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Increased expression of the RIα subunit of the cAMP-dependent protein kinase A is associated with advanced stage ovarian cancer

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Summary The primary element in the cAMP signal transduction pathway is the cAMP-dependent protein kinase (PKA). Expression of the RIα subunit of type I PKA is elevated in a variety of human tumours and cancer cell lines. The purpose of this study was to assess the prognostic importance of RIα expression in patients with ovarian cancer. We have evaluated the expression of RIα in a panel of human ovarian tumours (n = 40) and five human ovarian cancer cell lines using quantitative reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis. The human ovarian cell lines OAW42 and OTN14 express high endogenous levels of RIα mRNA and protein (at significantly higher mRNA levels than high tissue expressors, P < 0.05). The ovarian cell line A2780 expresses low endogenous levels of RIα mRNA and protein (also at higher mRNA levels than low tissue expressors, P < 0.05). Quantitative RT-PCR revealed no significant difference in RIα mRNA expression between different ovarian histological subtypes in this study. No associations were found between RIα mRNA expression and differentiation state. RIα mRNA expression was significantly associated with tumour stage (P = 0.0036), and this remained significant in univariate analysis (P = 0.0002). A trend emerged between RIα mRNA expression levels and overall survival in univariate analysis (P = 0.051), however, by multivariate analysis, stage remained the major determinant of overall survival (P = 0.0001). This study indicates that in ovarian epithelial tumours high RIα mRNA expression is associated with advanced stage disease. RIα expression may be of predictive value in ovarian cancer and may be associated with dysfunctional signalling pathways in this cancer type.

Keywords: type I-cAMP-dependent protein kinase; reverse transcription polymerase chain reaction; ovarian cancer; cAMP

Ovarian cancer is the leading cause of death from gynaecological malignancies in the US (Parker et al, 1996) and occurs with highest frequency in Northern and Western Europe and North America (Whelan et al, 1992). Five-year survival rates for patients with stage III disease are approximately 15–20%, and less than 5% for patients with stage IV disease (NIH Consensus Statement, 1994). The diagnosis of ovarian disease at an advanced tumour stage hinders the prospects of survival and cure. The majority of patients respond well to platinum-based therapies, but most subsequently relapse and develop progressive disease. Although early diagnosis is a primary goal, secondary aims are to identify those patients with borderline and early stage tumours who will rapidly progress and develop metastatic disease. A marker of such prognostic value could identify those patients who may benefit from aggressive therapy.

The primary mediator of cAMP events in eukaryotes is the cAMP-dependent protein kinase (PKA) (Walsh et al, 1968), which is composed of an inactive tetramer containing two regulatory (R) subunits associated as a dimer and two catalytic subunits (C). There are two types of PKA, type I and type II, which contain the same C subunit but differ in the R subunit they contain (RI and RII respectively) (Reimann et al, 1971). Evidence suggests that overexpression of the type I PKA is associated with proliferation and cell transformation (Cho-Chung, 1990), therefore type I RI isoforms are thought to induce cell growth. Conversely, low expression of type I PKA (with or without alterations in the level of RII isoforms) correlates with terminal differentiation and growth inhibition (Cho-Chung, 1993). The mammalian ovary is under constant stimulation by pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which modulate the expression of numerous gonadal genes and exert their effects by activating cAMP synthesis through adenylyl cyclase (Land, 1987). In view of the role of type I PKA in the regulation of cellular growth and differentiation, we have speculated that dysregulation of the PKA pathway may have profound effects on ovarian pathology. In this study, we have evaluated the expression of the RIα subunit of the type I cAMP-dependent protein kinase (PKA) in patients with ovarian cancer and in human ovarian cancer cell lines.

MATERIALS AND METHODS

Cell culture

All ovarian cell lines were derived from serous cystadenocarcinomas of the ovary, except for the OTN14 cell line which was derived from a mucinous cystadenocarcinoma. The PEA1 and PEA2 ovarian cell lines were kindly provided by Dr S Langdon (Langdon et al, 1988). OTN14 was kindly provided by Dr L Poels (Van Niekerk et al, 1988). OAW42 cells from Dr R Wilson and A Wilson (Wilson, 1984) and A2780 cells from Dr RI Freshney. All cell lines were maintained in RPMI (GibcoBRL) supplemented with 10% heat-inactivated fetal calf serum (Imperial), penicillin–streptomycin (100 mg ml⁻¹), sodium pyruvate (100 mg ml⁻¹) and insulin (10 mg ml⁻¹) in a humidified 5% carbon dioxide atmosphere.
Tumour collection

Tissue samples from patients with suspected ovarian epithelial carcinoma were collected at initial debulking surgery. Samples were stored in liquid nitrogen upon collection and transferred to vials for long-term storage at −70°C, or in vials suspended in liquid nitrogen. Clinicopathological details, such as histology, grade and differentiation state were determined by an ovarian pathologist and retained on a computer database for future reference.

RNA extraction

Cells (10^7) were lysed by passing through a fine needle and syringe in 0.5–1 ml of Total RNA Isolation Reagent (Advanced Biotechnologies), followed by chloroform extraction and precipitation according to the manufacturer’s instructions. The isolation procedure extracted DNA and proteins into an organic phase and interphase which was discarded, eliminating the need for DNAAse treatment before polymerase chain reaction (PCR). Negative PCR controls containing RNA instead of cDNA confirmed the absence of contaminating genomic DNA. Approximately 100 mg of tissue samples were homogenized (Ultra-turrax T8, IKA laboratories, Germany) in 1 ml of isolation reagent at 4°C. Pelleted RNA was resuspended in DNase-RNase-free distilled water (Sigma) and quantified by measuring absorbance at 260 and 280 nm (LKB Ultraspec II). Samples were also electrophoresed on 1% agarose gels containing ethidium bromide (10 mg ml⁻¹) to verify sample integrity and the exclusion of genomic DNA. RNA was stored at −70°C.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA (5 μg) was reverse transcribed in a 50-μl reaction volume containing 300 U of MMLV reverse transcriptase (GibcoBRL) in the presence of oligo(dT) at a final concentration of 0.02 μg μl⁻¹ (Pharmacia Biotech), 60 μg RNAGuard (Pharmacia Biotech), 0.5 mM dNTPs, 10 mM DTT and 1 μM RT buffer at 37°C for 1 h, followed by 95°C for 5 min. All reactions used DNase-/RNase-free distilled water and cDNAs were stored at −70°C. The gene for the S14 ribosomal protein was used as an internal standard for quantification of RItα mRNA (Leonard et al, 1993) (accession number M13934). S14 primers amplify a fragment of 143 bp from cDNA. S14HumS sense strand (nucleotides 187–208): 5′-GTC AGA CCG AGA TGA ATC ATA A-3′. S14HumA antisense strand (nucleotides 331–311): 5′-CAG GTC CAG GGG TCT TGG TCC-3′.

RItα primers were designed as areas of minimal homology with other R isoforms and yield an amplicon of 338 bp with cDNA. This fragment codes entirely for mRNA, which is translated into protein (accession no M33336).

RItαP1 sense strand (nucleotides 648–667): 5′-AAA GAA TGG GCA ACC AGT G-3′. RItαP2 antisense strand (nucleotides 986–976): 5′-GCT GAC CCC TCT AAA ATA ATG-3′. All primer sets were manufactured by R & D systems, UK. Expression of RItα mRNA was described as a ratio of S14 expression levels.

cDNA (2 μl) was amplified in an 18-μl PCR mix containing 0.25 U Taq DNA polymerase, 1.5 mM magnesium chloride and 1 × Taq buffer (all Thermoprime Plus, Advanced Biotechnologies), 1 μM S14HumA, S14HumS, RItαP1 and RItαP2, and 2.5 μCi [³²P]-dCTP (3000 Ci mmol⁻¹). Thermocycling was carried out on a Perkin Elmer Cetus 480: after an initial melt at 94°C for 4 min, samples were amplified through 18 cycles of 94°C for 1 min, 54°C for 2 min and 72°C for 30 s, with a final incubation at 72°C for 5 min. The linear range of amplification was established at 18 cycles. Samples were amplified in duplicate and PCR products were denatured at 95°C and electrophoresed on 6% polyacrylamide gels (Ultragel Sequagel Complete, National Diagnostics). Gels were dried at 80°C for 40 min (Rapidry-electrophoresis ATTO, Japan) and exposed to radiographic film in a cassette with intensifying screens at −70°C for a period of 24–96 h. Radiolabelled products were analysed using a densitometer (Bio-rad GS670, Molecular Analyst software).

Optimization of RT-PCR

The series of cell lines described earlier were used to optimize the assay conditions. PCR standardization was found to be an important factor in obtaining reliable results, and extensive validations of the technique with regard to reproducibility and PCR amplification efficiency were carried out. Cycle optimization determined the linear range of amplification to be 18 cycles (assuming a standard cDNA concentration of 5 μg and taking into account the variability in RItα mRNA expression in the cell lines). Reproducibility was achieved by preparing all reactions from a ‘master mix’ of appropriate reagents. Negative controls were routinely included by amplifying blanks containing distilled water, and genomic DNA, to verify primer specificity and amplicon size. Exposure times of dried gels were monitored and analysis ratios for which S14 values exceeded 6.0 were excluded in the calculation of the overall value.

Western blot analysis

Cell pellets were sonicated in 50–200 μl of 0.1 M KPhos lysis buffer, pH 7.4 (1 M potassium dihydrogen phosphate; 1 M dipotassium hydrogen phosphate; 4:1) in the presence of protease inhibitors (0.1 mM phenylmethylsulphonyl fluoride, 3.3 μg μl⁻¹ aprotinin and 10 μg μl⁻¹ leupeptin). Lysates were centrifuged at 14 000 r.p.m. for 5 min at 4°C to remove cell debris and the supernatant recovered to fresh tubes. Total protein was quantified against a bovine serum albumin standard using the method of Bradford (1976). Equal quantities of protein (10–100 μg) were resolved on 7.5–10% polyacrylamide gels using the Mini-PROTEAN II electrophoresis cell and transfer system (Bio-rad). Ten microlitres of prestained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) high-range standards (Bio-rad) was also loaded onto gels to determine optimal electrophoretic separation of proteins. Proteins were transferred for 2 h in transfer buffer (48 mM Tris, 39 mM glycine and 0.5 M EDTA in 20% methanol). Nitrocellulose blots were incubated for 30 min at room temperature in blocking solution [phosphate-buffered saline (PBS)/0.1% Tween/5% blocking agent]. Filters were washed three times in PBS/0.1% Tween, and incubated with a 1:1000 dilution of anti-protein kinase A RItα monoclonal antibody (Transduction Laboratories, UK) in PBS/0.1% Tween/5% block overnight at 4°C (or at room temperature for 1 h). Blots were washed three times in PBS/0.1% Tween and secondary antibody applied [anti-mouse horseradish peroxidase conjugated (Amersham, UK)] at a 1:2000 dilution at room temperature for 1 h in PBS/0.1% Tween/5% block. Blots were washed three times in PBS/0.1% Tween. The chemiluminescent substrate based on the enhanced chemiluminescence (ECL) method of Amersham was applied to blots for 1 min.
RESULTS

Expression of Rlα mRNA and protein in six human cancer cell lines

Quantifiable levels of Rlα mRNA were detected in all five cell lines (mean Rlα mRNA expression = 0.161 ± 0.104) with a 4.8-fold difference in Rlα mRNA expression ratios between the highest and lowest expressors, OAW42 and A2780 respectively (Figure 1). Rlα mRNA expression was highest in the cell line OAW42 (mean Rlα mRNA expression = 0.295) and lowest in the cell lines A2780 and PEA1 (mean Rlα mRNA expression = 0.055). The cell lines PE2 and OTN14 also expressed high levels of Rlα mRNA. Mann–Whitney U-test analysis of the data confirms a significant difference in the Rlα mRNA expression levels of OAW42 cells (high expressors) compared with A2780 and PEA1 (low expressors) (P < 0.05, n = 5).

Rlα protein levels were determined using a monoclonal antibody (Figure 2) (mean Rlα protein expression = 0.847 ± 0.584). The cell lines A2780 and PEA2 expressed the lowest endogenous levels of Rlα protein, whereas OAW42 cells expressed the highest levels. Rlα protein expression was also relatively high for the cell lines PEA1 and OTN14. The levels of Rlα mRNA and protein expression are consistent with respect to the cell lines A2780, OAW42 and OTN14, however there is an inverse relationship between Rlα mRNA and protein expression in the cell lines PEA1 and PE2 and, therefore, a poor statistical correlation in a comparison of all cell lines (r² = 0.266; P > 0.5). In a comparison of cell line data, excluding PEA1 and PE2, there is a good statistical correlation between Rlα mRNA and protein expression levels (r² = 0.932). Moreover, in a preliminary investigation of Rlα protein expression levels in the tumours examined, there was also a good correlation between Rlα mRNA and protein expression levels (r² = 0.86).

Expression of Rlα mRNA in human ovarian epithelial tissue

Quantifiable levels of Rlα mRNA were detected in all tumours: serous, n = 15; mucinous, n = 14; endometroid, n = 7; clear cell,
$n = 4$. Rlα mRNA was detectable at low levels in one benign serous tumour. Unfortunately, the number of benign and borderline ovarian tumours were underrepresented in this study because of poor availability. Expression ratios were found to vary extensively among samples with a 1192-fold difference in Rlα expression between the highest and lowest expressors (ratios ranging from 0.0002 to 0.2384), and data was skewed towards the lower levels. Rlα mRNA expression levels were divided into two malignant groups for the analysis of survival data, Rlα high ($n = 15$) compared with Rlα low ($n = 16$), based on expression levels below and above the median Rlα mRNA expression level.

**Histopathological correlations**

There was a trend for higher Rlα mRNA expression levels in malignant ovarian tumours than benign and borderline tumours, however, this was not significant ($P = 0.0871$) (Figure 3; Table 1). The pooled median Rlα mRNA expression levels for serous tumours was $0.058 \pm 0.0224$, for mucinous tumours $0.0227 \pm 0.006$, for endometrioid tumours $0.035 \pm 0.012$ and for clear cell tumours $0.023 \pm 0.031$. There was also a trend for serous tumours to express higher levels of Rlα mRNA than mucinous tumours, however this difference was not significant ($P = 0.068$) nor were there significant statistical differences in Rlα mRNA expression of all histological subtypes ($P = 0.201$) (Figure 4). Figure 5A and B depicts the RT-PCR autoradiographs obtained from serous and mucinous tumour histologies respectively.

No significant difference was detected between Rlα mRNA expression levels of well/moderately and poorly differentiated tumours of all histological subgroups ($P = 0.568$), nor was any association observed in a subset analysis of serous and mucinous
tumour grades. Tumour grade was not associated with RItα mRNA expression in ovarian tumours. Univariate analysis of grade and histological subtype (for serous and mucinous only) indicated significant associations with overall survival \( (P = 0.0375 \text{ and } P = 0.0205 \text{ respectively}) \).

**Relationship to tumour stage**

RItα mRNA expression levels were highest in stage III and IV tumours \( (n = 17; \text{median RItα mRNA expression} = 0.041 \pm 0.0628) \), regardless of histology, and lowest in benign, borderline and stage I and II ovarian tumours \( (n = 23; \text{median RItα mRNA expression} = 0.0151 \pm 0.0322) \). These differences were true for all histological types \( (P = 0.0036; \text{Figure 6}) \). This significance increases in a comparison of stage III/IV and stage I/II tumours \( (P = 0.001) \). Therefore, regardless of histology, increased RItα mRNA expression was associated with advanced stage ovarian tumours. The association of RItα mRNA expression with tumour stage remains significant in an analysis of serous and mucinous histologies individually \( (P < 0.01) \).

**Clinical associations**

The median survival time for RItα low mRNA expressors was 142 weeks compared with 60 weeks for the RItα high subgroup (Figure 7). Log-rank analysis of the data indicated a trend towards statistical significance, with patients expressing higher levels of RItα mRNA exhibiting shorter overall survival rates. Low RItα expressor: number of cases = 16, number of events = 11; high RItα expressor: number of cases = 15, number of events = 13.

### Table 2

| Variable          | Classification | RItα low \( n \) | RItα high \( n \) | Univariate \( P \)-value* |
|-------------------|----------------|-----------------|-----------------|--------------------------|
| Histology         | Serous         | 3               | 8               | 0.737                    |
|                   | Mucinous       | 5               | 4               |                          |
|                   | Endometrioid   | 3               | 4               |                          |
|                   | Clear cell     | 2               | 2               |                          |
| Histology         | Serous         | 3               | 8               | 0.0205                   |
|                   | Mucinous       | 5               | 4               |                          |
| Stage             | Benign         | 3               | 2               | 0.0002                   |
|                   | Borderline     | 4               | 0               |                          |
|                   | Stage I/II     | 10              | 4               |                          |
|                   | Stage III/IV   | 3               | 14              |                          |
| Differentiation   | Well/moderate  | 7               | 6               | 0.0375                   |
|                   | Poor           | 6               | 10              |                          |
| RItα status       | Low            | \( n = 16 \)    | –               | 0.051                    |
|                   | High           | \( n = 15 \)    | –               |                          |

*Log-rank statistic based on Cox models. RItα mRNA expression divided into two categories, RItα high/RItα low, which correspond to values below and above the median value.
after adjusting for \(R\alpha\) mRNA status, histology and grade. Thus, although \(R\alpha\) expression is associated with stage and survival, tumour stage was the most significant predictor of survival in this cohort.

**DISCUSSION**

In this study, we have examined the expression of \(R\alpha\) in patients with ovarian cancer. The results indicate that elevated levels of \(R\alpha\) mRNA show a very significant correlation with advanced stage ovarian cancer \((P = 0.0036)\). There was no statistically significant relationship between \(R\alpha\) mRNA expression and patient survival. However, there was a trend towards this, in which those patients whose tumours expressed lower levels of \(R\alpha\) mRNA expression had longer overall survival rates \((P = 0.051)\).

In contrast to this study, an analysis of PKA binding proteins from 107 human ovarian tumours suggests that both total cAMP-binding protein levels and photoaffinity-labelled RI protein levels were not significantly associated with stage \((P > 0.5)\) (Simpson et al, 1996). Given the poor correlation between \(R\alpha\) mRNA and protein found with our panel of cell lines, this may provide a possible explanation for the different findings of both studies. The findings presented here underline the potential role of the \(R\alpha\) subunit overexpression with respect to advanced tumour stage and subsequent poor prognosis. The accurate determination of the prognostic significance of \(R\alpha\) mRNA expression will require a prospective study utilizing large-sample analysis.

\(R\alpha\) overexpression may also be useful as a tool for monitoring disease progression and response to treatment. Unfortunately, there was only a small number of matched pairs available in our ovarian tumour bank which did not reveal a significant trend. A more extensive study of \(R\alpha\) expression in breast cancer patients after treatment with tamoxifen has been reported (Miller et al, 1996). From the 37 matched tumour pairs available, decreases in \(R\alpha\) expression levels were observed in 18 patients, increases in ten patients, and no change in \(R\alpha\) status in nine patients. Seventeen of the 18 patients with decreased levels of \(R\alpha\) expression showed tumour regression, indicating that \(R\alpha\) expression may have potential as a prognosticator of clinical response. Current therapeutic strategies which target PKA include the cAMP analogue 8-Cl-cAMP, which is currently in phase I/II clinical trial (Tortora et al, 1995), and \(R\alpha\) antisense oligonucleotides (Nesterova et al, 1995). Both approaches involve the modulation of endogenous PKA II intracellular ratios via the down-regulation of RI isofoms. Because endogenous expression levels of \(R\alpha\) determine the concentration of 8-Cl-cAMP required to induce growth inhibition (McDaid et al, 1997), the future therapeutic efficacy of both may rely on the determination of endogenous \(R\alpha\) expression levels in patients who are most likely to benefit from PKA-targeted treatment.

In this study, \(R\alpha\) mRNA expression was not associated with tumour pathology or grade and we did not find statistically significant differences between the \(R\alpha\) mRNA expression levels of serous compared with mucinous, endometrioid or clear cell tumours, although a trend was apparent for serous tumours to express higher levels of \(R\alpha\) mRNA. Simpson et al (1996) have found an association between elevated RI protein and the serous histological subgroup \((P = 0.01)\). The consequence of elevated levels of \(R\alpha\) expression for serous tumours would be a relative up-regulation of PKAI activity compared with PKAII activity, but, overall, a decrease in free catalytic subunits and a subsequent reduction in phosphorylation of proteins by this pathway. Because it is believed that the cAMP pathway generally antagonizes the growth factor-stimulated pathway (Beebe, 1994), this decrease in downstream signalling by the cAMP pathway may remove this inhibition and so promote cell proliferation in an already dysfunctional cell.

This study has also addressed the expression of \(R\alpha\) mRNA in a panel of ovarian cell lines. Endogenous \(R\alpha\) in these ovarian cell lines can be divided into two categories, low expressors (PEA1 and A2780), which express at levels comparable to those found in tissue samples expressing high levels, and high expressors (PEA2, OAW42 and OTNI4), whose expression greatly exceeds levels determined in all tissue samples \((P < 0.05)\). One simple observation for the difference in expression levels between the two groups is a difference in the DNA content of the cells. The two low expressors, PEA1 and A2780, have pseudodiploid modal chromosome numbers of between 41 and 50 whereas those cell lines which displayed greatly elevated levels of \(R\alpha\) have modal chromosome numbers of \(\geq 80\), therefore gene amplification may explain this observation.

In conclusion, the level of \(R\alpha\) mRNA expression is associated with advanced stage ovarian cancer, but not with overall survival, although there is a trend by univariate analysis between high levels of \(R\alpha\) mRNA expression and poor patient survival. A prospective study exploiting larger sample numbers will be necessary to fully evaluate the role of \(R\alpha\) as a prognosticator of survival.

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