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Epac1 is involved in cell cycle progression in lung cancer through PKC and Cx43 regulation

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Abstract

Introduction. The exchange protein directly activated by cAMP (Epac1), a downstream target of the second messenger cAMP, modulates multiple biological effects of cAMP, alone or in cooperation with protein kinase A (PKC). Epac1 is necessary for promoting protein kinase C (PKC) translocation and activation. The aim of the study was to assess the intensity of Epac1 and protein kinase C (PKC) immunoreactivity in lung cancer and para-carcinoma tissues, and their associations with clinical-pathological indexes. Correlations between the immunoreactivity of Epac1, PKC, A-kinase anchor protein 95 (AKAP95) and connexin43 (Cx43) were also examined.

Material and methods. Epac1, Cx43 (46 cases) and PKC, AKAP95 (45 cases) immunoexpression levels were determined in tissue samples of lung cancer and in 12 samples of neighboring para-carcinoma specimens by the PV-9000 Two-step immunohistochemical technique.

Results. The percentage of Epac1 positive samples was significantly lower in lung cancer tissue than in neighboring para-carcinoma specimens (37% *vs.* 83.3%, $p < 0.05$); the difference in PKC immunoreactivity was not significant (64.4% *vs.* 91.7%). Epac1 expression was associated with the degree of malignancy and lymph node metastasis ($P < 0.05$), but not with histological type ($P > 0.05$), whereas PKC expression was not related to these parameters. Interestingly, Epac1 expression was correlated with PKC and Cx43 expression. Moreover, PKC expression was correlated with AKAP95 expression.

Conclusion. Normal Epac1 expression may suppress lung cancer occurrence and metastasis, and its downregulation is involved in cell cycle progression in lung cancer through PKC and Cx43 regulation. *(Folia Histochemica et Cytobiologica 2018, Vol. 56, No. 1, 21–26)*

Key words: lung cancer; Epac1; PKC; AKAP95; Cx43; IHC

Introduction

The exchange protein directly activated by cAMP (Epac1), a downstream target of the second messenger cAMP, modulates multiple biological effects of cAMP, alone or in cooperation with protein kinase A (PKA) [1]. Epac1, in turn, acts upstream of phospholipase C (PLC) and phospholipase D (PLD), both of which were necessary for promoting PKCϵ translocation to the plasma membrane and activation [2]. AKAP95, protein kinase A-anchoring protein 95, mediates phosphorylation of target proteins by combination with the type II R subunit of PKA [3]. Connexin 43 (Cx43) is a gap junction (GJ) protein that forms a transmembrane protein channel between cells, and promotes communication and exchange of molecules between adjacent cells. Cx43 plays an important regulatory role in cell proliferation, differentiation and homeostasis [4, 5]. Cx43 expression is decreased or

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lost in many cancers and is significantly associated with disease progression and unfavorable prognosis [6]. The AKAP95 and Cx43 proteins interact with each other and participate in cell cycle regulation; their binding and separation show periodic and dynamic changes with cell cycle progression [7]. Epac1 and PKA cooperatively enhance functional GJ neo-formation in cardiomyocytes by the accumulation of Cx43 [8]. These findings collectively suggested that Epac1, PKC, AKAP95 and Cx43 may act synergistically in lung cancer to regulate cell cycle progression. Therefore, the current study aimed to assess the immunoexpression of these proteins in lung cancer and analyze associations between them.

Materials and methods

Patients. A total of 51 lung cancer tissue specimens were obtained from patients with lung cancer in Shengjing Hospital affiliated to China Medical University TOWN between 2007 and 2009. The study was approved by the ethics committee of the Xiamen University (Xiamen, China), and the written informed patient consents were obtained from the patients or the patients' family. Pathological diagnosis was definite in all patients. Of these, 46 cancer tissue samples alongside 12 control specimens were assessed by the immunohistochemical method for Epac1, and 45 for PKC expression. Patients' age was 59 ± 12 years (mean ± SD, range 38–79). The 12 control samples were specimens from the 51 above-mentioned lung cancer patients that were located more than 3 cm away from the cancerous tissue, with no cancer cells detected.

Immunochemistry. The specimens were fixed with 10% neutral formaldehyde, paraffin embedded, and sectioned at 4 μm. Citrate buffer (pH 6.0) was used for antigen retrieval at high pressure, and the PV-9000 2-step plus Poly-HRP Anti-Mouse/Rabbit IgG Detection System (Zhongshan Jinqiao Biotechnology Company, Beijing, China) was employed to assess protein expression. Hematoxylin was used to stain cell nuclei. Rabbit anti-human primary monoclonal antibodies against Epac1 (1:300; cat. no. ab21236) and PKC (1:300; cat. no. ab32376) were obtained from Abcam (Cambridge, UK). Phosphate-buffered saline (PBS) was used to dilute the antibodies. The primary antibody was incubated with histological sections at 4°C overnight. PBS was used as a negative control for the antibody.

Assessment of protein immunoexpression. Brown-yellow staining was considered positive protein expression, with the lack there of indicating no protein expression. Ten different high power fields were assessed per section, with 200 tumor cells counted per field. The percentage of positive cells that showed presence of brown deposits was used as a metric to evaluate protein expression. The criteria for protein expression were as follows: "–", no brown; "+/–", < 25%; "+", ≥ 25% and < 75%; "++", ≥ 75%. For data analysis, "+/–" and "–" were considered to indicate negative expression, and "+"and "++" indicated positive expression.

Table 1. Epac1 and PKC expression in para-carcinoma and lung cancer tissues

		Para-carcinoma	Lung cancer	χ^2	P
Epac1	Positive	10	17	8.227	0.004
	Negative	2	29		
PKC	Positive	11	29	2.180	0.140
	Negative	1	16		

Epac1 and PKC expression was determined by PV-9000 Two-step immunohistochemical technique as described in Methods. The difference in Epac1 positive expression rates between para-carcinoma and lung cancer tissue samples was statistically significant. No statistically significant difference in PKC expression was found between lung cancer and para-carcinoma tissue specimens.

Statistical analysis. The SPSS17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Positive rates were compared by the χ^2 test; associations were analyzed by Spearman's rank correlation analysis. $P < 0.05$ was considered statistically significant.

Results

Epac1 and PKC expression levels in lung cancer and para-carcinoma tissues

In our previous study, we assessed AKAP95 and Cx43 expression levels in 51 lung cancer tissue samples [9]. The positive expression rate for AKAP95 was significantly higher in lung cancer than in para-carcinoma tissues (82.35% *vs.* 33.33%, $P < 0.05$); meanwhile, the positive expression rate for Cx43 was lower in lung cancer tissues than in para-carcinoma tissues (60.78% *vs.* 80%, $P > 0.05$). In the present study 46 and 45 patients of the above 51 cases were assessed for the Epac1 and PKC immunoreactivity, respectively. The positive expression rate for AKAP95 immunoreactivity of the 45 same samples as for PKC was 84.4%; meanwhile, the positive expression rate for Cx43 of the 46 same samples as for Epac1 was 58.7%. The immunoexpression pattern of AKAP95 and Cx43 was shown in the previous report [9], and therefore, the respective microphotographs were here not presented.

In the current study a total of 17/46 lung cancer patients showed positive Epac1 expression, representing a positive expression rate of 36.96%; meanwhile, 10 cases showed positive Epac1 expression among the 12 control, para-carcinoma tissues, representing a positive expression rate of 83.33%. The difference was statistically significant between the two groups ($P < 0.05$) (Table 1 and Fig. 1).

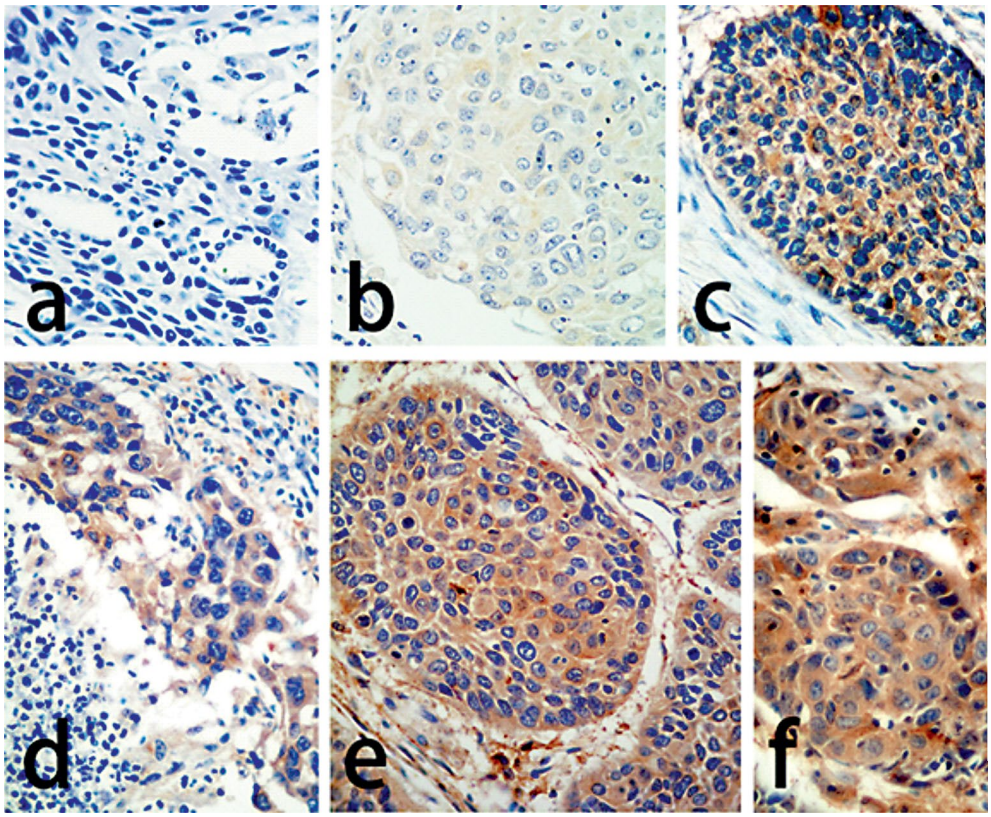

Figure 1. Epac1 **(a, b, c)** and PKC **(d, e, f)** protein expression in lung cancer tissues was assessed by immunohistochemistry as described in Methods. Epac1 negative expression in **(a)** and **(b)**; moderate intensity of cytoplasmic Epac1 immunoreactivity in **(c)**. PKC was mainly expressed in the cytoplasm, with low expression in the nucleus: **(d)** minimal; **(e)** and **(f)** high immunoexpression. Magnification 400×.

A total of 29/45 lung cancer tissue samples showed positive PKC expression, indicating a positive expression rate of 64.44%; 11 cases showed positive expression of the PKC protein among the 12 para-carcinoma tissue samples, representing a positive rate of 91.67%; no statistically significant difference in PKC expression was obtained between lung cancer and para-carcinoma tissue specimens ($P > 0.05$) (Table 1 and Fig. 1).

Associations of Epac1 and PKC with clinical--pathological parameters in lung cancer

The associations of Epac1 and PKC with clinical-pathological parameters are summarized in Table 2. Epac1 expression in lung cancer tissues was associated with the degree of differentiation and lymph node metastasis ($P < 0.05$); there was no relationship between Epac1 expression and the histological type ($P > 0.05$). We further assessed the associations of PKC expression in lung cancer with the degree of differentiation, histological type and lymph node metastasis. There were no significant associations of PKC expression with the degree of differentiation, histological type and lymph node metastasis ($P > 0.05$).

Associations of Epac1, PKC, AKAP95 and Cx43 in lung cancer

In the present study, the correlation between Epac1 and PKC protein levels in lung cancer tissues was analyzed. In addition, the associations of these two proteins with AKAP95 and Cx43 were also assessed. Significant correlations between Epac1 and PKC, Epac1 and Cx43, and PKC and AKAP95 were found (all $P < 0.05$), with Spearman rank correlation coefficients of 0.326, 0.367 and 0.348, respectively (Tables 3–5). No correlations were found for other protein pairs ($P > 0.05$), *e.g.* Epac1 and AKAP95, and PKC and Cx43 (data not shown).

Discussion

Finding molecular markers with predictive and prognostic values is critical for precise treatment in cancer. Expression of the Epac1 protein, which may

Table 2. Associations of Epac1 and PKC protein expression with clinical-pathological parameters

Item	Cases		Positive		Negative		χ^2		*P*	
	Epac1	PKC	Epac1	PKC	Epac1	PKC	Epac1	PKC	Epac1	PKC
Degree of differentiation										
Highly	4	4	3	3	1	1				
Moderately	24	24	13	15	11	9	**6.220**	0.245	**0.045**	0.884
Poorly	17	17	3	11	14	6				
Histological type										
Small cell lung cancer	9	9	4	6	5	3				
Lung squamous carcinoma	18	17	6	13	12	4	0.546	1.79	0.909	0.617
Lung adenocarcinoma	14	14	5	8	9	6				
Alveolar cell carcinoma	4	4	1	2	3	2				
Lymph node										
Positive	24	24	5	13	19	11	**5.599**	2.371	**0.018**	0.124
Negative	22	21	12	16	10	15				

Table 3. Correlation between Epac1 and PKC immunoexpression levels in lung cancer

Epac1	PKC				rs	P
	–	+/–	+	++		
–	3	2	4	2	0.326	0.029
+/–	3	6	5	3		
+	0	2	9	1		
++	0	0	2	3		

rs — Spearman's rank correlation coefficient; n = 45 patients.

Table 4. Correlation between Cx43 and Epac1 immunoexpression levels in lung cancer

Cx43	Epac1				rs	P
	–	+/–	+	++		
–	1	1	1	0	0.367	0.012
+/–	6	9	1	0		
+	1	4	4	1		
++	4	3	6	4		

rs — Spearman's rank correlation coefficient; n = 46 patients.

Table 5. Correlation between AKAP95 and PKC immunoexpression levels in lung cancer

AKAP95	PKC				rs	P
	–	+/–	+	++		
+/–	3	2	2	0	0.348	0.019
+	1	4	8	2		
++	2	4	10	7		

rs — Spearman's rank correlation coefficient; n = 45 patients.

be involved in cell cycle regulation, was detected in lung cancer tissues in the current study. Epac has 2 known isoforms, including Epac1 and Epac2, with little functional difference ascribed to their effects. Epac is a family member of guanine nucleotide exchange factors targeting the monomeric G-protein Rap1 [10]. The Epac-Rap1 pathway is intimately involved in the regulation of cell migration and cell-cell interactions in a cell type dependent manner [11, 12]. Both Epac/Rap1 and PKA may be involved in smooth muscle relaxation and could inhibit proliferation of vascular smooth muscle cells [13, 14]. Epac activation through inhibition of MAP kinases and RhoA in human prostate cancer cells suggests anti-proliferative and anti-migratory effects for this protein [15]. In the present study we found that the positive rate of Epac1 expression was significantly higher in para-carcinoma tissue samples than in lung cancer specimens, suggesting that Epac1 may inhibit the proliferation of lung cancer cells, corroborating previous findings that Epac reduces proliferation in smooth muscle cells and prostate cancer cells [13–15]. However, other authors proposed that Epac may promote proliferation, invasion and migration of prostate cancer and pancreatic cancer cells [16, 17]. These discrepancies suggest that the regulatory mechanism of the Epac protein in cell cycle progression may depend on cancer type.

PKC is involved in the regulation of cell proliferation, apoptosis, and migration, by catalyzing the phosphorylation of target proteins [18]. At the molecular level PKC was shown to be a tumor suppressor [19]. Indeed, a meta-analysis of controlled trials assessing PKC inhibitors combined with chemotherapy *versus* chemotherapy alone revealed that PKC inhibitors significantly decrease response and disease control rates in non-small cell lung cancer [20]. Clinical data revealed lower PKC protein levels and activity in tumor tissue samples compared with cognate normal tissue specimens [21]. In our study, the positive rate of PKC expression in lung cancer tissues showed a tendency to be lower than that of adjacent 'normal' tissues, also supporting a tumor-suppressive role for PKC; however, the difference was not statistically significant.

Epac is involved in the regulation of gap junction formation [22–23]. PKC phosphorylates a number of targets, including serine residues 262, 364, 368 and 372 of Cx43 [24–26], and may play a major role in intercellular communication. Many studies have shown that Cx43 phosphorylation promotes intercellular communication [27]. It was shown that Epac can induce PKC activation and Cx43 phosphorylation [28]. The complex cAMP-Epac2 increases Cx43 expression, and Epac2 overexpression inhibits glioma cell proliferation [29]. In the present study, significant correlations between Epac1 and PKC immunoreactivity on one hand, and between Epac1 and Cx43 immunoexpression on the other hand, were observed in lung cancer tissues, indicating that Epac1 may be involved in the regulation of lung cancer cell proliferation through the PKC and Cx43 proteins. These findings were consistent with previous reports [28, 29].

AKAP95 can anchor protein kinase A in the nucleus, and was shown to transfer PKA to a specific substrate to facilitate PKA-mediated phosphorylation [30]. The current study showed that the levels of PKC and AKAP95 immunoreactivity are correlated in lung cancer tissues, suggesting that AKAP95 may also transfer PKC to the specific location; alternatively, PKC may be involved in AKAP95 phosphorylation.

Epac can be directly activated by cAMP, and AKAP95 is a cAMP-dependent protein, suggesting that the Epac and AKAP95 proteins may have synergistic functions in cell cycle regulation. However, since the expression levels of Epac1 and AKAP95 were not correlated in lung cancer tissue in our study we propose that Epac1 and AKAP95 protein expression in lung cancer may be independently disturbed; alternatively, the observed abnormal expression levels may result from cell deterioration. The elucidation of exact mechanisms remains further studies.

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Disclosure of conflict of interest

None.

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