ng RNA to 22308.0 copies/ng RNA). It may be the result of high-sensitive Q-PCR analysis. Combined with the result of Q-PCR and the images of the electrophoresis, we defined the sample with more than 2×10^2 copies/ng target genes as RNA positive-expression. LightCycler RT-PCR analysis revealed that the presences of ERα, ERβ, ERRα, ERRγ transcripts in ovarian cancer cell lines are very common phenomena. In contrast to their high expression in ovarian cancers, the mRNA levels of ERRβ seem lower in the established ovarian cancer cell lines as well as in primary ovarian cancer samples (12.1%, 4/33). By using ERα special primer set an amplified product of 115 bp corresponding to nucleotides 821-936 of the full-length ERα cDNA sequence was detected in 20 samples of ovarian primary cancer (66.6%, 22/23). Expression of ERα mRNA exhibited the highest mRNA levels (mean level, 4224 copies, 48-22308 copies/ng RNA) among evaluated nuclear receptors in approximately 67% ovarian cancer samples and 60% normal ovarian tissues. We also demonstrated expression of hERRα transcript in 60.6% (20/33) ovarian cancer samples and 25% (3/12) normal ovarian tissues. Expression of hERRα was the second highest genes in these samples. The mRNA expressions of other genes were summarized in the Figure 3.14 and Table 3-5. Compared with the normal ovarian tissues, the mRNA concentration of hERRα and hERα increased in the ovarian cancers. Although positive-expression rate of hERRγ in the ovarian cancer was significant different with the rate in normal ovarian tissues, the immunoreactivity and mRNA concentration of hERRγ were no significant different between these two groups.

3.5 Different expression pattern of ERRα and ERα

I also compared the *in vivo* mRNA expression pattern of hERα and hERRα. hERα and hERRα co-expression (defined as both of their mRNA levels are higher than 2×10^2 copies/ng RNA) was detected in 15 ovarian cancer samples and 2 normal ovaries, hERβ and hERRα co-expression was detected in 13 ovarian cancer samples. In all 33 ovarian cancer samples, there are only 5 samples of hERs+/hERRs- expression pattern and 8 samples without any expression of either hERα or hERRα. In this work, 72% ovarian cancer samples (24/33) were detected an hER-positive expression (including hERα+, hERβ+ and hERα+/hERβ+). An increasing expression of hERRα tends to be associated with a decreasing expression of ERα. However, this association is not significant (p=0.292). Similarly, an increasing expression of hERRα tends to be associated with and a decreasing expression of hERRγ. Of all 33 ovarian cancers, 2 (6.1%) samples showed expression of all the 3 members of ERRs, 4 (12.1%) samples co-expression of hERRα and hERRγ, 1 (3.0%) sample co-expression of hERRα and hERRβ, 12 (36.4%) samples only expression of hERRα and 10 (30.3%) samples only expression of hERRγ.

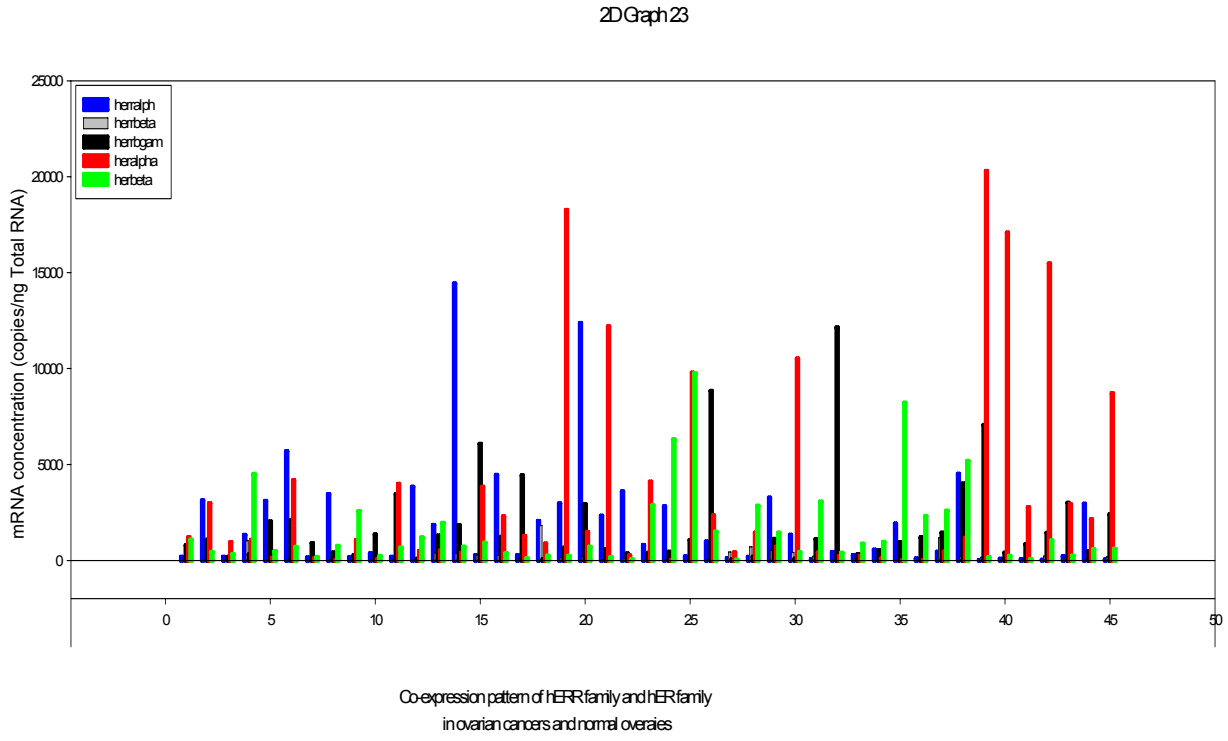


Figure 3.14: Co-expression pattern of ER family and ERR family in ovarian cancer and normal ovaries. Increasing expression of hERRα seemed to be associated with decreasing expression of ERα and ERβ (although there is no statistical significance).

Table 3-6: Co-expression pattern of ERs and ERRs in cell lines

	hERα	hERβ	hERRα-1	hERRβ-1	hERRγ
ES-2	(-)	(+)	(+)	(-)	(-)
Mdah-2774	(+)	(-)	(+)	(-)	(+)
SKOV-3	(+)	(+)	(+)	(+)	(+)
OVCAR-3	(+)	(+)	(+)	(+)	(+)
OAW-42	(-)	(-)	(+)	(-)	(-)
MCF-7	(+)	(+)	(+)	(-)	(+)

MCV-7 cell lines were used as a control

3.6 CA125 in patients with different ERR expression

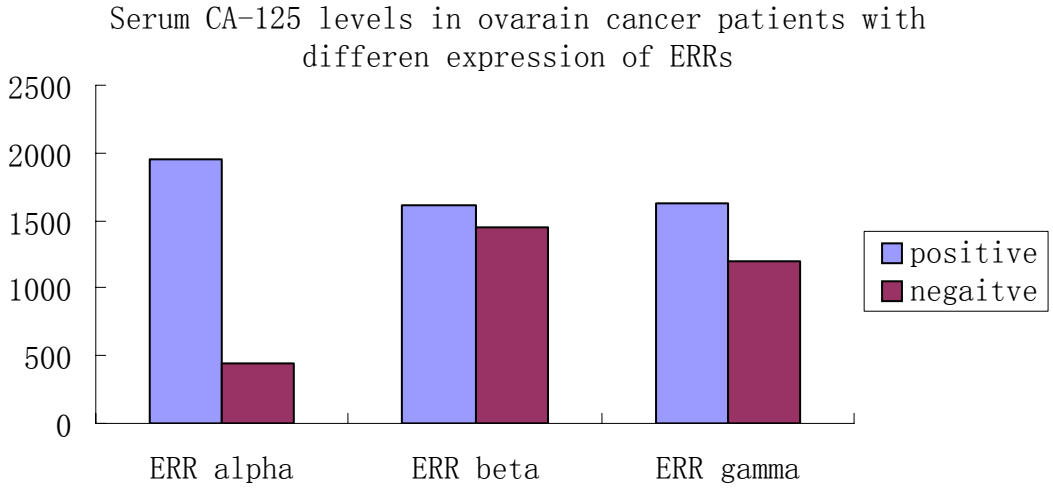
3.6.1 Definition of ERRs positive-expression

CA-125 is the most important well-established tumor marker in the clinical management of ovarian cancer [74]. To explore the potential clinical use of ERRs as tumor markers, I also analyzed the association of the serum CA-125 levels with the expression of ERRs (Figure 3.15). In the above part of this study, I had analyzed the expression of ERRs both in the protein level and in mRNA level. In the following studies, ERRs positive-expression was defined as both the protein level (by ICH method, immunoreactivity >10% positive staining cells) and mRNA level (by Q-PCR analysis, RNA concentration > 2×10^2 copies/ng total RNA) are positive. According to this definition, there are 19 hERRα positive-expression samples, 16 hERRγ positive-expression samples and 3 hERRβ positive-expression samples in the total 33 ovarian cancer samples. In normal ovaries, there are 2 hERRα positive-expression cases and 4 hERRγ positive-expression cases.

3.6.2 Association between the expression of ERRs and serum CA-125

According to the protocol provided by the kit, serum CA-125 level higher than 35U/ml is defined as abnormal level, which indicates a potential malignancy. In this study population of ovarian cancer, the average value of CA-125 was 1303.5±466.8 U/ml (5.0-15489.0 U/ml). The correlation between of the serum level and ovarian malignancy was analyzed by the SPSS software. A good correlation was set in this research (r=0.573, p=0.037).So, the serum CA-125 level could be thought as a good tumor marker in this study. The mean of the CA-125 level in hERRα-positive group (1954.8 U/ml) was higher than hERRα-negative group (448.6 U/ml) (p=0.012). In the hERRα-positive group, 2 cases were detected with very high levels of CA-125. To exclude the impact made by these 2 cases, we performed a new analysis excluding

them. The results showed that there still was a significant difference (p=0.016). Thus, we think the significant difference of CA-125 levels was not due to a few outliers, but due to the different expression of hERRα. In contrast to hERRα, CA-125 levels in the hERRβ-positive group (1675.8 U/ml) showed no difference from the hERRβ-negative group (1454.5 U/ml) (p=0.795). Similar to hERRβ, the hERRγ-positive group and hERRγ-negative group did not show a significant difference in the serum CA-125 levels (1622.0 U/ml vs. 1201.1 U/ml, p=0.515). Correlation analysis showed the increasing expression of hERRα was positive associated with the increasing CA-125 serum level (r=0.472, p=0.048) However, although the increasing expression of hERRγ seemed to be associated with a decreasing serum CA-125 level, there is no significance (r=0.793, p=0.088)



group	+	-	+	-	+	-
case	19	14	3	30	16	17
p	0.012		0.795		0.515	

Figure 3.15: Serum CA-125 levels in ovarian cancer patients with different expression of ERRs. Independent T-test was used to analyze the parametric data. In the hERRα positive group, 2 cases were outside the serum CA-125 levels of the hERRα negative group. There is still a significant difference between the hERRα positive group without these 2 cases and the negative groups (p=0.016)

3.7 Survival analysis of patients with different ERRs expression

All patients were enrolled in a follow-up program as soon as they received the primary anticancer therapy. The median follow-up time was 31.54 months (2.0- 76 months). Valid follow-up data were available for 29 cases (87.8%) of 33 ovarian cancer patients. The median overall survival (N=29) was 26.8 months (2.0 to 65.0 months), and the median

progression-free survival time was 13.8 months (1.0 to 40.0 months; other details can be seen in Table 3-6 and Figure 3.16). The median overall survival time of ovarian cancer patients with hERRα-positive expression was 19.0 months, compared to hERRα-negative group, the overall survival time was significantly reduced (log rank test, p=0.015). The median progression-free survival of hERRα-positive group and hERRα-negative group was 12.6 months and 14.5 months, respectively (p=0.820). The median overall survival showed no significant difference between hERRγ-positive group (23.4 months) and negative group (19.6 months, log rank test p=0.092). However, the hERRγ-positive ovarian cancer patients had a longer progression-free survival time (18.0 months) than the hERRγ-negative group (13.5 months, log rank test p=0.020). We used a multivariate analysis to test the independent value of each parameter predicting overall survival and progression-free survival. Expression of hERRα was an independent prognostic factor for poor survival (relative risk, 3.032: 95% CI: 1.27-6.06). Other independent prognostic factors associated with poor prognosis were histological grade and FIGO stage (Table 3-7). Volume of ascites and expression of hERRγ were not independent prognostic factors for poor survival.

Table 3-6: Overall survival time and progression-free survival time of patients with different expression of ERRs (n=29)

	Cases	Median overall survival Months (95%CI)	p*	Median progression-free survival Months (95%CI)	p*
hERRα					
positive	15	19.0(6.9-27.4)	P_o=0.015	12.6(9.1-16.1)	P_f =0.820
negative	14	31.5(13.1-54.2)		14.5(10.9-17.1)	
hERRβ					
positive	2	28.2(20.4-38.6)	**	16.8(4.8-27.6)	**
negative	27	30.4(20.9-45.7)		22.4(12.7-31.6)	
hERRγ					
positive	14	23.4(12.3-37.1)	p_o=0.093	18.0(14.5-21.5)	p_f =0.020
negative	15	19.6(13.6-28.4)		13.5(9.8-17.0)	

*p_o=*overall survival,* p_f=*progression survival,***the number of ERRβ positive cases were too small to analyse*

3.8 The association between expression of ERRs and clinical parameters

Talbe3-7: Multivariate survival analysis (Cox regression model, N=29)

	Beta	Standard error	Wald	df	Relative Risk	95% CI of RI	P Value
hERRα			9.172	1			0.044
negative					1.00		
positive	1.159	0.593	9.172	1	3.03	1.27-6.06	0.044
hERRγ			6.748	1			0.343
negative					1.00		
positive	0.809	0.474	6.748	1	1.371	0.588-2.199	0.343
histology			0.958				0.477
serous					1.00		
non-serous	-0.581	0.593	0.958	1	0.930	0.175-1.142	0.477
FIGO			10.306	3			0.012
I					1.00		
II	-0.144	0.794	4.679	1	2.332	0.274-2.199	0.038
III	1.084	0.889	9.203	1	7.068	0.607-13.403	0.002
IV	3.133	0.903	13.064	1	22.938	2.326-59.55	0.001
grade			3.843	2			0.048
I					1.00		
II	0.826	0.582	3.420	1	3.285	1.147-9.892	0.044
III	0.944	0.580	4.843	1	4.326	2.173-10.395	0.020
ascites			0.985	2			0.685
0					1.00		
<500	0.128	0.337	0.145	1	1.137	0.588-2.199	0.737
≥500	-0.407	0.391	1.081	1	0.845	0.309-1.433	0.666

** The number of ERRβ positive cases was too small to analyse*

The association between expression of ERRs and clinicopathological parameters such as FIGO stage, grading ascites and histological types was analyzed by SPSS software (version 11.0, CA, USA). Bivariate correlation analysis showed that the expression of hERRα mRNA is significant correlation with the FIGO stage (p=0.017) and histological

grading (p=0.022). Further details can be seen in Table 3-7.

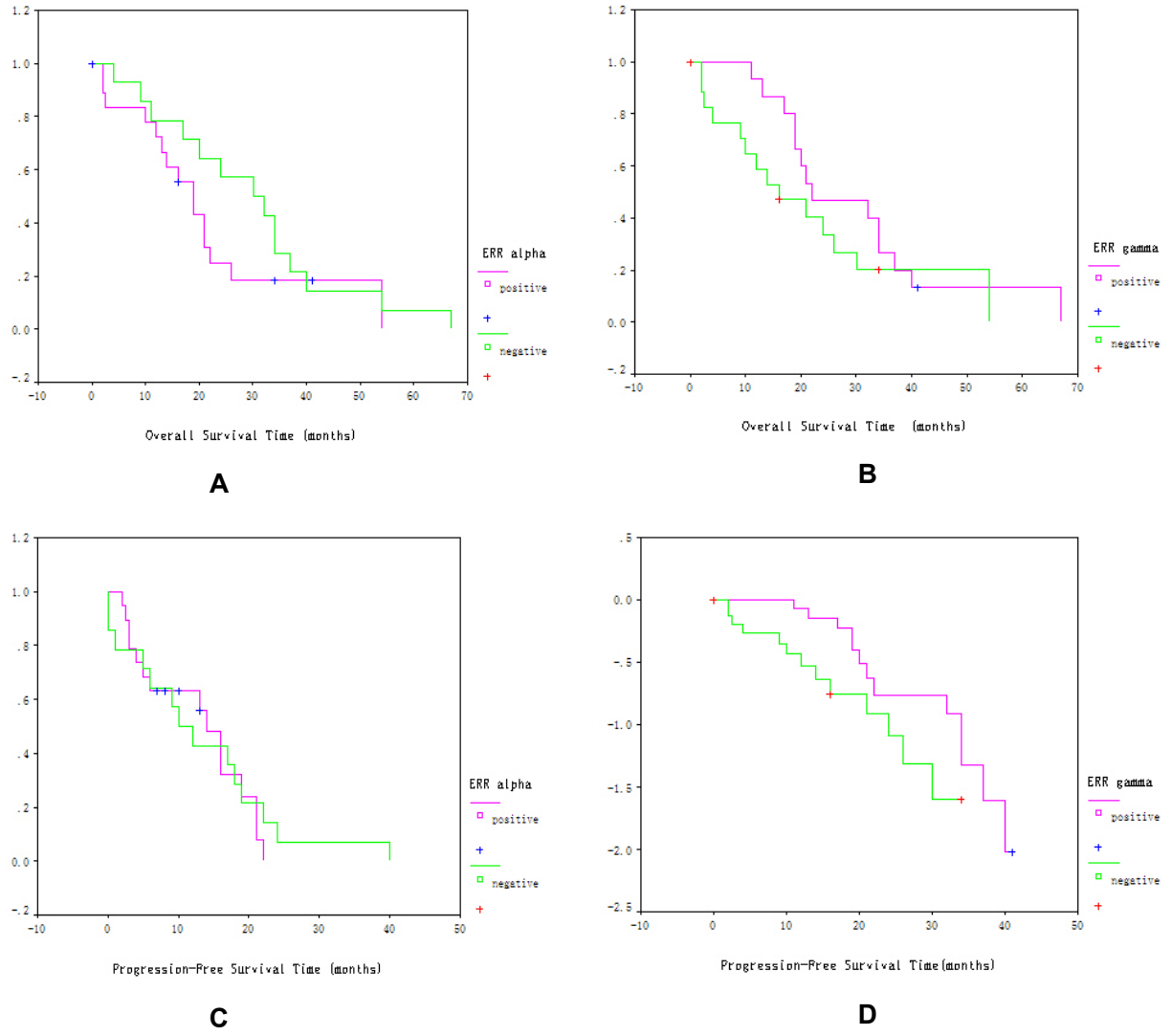


Figure 3.16: Overall survival and progression-free survival curve, analyzed by the Kaplan-Meier method. **A**: The overall survival time of ovarian cancer patients with hERRα expression (N=15, 19.0 months, 95% CI: 6.9-27.4) was significant reduced compared with the hERRα negative group (N=14, 31.5 months, 95% CI: 13.1-54.2) (log rank test, p=0.015). **B:** The progression-free survival time was not difference between the hERRα positive- and negative- groups. **C:** The overall survival time of ovarian cancer patients was not different between the hERRγ positive and negative groups. **D:** hERRγ positive ovarian cancer patients has a longer progression-free survival time (N=14, 18.0 months, 95% CI: 14.5-21.5) than hERRγ negative patients (N=15, 13.5 months, 95% CI: 9.8-17.0) (log rank test, p=0.020).

Expression of hERRα mRNA was associated with more advanced FIGO stage and grading. A positive correlation was also observed between the FIGO stage and

expression of hERRγ (p=0.040). In comparison to hERRα and hERRγ, the number of hERRβ positive samples was not enough to perform an analysis. Moreover, the survival analysis was not possible for the ERRα/ERRβ/ERRγ all negative ovarian cancer patients, because there are only 5 (12.1%) samples.

3.9 Summary of the results

In brief, the results of this work were summarized as follows;

- ► Exogenous and endogenous protein analysis showed that the expressions of human ERRs protein are chiefly located in the cell nucleus.
- ► Both the results of quantitative PCR and immunoreactivity demonstrate an increasing expression of hERRα in the ovarian cancers.
- ► Positive-expression rate of hERRγ was found increasing in the ovarian cancers, however, both the quantitative mRNA levels and immunoreactivity of hERRγ did not show a significant difference between in the ovarian cancers and normal ovaries
- ► A high co-expression of hERα and hERRα was found in the ovarian cancers. Compared with normal ovaries, the ratio of hERα/hERRα seems decreased in the ovarian cancer.
- ► Increasing expression of hERRα is associated with increasing serum CA-125, advanced FIGO stage, poor differentiation (grade) and reduced overall survival time.
- ► Increasing expression of hERRγ is associated with a lower FIGO stage and longer progression-free survival time. Moreover, increasing expression of hERRγ tends to be correlated with the decreasing serum CA-125 and less ascites.

Table 3-8. Clinicopathological characteristics of patients with different expression of ERRs

	ERRα			ERRβ			ERRγ		
	Positive	negative	Correlation*	Positive	negative	Correlation*	positive	negative	Correlation*
Cases (N)	21	24		3#	42		20	25	
Normal ovaries	2	10		0	12		4	8	
Ovarian cancer	19(100%)	14(100%)		3(100%)#	30(100%)		16(100%)	17(100%)	
FIGO stage			0.017			##			0.040
FIGO I	0(0%)	2(14.3%)		0(0%)	2(6.7%)		2(12.5%)	0(0%)	
FIGO II	0(0%)	3(21.4%)		0(0%)	3(10.0%)		3(18.7%)	0(0%)	
FIGO III	13(68.4%)	5(35.7%)		2(66.7%)	16(53.3%)		7(43.8%)	11(64.7%)	
FIGO IV	6(31.6%)	4(28.6%)		1(33.3%)	9(30.0%)		4(25.0%)	6(35.3%)	
Grading			0.022			##			
G I	1(5.3%)	3(21.4%)		1(33.3%)	3(10.0%)		2(12.5%)	2(11.8%)	0.479
G II	7(36.8%)	4(28.6%)		1(33.3%)	10(33.3%)		6(37.5%)	6(35.3%)	
G III	11(57.9%)	7(50.0%)		1(33.3%)	17(56.7%)		8(50.0%)	9(52.9%)	
Histology			0.183			##			0.297
Serous	12(63.2%)	8(57.1%)		3(100%)	17(56.7%)		10(62.5%)	10(58.8%)	
nonserous	7(36.8%)	6(42.9%)		0(0%)	13(43.3%)		6(37.5%)	7(41.2%)	
Ascites			0.059			##			0.095
no	2(10.6%)	6(42.9%)		0(0%)	8(26.7%)		4(25.0%)	4(23.5%)	
<500ml	10(52.6%)	5(35.7%)		2(66.7%)	13(43.3%)		8(50.0%)	7(41.2%)	
≥500ml	7(36.8%)	3(21.4%)		1(33.3%)	9(30.0%)		4(25.0%)	6(35.3%)	

*p value of Bivariate Correlate analysis, by Chi-square test, # Including hERRβ-1 positive and hERRβ-2 positive, ## the number of ERRβ positive cases were too small to analyze