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Endogenous Gene Expression of p53 and Regulatory Subunits of Cyclic AMP-dependent Protein Kinase in Ovarian Cancer Cells

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In an effort to develop a new therapeutic strategy for human gene therapy of solid ovarian tumor, we studied the expression of the p53 tumor suppressor gene as well as regulatory subunits of cyclic AMP (cAMP)-dependent protein kinase in human ovarian carcinoma cells. Four cell lines (2774, Caov-3, SK-OV-3 and OVCAR-3) were selected for the analyses. The p53 transcript and protein were detected only in the 2774 cell line by Northern and Western analysis. In the relatively fast growing cell line, SK-OV-3, the type I α regulatory subunit (RI_{α}) of cAMP-dependent protein kinase was the highest among the four cell lines. The expression level of RII_{β} protein was low in the four cell lines examined. These results may point to a direction to select the target gene(s) to be employed for gene therapy to control the ovarian cancer.

KEY WORDS: Gene Expression, p53, PKA RI_{α}, PKA RII_{β}, Gene Therapy

Ovarian cancer accounts for more than half of the deaths due to gynecological malignancy with a 5-year survival rate of only 20-30% (Bast *et al.*, 1990). Multiple genetic changes have been noted to occur during carcinogenesis, which include the activation of oncogenes and the inactivation of tumor suppressor genes (Bishop, 1991; Hollingsworth and Lee, 1991). Among many chromosomal abnormalities observed in ovarian carcinomas are those affecting the p arm of chromosome 17, where the p53 tumor suppressor gene is located (Sato *et al.*, 1991; Lee *et al.*, 1990; Lee *et al.*, 1989).

Cyclic AMP (cAMP)-dependent protein kinase is composed of two catalytic (C) and two regulatory (R) subunits, i.e., R_2C_2 configuration (Beebe and Corbin, 1983). There are two different classes of cyclic AMP-dependent protein kinase designated as type I and type II, where a common C subunit is associated with distinct R subunits, RI and RII, respectively. These two isoforms of the regulatory subunits are inversely expressed during ontogeny and differentiation of normal cells and the disruption in normal patterns of these cAMP-receptor proteins has been shown to correlate with the malignant transformation (Cho-Chung, 1990). It has also been known that cAMP-dependent signal transduction, which is mediated by cAMP-dependent protein kinase, plays important role(s) in the regulation of cellular growth and differentiation.

As a first step to look for the possible target gene(s) to be used for the gene therapy of human ovarian cancers, we decided to examine some ovarian cancer cell lines for any altered expression patterns of the p53, RI_{α} and RII_{β} genes at the RNA and the protein level and their correlation with the cellular growth rate.

Materials and Methods

Cell culture

Cells were grown at 37°C under 5% CO_2 in medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G (Sigma Chemical Company) and 100 μg/ml streptomycin (Sigma Chemical Company). SK-OV-3 and Caov-3 cells were grown in Dulbecco's modified Eagle's medium. 2774 cells were grown in RPMI 1640 and OVCAR-3 cells were grown in RPMI 1640 supplemented with 10 μg/ml insulin.

Analysis of cellular growth

To compare growth rate in each cell line, we use microculture tetrazolium assay (Alley *et al.*, 1988) using 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Chemical Company). About 4 X 10^3 cells were seeded into a flat-bottomed 96-well plate. The plates were incubated in a humidified atmosphere at 37°C in 5% CO_2 for 0 to 5 days. At each day, cells were washed with PBS and MTT (2 mg/ml in PBS) was added to the wells in the plate. After incubation for 4 hours, the resulting colored reaction product, MTT formazan, was extracted with dimethyl sulfoxide and the absorbance was measured at 570 nm. The absorbance at day 0 was measured 4 hours after seeding the cells in order to allow cells to attach on the bottom.

RNA isolation and Northern blot analysis

Total RNA was extracted from cells by the guanidium thiocyanate extraction procedure (Chomczynski and Sacchi, 1987) with a slight modification. Total RNA samples (30 μg) were denatured at 65°C in 16% formaldehyde and 50% formamide, subjected to electrophoresis on a 1% agarose gel containing formaldehyde and transferred to a nitrocellulose filter for hybridization. DNA probes for Northern blot analysis were synthesized using random oligonucleotide primers. The filter was prehybridized for 6 hours in 50% formamide, 6X SSC, and 300 μg/ml of denatured calf thymus DNA. Radiolabelled probes were added to the prehybridization buffer and hybridization was proceeded for 20 hours at 42°C. The filter was washed twice in 1X SSC/0.1% SDS at 25°C and twice in 0.1X SSC/0.1% SDS at 37°C.

Western blot analysis

Cells were harvested and incubated in lysis buffer (20 mM Tris-Cl pH 7.4, 100 mM NaCl, 1% NP40, 0.5% Sodium deoxycholate, 5 mM $MgCl_2$, 100 μg/ml PMSF, 1 μg/ml aprotinin, 0.5 mg/ml soybean trypsin inhibitor, 5 mM benzamidine, 5 mM ε-amino-n-caproic acid). The lysates were centrifuged and the supernatant was assayed for protein concentration using the Bradford assay kit (Bio-Rad Laboratories) with BSA as a standard. For detection, 100 μg of the cell extract was electrophoresed on a 10% polyacrylamide/SDS gel and transferred to nitrocellulose membrane using semi-dry electrotransfer chamber (Hoefer Scientific Instruments). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline-Tween (TBS-T), washed with TBS-T and incubated with affinity-purified anti RI_α (1:1000 in TBS-T), RII_β (1:500 in TBS-T) and p53 (1:1000 in TBS-T) sera. The membrane was washed and then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:2000) in TBS-T. Detection was accomplished using the enhanced chemiluminescence detection kit (Amersham International plc.).

Results

The level of p53 tumor suppressor gene expression was measured in 4 different human ovarian carcinoma cell lines to see if there is any defect of the gene associated with the tumor. As seen in the Fig. 1, the 2.5-kilobase transcripts of p53 gene lighted up only in the 2774 cell line. This result suggests that the p53 gene is either deleted or rearranged not to be transcribed in Caov-3, SK-OV-3 and OVCAR-3 cell lines. The result of Western blot analysis (Fig. 2) was consistent with that of the Northern blot analysis. Only the 2774 cell line contained a distinct p53 band, whereas the p53 peptide was not detectable in SK-OV-3, Caov-3 and OVCAR-3 cell lines. However, these results contradict the previously published data. (Yaginuma and Westphal, 1992)

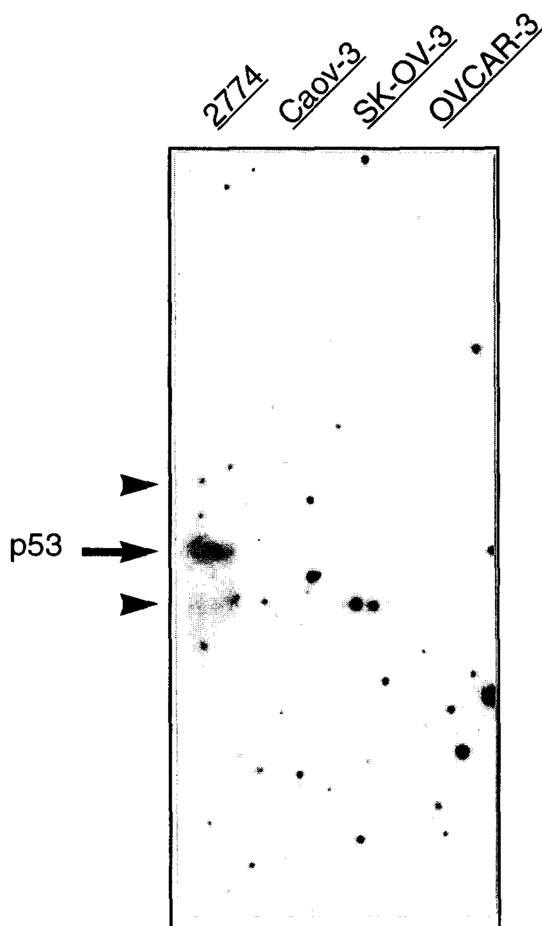

Fig. 1. Northern blot analysis of ovarian cancer cell lines for p53 gene. Thirty μg of total cellular RNA was fractionated on 1% agarose gel and transferred to nitrocellulose filter. The blot was probed with full sequence of p53 cDNA. The positions of 28S and 18S rRNA markers are indicated with arrowheads and that of p53 mRNA is marked with an arrow.

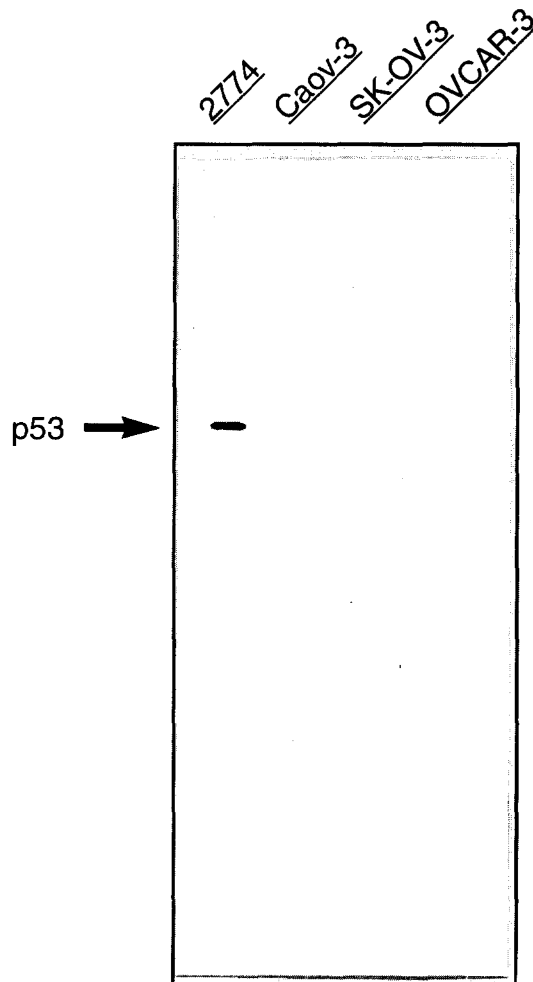

Fig. 2. Western blot analysis of endogenous p53. One hundred μg of cell lysate from four human ovarian carcinoma cell lines (2774, Caov-3, SK-OV-3 and OVCAR-3) were subjected to 10% SDS-PAGE, electrotransfered to nitrocellulose filter and probed with affinity-purified anti p53 polyclonal antibody (Novocastra Laboratories Ltd.). The p53 protein band is marked with an arrow.

To analyze the correlation between the intracellular RI_{α}/RII_{β} ratio of cAMP-dependent protein kinase and the cellular growth rate, we checked the expression levels of the two genes and growth rate by microculture tertazolium assay using MTT.

Transcript level of RI_{α} gene was the highest in SK-OV-3 cell line and the lowest in Caov-3 (Fig. 3). The 2774 and SK-OV-3 cell lines, relatively faster growing cells, had higher transcript level of RI than OVCAR-3 or Caov-3 cell lines. At the protein level, SK-OV-3 cell line contained the highest amount of RI_{α} subunits and Caov-3 cell line expressed the least RI_{α} subunits. However, OVCAR-3 cell line showed relatively large amount of RI_{α} subunits compared to the transcript level, whereas 2774 cell line expressed reasonable amount of RI_{α} subunits (Fig. 4).

Transcript level of RII_{β} was the highest in

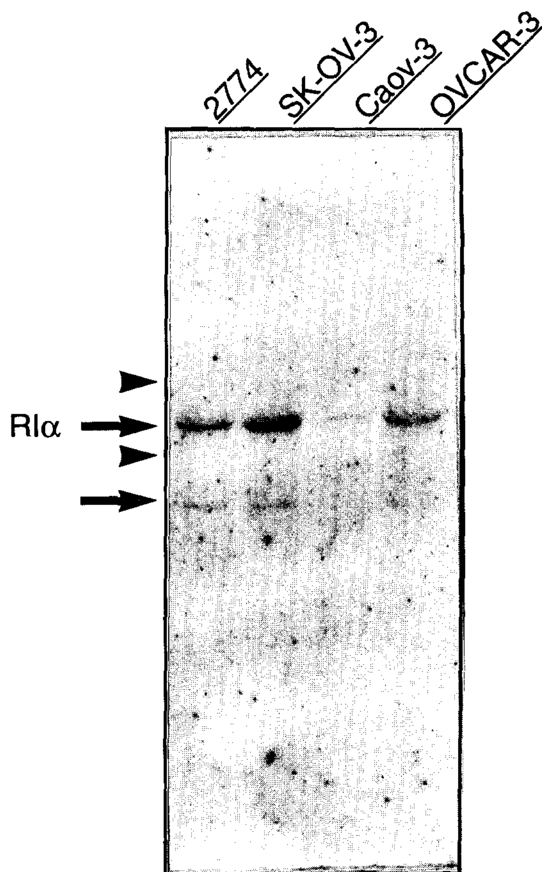

Fig. 3. Northern blot analysis of ovarian cancer cell lines for RI_α gene. Thirty μg of total cellular RNA was fractionated on 1% agarose gel and transferred to nitrocellulose filter. The blot was probed with PstI fragment of RI_α cDNA. The positions of 28S and 18S rRNA are indicated with arrowheads. Two kinds of endogenous RI_α mRNA are marked with arrows.

Fig. 4. Western blot analysis of endogenous RI_α. One hundred μg of cell lysate from four human ovarian carcinoma cell lines (2774, Caov-3, SK-OV-3, OVCAR-3) and breast cancer cell line, MCF7, overexpressing $RII\beta$ were subjected to 10% SDS-PAGE, electrotransfered to nitrocellulose filter and probed with polyclonal Ab against RI_α. The position of RI_α is indicated.

OVCAR-3 cell line (Fig. 5). In the 2774 and Caov-3 cell lines, very low level of RII_β transcript were detected. To detect the relatively low level of endogenous RII_β as compared with RI_α, X-ray film was exposed much longer time, and hence too many non-specific bands lighted up. To confirm the RII_β band, we used the breast cancer cell line, MCF7 overexpressing RII_α as a positive control. The 2774, SK-OV-3 and OVCAR-3 cell lines displayed relatively low amount of RII_β peptide band, whereas RII_β peptide was hardly detectable in Caov-3 cell line (Fig. 6).

Growth curve based on MTT assay showed that the 2774 and SK-OV-3 cell lines were growing faster than either the OVCAR-3 or Caov-3 cell lines (Fig. 7). Based on the Western blot analyses, the expression level of RI_α and RII_β level was quantitated (Table 1). The RI_α/RII_β ratio of the slowest growing Caov-3 cell line was taken as arbitrary number 1. In the 2774 and SK-OV-3 cell lines, RI_α/RII_β ratio was 1.98 and 2.96, respectively. These results were consistent with the previous published data that RI/RII ratio in transformed cell lines is greater than that of normal counterpart in terms of cellular growth (Cho-Chung *et al.*, 1991). In here, the RI_α/RII_β ratio of fast growing 2774 and SK-OV-3 cell lines was higher than that of the slowest growing Caov-3 cell lines. However, the RI_α/RII_β ratio was the highest in the relatively slow growing OVCAR-3

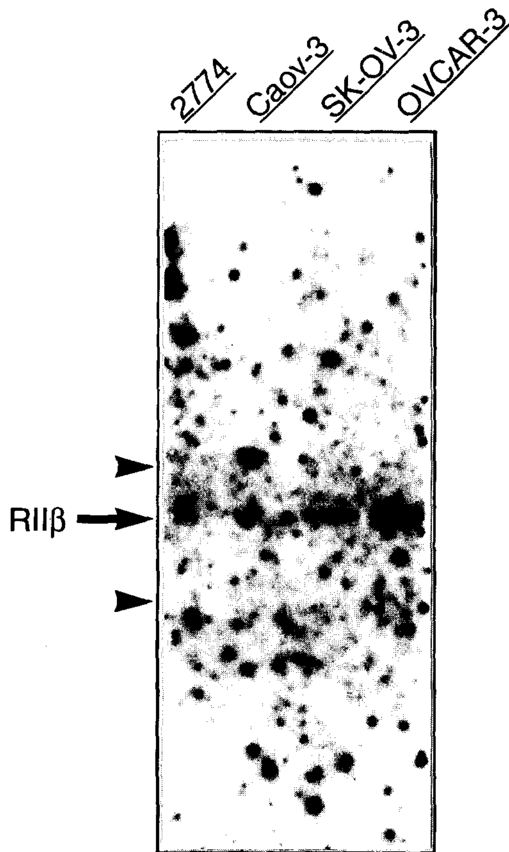

Fig. 5. Northern blot analysis of ovarian cancer cell lines for RII_β gene. Thirty μg of total cellular RNA was fractionated on 1% agarose gel and transferred to nitrocellulose filter. The blot was probed with full sequence of RII_β cDNA. The positions of 28S and 18S rRNA are marked with arrowheads and of RII_β mRNA with an arrow.

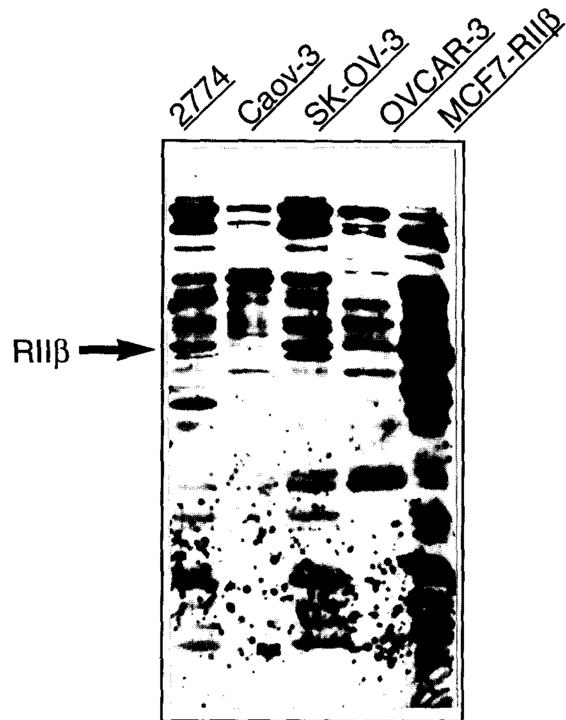

Fig. 6. Western blot analysis of endogenous RII_β. One hundred μg of cell lysate from four human ovarian carcinoma cell lines (2774, Caov-3, SK-OV-3, OVCAR-3) and breast cancer cell line, MCF7, overexpressing RII were subjected to 10% SDS-PAGE, electrotransferred to nitrocellulose filter and probed with polyclonal Ab against RII_β. The position of RII_β protein is indicated with an arrow.

cell line. The possible explanation for this apparent contradiction will be discussed later.

Discussion

Our studies suggest that mutations in the p53 tumor suppressor gene are frequently associated with cells derived from human ovarian cancer and the intracellular RI/RII ratio correlated with cellular growth rate. Oncogene-mediated cell transformation has been shown to be inhibited by increased levels of wild-type p53, (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989) and wild-type p53 when overexpressed can completely abolish the tumorigenicity of tumor-derived cell (Chen *et al.*, 1991). This suggests the possibility that p53 gene can be used as therapeutic target gene to cure for ovarian cancer that is frequently associated with the p53 gene mutation.

It has been shown that enhanced expression of regulatory subunit of type I cAMP-dependent protein kinase (RI_α) correlates with active cellular growth and cell transformation or early stages of differentiation, while a decrease in RI_α correlates with growth inhibition and differentiation-maturation (Cho-Chung, 1990). In many tumor or transformed cell lines, RI/RII ratio in comparison with that in normal counterpart cell lines or

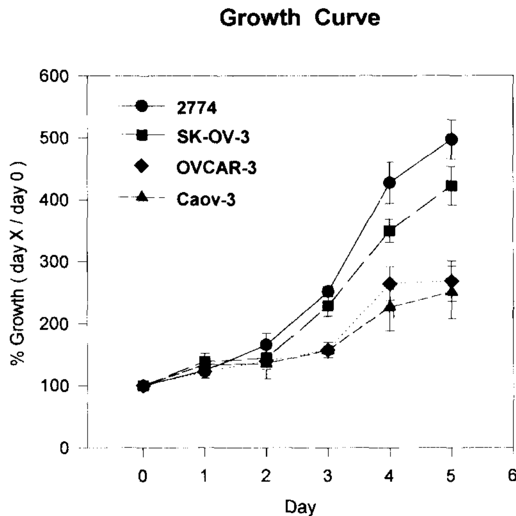

Fig. 7. Growth curves of four ovarian cancer cell lines based on MTT assay. MTT reduction was determined at 24 hour intervals after day 0. Day 0 is the time after 4 hours after seeding the cell into 96-well plate. Y-axis indicates % growth. % growth means OD_{570} on day (0, 1, 2, 3, 4 and 5) divided by OD_{570} on day 0. Each points are obtained from 5 separate determinations; bars indicate SD.

Table 1. Level of RI_α and RII_β of cAMP-dependent protein kinase in ovarian cancer lines. RI_α/RII_β ratio was calculated by scanning RI_α bands in Fig. 4 and RII_β bands in Fig. 6 using densitometer. In Caov-3 cell line where RI_α and RII_β were expressed the lowest among four cell lines, each expression level of RI_α and RII_β was taken as arbitrary number 1. In the other cell lines, expression level of RI_α and RII_β was calculated in comparison with that of Caov-3 cells, and written in the parenthesis for comparison.

Cell Line Related Genes	2774	Caov-3	SK-OV-3	OVCAR-3
RI_α	3.57 (8.55)	0.42 (1)	4.41 (10.56)	4.53 (10.86)
RII_β	5.96 (4.31)	1.38 (1)	4.93 (3.57)	2.68 (1.94)
RI_α/RII_β	1.98	1	2.96	5.60

growth-inhibited cells is increased (Cho-Chung *et al.*, 1991). The RI/RII ratio in ovarian cancer cell lines used in this study correlated well with cellular growth rate except for the OVCAR-3. However, this OVCAR-3 cell line is dependent upon the addition of insulin for the cellular growth and may contain multiple defects and/or mutations unexplainable so far. The RII_β gene is also a promising target gene for the gene therapy of the ovarian cancer. Especially, in the light that some site-selective cAMP analog such as 8-Cl-cAMP restored the growth control in various cancer cells (Ally *et al.*, 1988; Cho-Chung *et al.*, 1989), treatment of the site-selective cAMP analogs accompanied with RII_β gene transfer in ovarian cancer will be of great potential in ovarian cancer gene therapy. Further investigations on tumor suppressor genes and the various oncogenes in the signalling pathway in not only ovarian cancer cell lines but also ovarian primary tumors should be followed throughly to evaluate the therapeutic potential of the involved genes in the gene therapy of ovarian cancer.

Acknowledgements

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난소암 세포주에서 p53과 Cyclic AMP-dependent Protein Kinase의 Regulatory Subunit 유전자들의 발현에 관한 연구

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난소 고형암 치료에 유전자 요법을 활용하기 위한 새로운 전략을 찾기 위해, 사람의 난소암 세포주에서 p53 암 억제 유전자와 cyclic AMP-dependent protein kinase (PKA)의 두regulatory subunit들의 발현에 관해 연구하였다. 네 종류의 난소암 세포주(2774, Caov-3, SK-OV-3, OVCAR-3)들을 선택하여 Northern 및 Western blot 방법으로 분석하여본 결과, p53 mRNA와 그 단백질은 단지 2774 세포주에서만 발현되었다. cAMP-dependent protein kinase의 type I α regulatory subunit(RI_{α})의 발현은 다른 세포주들에 비해 상대적으로 빨리 자라는 세포주인 SK-OV-3 세포주에서 가장 높은 것으로 나타났다. 또한, 연구대상이 되었던 4개의 세포주 모두에서 RII_{β} 단백질 발현 정도는 상당히 낮은 것으로 나타났다. 이러한 결과들은 유전자 요법에 의해 난소암 세포에 도입할 유전자를 선택함에 있어 한 방향을 제시해 준다고 하겠다.