

ORIGINAL ARTICLE

Epac1, PDE4, and PKC protein expression and their correlation with AKAP95 and Cx43 in esophagus cancer tissues

Zhiyu Guan¹, Winxin Zhuang², Hui Lei², Dai Wang², Youliang Yao², Dongbei Guo², Qian Sun², Yun Chen², Xiaoyi Chen², Hongyan Lin², Bogang Teng² & Yongxing Zhang²

1 Department of Thoracic Surgery, The Second Hospital of Tianjin Medical University, Tianjin, China

2 State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, China

Keywords

Correlation analysis; Epac1; esophageal cancer; PDE4; PKC.

Correspondence

Yongxing Zhang, State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, 442 Simingnan Road, Xiamen, Fujian 361102, China.
Tel: +86 189 5924 4508
Fax: +86 59 2218 8679
Email: z63y94x@xmu.edu.cn

Received: 17 May 2017;

Accepted: 13 June 2017.

doi: 10.1111/1759-7714.12479

Thoracic Cancer 8 (2017) 572–576

Abstract

Background: This study examined the expression of exchange protein directly activated by cAMP1 (Epac1), PDE4, and PKC in esophageal cancer tissues, and analyzed the association of each protein with the pathological parameters of the samples.

Methods: Epac1, PDE4, and PKC protein expression was evaluated by PV-9000 two-step immunohistochemical techniques in 51 esophageal cancer specimens and 10 para-carcinoma tissues.

Results: The positive expression rates of Epac1 and PKC in esophageal cancer tissues (62.7% and 68.6%, respectively) were higher compared to those in para-carcinoma tissues (20% and 20%, respectively) ($P < 0.05$). The positive expression rate of PDE4 in esophageal cancer tissues (54.1%) was higher than in para-carcinoma tissues (30%), ($P > 0.05$). Epac1, PDE4, and PKC protein expression levels were not associated with the extent of tumor differentiation and/or lymph node metastasis ($P > 0.05$). Epac1 protein expression levels correlated with PDE4, PKC, and AKAP95 protein expression levels. In addition, there was a correlation between PKC and Cx43 protein levels ($P < 0.05$).

Conclusion: The expression rates of Epac1, PDE4, and PKC protein in esophageal cancer tissues were significantly higher compared to the rates in para-carcinoma tissues, suggesting an association between these proteins and the development and progression of esophageal cancer. The correlations between these proteins also revealed that they may exert a synergistic effect during the development of esophageal cancer.

Introduction

The underlying mechanism of esophageal cancer progression remains unclear; thus several research studies have investigated the potential signaling proteins that contribute to the development of esophageal cancer.

Epac1 acts as a cAMP dependent downstream protein and plays an important role in the progression of the cell cycle.¹ Studies have shown that cAMP/Epac1/Rapl signaling pathways are involved in the regulation of various cellular functions, including cell proliferation.² PDE4 is responsible for the degradation of cAMP.³ Changes in cAMP concentrations can activate different cAMP-dependent proteins and protein kinases, namely, Epac1, AKAP95, and PKC. PDE4 may play

an important role in the regulation of cAMP-dependent protein and protein kinase activity by regulating intracellular cAMP concentration. It has further been reported that AKAP95 can competitively replace the binding of CDK4 and cyclin D3, and the binding of CDK2 and cyclin E1, suggesting that this protein may regulate cell cycle progression via cyclin D/E proteins.⁴

A synergistic effect may exist between PDE4, Epac1, AKAP95, Cx43, PKC, and cyclin E/D proteins in the regulation of cell cycle progression. Therefore we investigated the expression of PDE4, Epac1, and PKC proteins in esophageal cancer tissues and their relationship to each other, as well as their corresponding association with AKAP95 and Cx43 expression.

Methods

Specimen source

Tissue samples were obtained from the First Affiliated Hospital of Liaoning Medical University between 2010 and 2011. Fifty-one cases (50 men, 1 woman) of invasive ductal esophagus cancer were all definitively pathologically diagnosed. Twenty-three cases exhibited lymph node metastases, while 27 cases had no lymph node metastases. Identification of metastasis in the remaining case was unclear. In addition, 19, 27, and six cases displayed high, moderate, and low differentiation, respectively. The control group samples ($n = 10$) were collected from tissues over 3 cm distant from esophageal cancer tissues.

Reagents and methods

All samples were fixed in 10% neutral formaldehyde, embedded in paraffin, and sectioned in 4 μ m diameter specimens. A PV-9000 two-step immunohistochemical staining kit was used (Zhongshan Jinqiao Biotechnology Co. Ltd., Beijing, China) and 3,3'-diaminobenzidine-tetrahydrochloride coloring and hematoxylin counterstaining were conducted according to the manufacturer's instructions. Mouse anti-human PDE4 rabbit monoclonal antibody was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), and the Epac1 and PKC monoclonal antibodies were purchased from Abcam (Cambridge, UK). The positive controls were set up particularly according to the specification of the antibodies. Phosphate buffered saline was used for the control.

Criteria for judging positive expression

A brown-yellow stain was considered positive protein expression, whereas the absence of a brown-yellow stain indicated negative protein expression. Each section consisted of 10 different microscopic points of view, and 200 tumor cells in each view were counted. The ratio of positive to total cells was used as a parameter to evaluate protein expression and was presented as a percentage. The criteria for positive expression are as follows: "negative, -," < 10% brown; "positive and/or negative, \pm ," $\geq 10\%$ and < 20% brown; "positive, +," $\geq 20\%$ and < 50% brown; "positive, ++," $\geq 50\%$ and < 70% brown; and "positive, +++, " $\geq 70\%$ brown. When the data were statistically processed, "+ -" and "-" were regarded as negative expression, and "+," "++," and "+++" were regarded as positive.

Statistical analyses

SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data by χ^2 test. Fisher's exact test and Spearman rank correlation analysis were employed to analyze the clinicopathological characteristics. The test level was set at $\alpha = 0.05$.

Results

Epac1, PDE4, and PKC protein expression in esophageal cancer and para-carcinoma tissues

Our group has previously reported the expression levels of AKAP95 and Cx43 proteins in 54 cases of esophageal cancer.⁵ The positive rate of AKAP95 protein expression in esophageal cancer tissues was higher compared to that in para-carcinoma tissues, suggesting that the AKAP95 protein may play an important role in promoting the development of esophageal cancer. The positive rate of Cx43 protein expression in esophageal carcinoma tissues was significantly lower compared to that in para-carcinoma tissues, suggesting that Cx43 protein may have an antitumor effect.

Epac1 and PKC protein expression was detected in 51 esophageal squamous cell cancer tissues from the aforementioned 54 cases. Furthermore, PDE4 protein expression was detected in 37 cases of esophageal cancer. Epac1 and PKC protein expression levels were 62.7% (32/51) and 68.6% (35/51), respectively, in the esophageal cancer tissues (Table 1), which were significantly higher than those noted in the para-carcinoma tissues (20% [2/10], 10% [1/10], respectively; $P < 0.05$). The positive expression level of PDE4 in esophageal cancer tissues was 54.1% (20/37), not significantly higher compared to the para-carcinoma tissues (30% [3/10]) ($P > 0.05$). Epac1 and PKC proteins were predominantly localized in the cytoplasm and minimal nuclear expression was noted. PDE4 protein was predominantly localized to the nuclear region (Fig 1).

Table 1 Epac1, PDE4, and PKC protein expression in esophageal cancer tissues

Protein	Characteristics	Cancer	Pericarcinoma	χ^2	P
Epac1	Positive	32	2	4.581	0.032
	Negative	19	8		
PDE4	Positive	20	3	-	0.286*
	Negative	17	7		
PKC	Positive	35	2	6.372	0.012
	Negative	16	8		

*Fisher's exact test was used to determine the P value of PDE4.

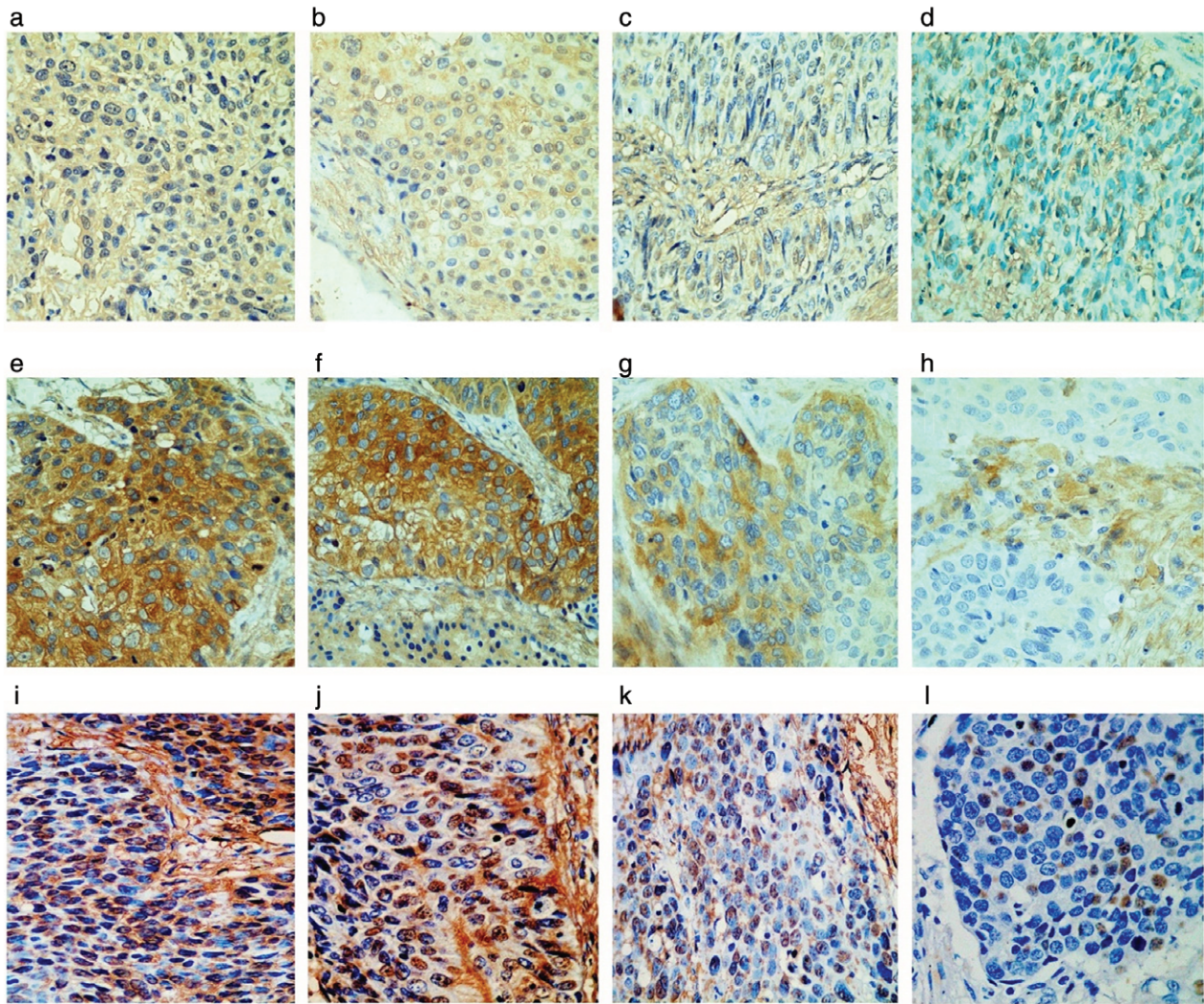


Figure 1 (a,b) Epac1 and (e,f) PKC were highly expressed in the cytoplasm. (c,d) Epac1 and (g,h) PKC were negatively expressed and minimal nuclear expression was noted. PDE4 was predominantly localized to the nuclear region, (i,j) highly expressed in the nucleus, and (k,l) negatively expressed (magnification, $\times 400$).

Epac1, PDE4, and PKC protein expression in esophageal carcinoma, and their correlation with clinical and pathological parameters

Epac1, PDE4, and PKC protein expression levels were not associated with the extent of tumor differentiation and/or lymph node metastasis (Table 2; $P > 0.05$).

Correlation between Epac1, PDE4, PKC, AKAP95, and Cx43 proteins in esophageal cancer

We analyzed the correlation between Epac1, PDE4, and PKC protein expression and their correlation with AKAP95 and Cx43 protein expression. The results indicated a significant

correlation between Epac1 and PDE4, Epac1 and PKC, Epac1 and AKAP95, and PKC and Cx43 expression ($P < 0.05$) (Tables 3–6). No correlation was observed between the other proteins examined ($P > 0.05$, data not shown).

Discussion

Epac1 can promote the migration and invasion of pancreatic cancer cells,⁶ and has been shown to increase prostate cancer proliferation via the serine-threonine-protein/extracellular-signal-regulated-kinase and mammalian target of rapamycin signaling pathways.⁷ In the present study, 51 cases of esophageal cancer tissues indicated positive expression of Epac1. Epac1 protein expression was higher in the cancerous compared to the para-carcinoma tissues,

Table 2 Epac1, PDE4, and PKC protein expression in esophageal carcinoma and their correlation with clinical and pathological parameters

Protein		Differentiation (%)			N	Lymph node (%)		N
		High	Moderate	Low		Positive	Negative	
EPAC1	Negative	8 (42.1)	9 (34.6)	2 (33.3)	19	8 (34.8)	11 (40.7)	19
	Positive	11 (57.9)	17 (65.4)	4 (66.7)	32	15 (65.2)	16 (59.3)	31
	χ^2		0.308			0.187		
	P		0.857			0.665		
PKC	Negative	6 (31.6)	9 (33.3)	1 (20.0)	16	8 (34.8)	8 (29.6)	16
	Positive	13 (68.4)	18 (66.7)	4 (80.0)	35	15 (65.2)	19 (70.4)	34
	χ^2		0.349			0.152		
	P		0.84			0.697		
PDE4	Negative	5 (38.5)	10 (50.0)	2 (50.0)	17	6 (35.3)	11 (57.9)	17
	Positive	8 (61.5)	10 (50.0)	2 (50.0)	20	11(64.7)	8 (42.1)	19
	χ^2		0.452			1.839		
	P		0.798			0.175		

Table 3 Epac1 and PDE4 correlation analysis in esophageal carcinoma tissues

Epac1	PDE4					rs	P
	-	+-	+	++	+++		
-	3	0	2	0	0	0.340	0.046
+-	3	2	3	1	0		
+	1	2	1	3	0		
++	2	1	3	1	2		
+++	0	2	1	2	0		

rs, Spearman's rank correlation coefficient.

Table 4 Epac1 and PKC correlation analysis in esophageal carcinoma tissues

Epac1	PKC					rs	P
	-	+-	+	++	+++		
-	1	3	3	0	1	0.356	0.013
+-	0	5	3	1	1		
+	1	2	5	2	2		
++	0	4	3	3	2		
+++	0	0	2	1	3		

rs, Spearman's rank correlation coefficient.

suggesting that Epac1 may play a role in promoting carcinogenesis and inhibiting tissue differentiation, consistent with the results of previous studies.

Epac1 and AKAP95 are cAMP-dependent proteins that are involved in a variety of cellular functions.^{8,9} PDE4 is cAMP-specific phosphodiesterase, which specifically reduces the levels of cAMP, suggesting that PDE4 may have a regulatory effect on cAMP-dependent proteins or protein kinases. cAMP levels are directly related to several pathological events observed in different tissues, such as tumor cell migration^{10,11} and proliferation.¹²

Our previous studies have shown that AKAP95 expression in esophageal cancer has a cancer-promoting effect.⁵

Table 5 Epac1 and AKAP95 correlation analysis in esophageal carcinoma tissues

Epac1	AKAP95					rs	P
	-	+-	+	++	+++		
-	3	1	5	0	0	-0.292	0.038
+-	3	3	4	0	0		
+	2	4	6	0	0		
++	4	8	2	0	0		
+++	4	2	0	0	0		

rs, Spearman's rank correlation coefficient.

Table 6 PKC and Cx43 correlation analysis in in esophageal carcinoma tissues

PKC	Cx43					rs	P
	-	+-	+	++	+++		
-	1	1	0	0	0	0.322	0.021
+-	9	4	1	0	0		
+	7	5	3	1	0		
++	2	5	2	0	0		
+++	3	3	3	0	1		

rs, Spearman's rank correlation coefficient.

A study reported that Epac1 downregulation could inhibit the activity of cyclin D1-CDK4, thereby inhibiting cell cycle progression and the proliferation of ovarian cancer cells.¹³ Therefore, it is hypothesized that Epac1 may play a role in ovarian cancer progression via regulation of cyclin D protein. Epac1 and AKAP95 proteins can regulate the cell cycle via cyclin D. Our results indicated a negative association between Epac1 and AKAP95 proteins, suggesting that Epac1 may compete with AKAP95 for binding with the cyclin D/Cdk4 complex, thus contributing to cancer progression. However the exact mechanism remains unknown and requires further experimental study.

Epac can increase the accumulation of Cx43 at the cell-cell junction, thereby regulating the formation of cell gap linkages.¹⁴ Cx43 protein is a tumor suppressor and the expression of the protein decreases in a variety of tumors.^{15–18} The cAMP/Epac/GSK-3 β pathway is an important pathway as it regulates Cx43 protein phosphorylation.¹⁴ However, we found no correlation between Epac1 and Cx43 proteins in esophageal cancer tissues. Whether this pattern of expression is related to the occurrence of esophageal cancer requires further validation.

PKC is a protein kinase that participates in various cellular functions, including proliferation and differentiation. The isoform of the protein PKC, PKC- α , is highly expressed in prostate, advanced bladder, and hepatocellular cancers.¹⁵ In the present study, PKC was highly expressed in esophageal cell cancer, and there were significant correlations between the expression of this protein and Epac1 and Cx43 expression levels, suggesting that this correlation may be associated with Epac1 and Cx43 protein phosphorylation by PKC.

Acknowledgments

The Natural Science Foundation of Fujian Province of China (No. 2016J01407), the National Natural Science Foundation (No. 31370166), Xiamen University Training Programs of Innovation and Entrepreneurship for Undergraduates (No. 2016X0433 and No. 2015X0453), and the Open Research Fund of the State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics (Grant No. 2016KF04) supported this study.

Disclosure

No authors report any conflict of interest.

References

- 1 Kawasaki H, Springett GM, Mochizuki N *et al.* A family of cAMP-binding proteins that directly activate Rap1. *Science* 1998; **282**: 2275–9.
- 2 de Rooij J, Zwartkruis FJ, Verheijen MH *et al.* Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 1998; **396**: 474–7.
- 3 Martinez A, Gil C. cAMP-specific phosphodiesterase inhibitors: Promising drugs for inflammatory and neurological diseases. *Expert Opin Ther Pat* 2014; **24**: 1311–21.
- 4 Arsenijevic T, Degraef C, Dumont JE, Roger PP, Pirson I. G1/S cyclins interact with regulatory subunit of PKA via A-kinase anchoring protein, AKAP95. *Cell Cycle* 2006; **5**: 1217–22.
- 5 Zhao S, Yi M, Yuan Y *et al.* Expression of AKAP95, Cx43, CyclinE1 and CyclinD1 in esophageal cancer and their association with the clinical and pathological parameters. *Int J Clin Exp Med* 2015; **8**: 7324–32.
- 6 Almahariq M, Tsalkova T, Mei FC *et al.* A novel Epac1-specific inhibitor suppresses pancreatic cancer cell migration and invasion. *Mol Pharmacol* 2013; **83**: 122–8.
- 7 Misra UK, Pizzo SV. Epac1-induced cellular proliferation in prostate cancer cells is mediated by B-Raf/ERK and mTOR signaling cascades. *J Cell Biochem* 2009; **108**: 998–1011.
- 8 Eide T, Coghlan V, Orstavik S *et al.* Molecular cloning, chromosomal localization, and cell cycle-dependent subcellular distribution of the A-kinase anchoring protein, AKAP95. *Exp Cell Res* 1998; **238**: 305–16.
- 9 Jeyaraj SC, Unger NT, Chotani MA. Rap1 GTPases: An emerging role in the cardiovascular system. *Life Sci* 2011; **88**: 645–52.
- 10 Zimmerman NP, Roy I, Hauser AD, Wilson JM, Williams CL, Dwinell MB. Cyclic AMP regulates the migration and invasion potential of human pancreatic cancer cells. *Mol Carcinog* 2015; **54**: 203–15.
- 11 Yang Z, Tsuchiya H, Zhang Y, Hartnett ME, Wang L. MicroRNA-433 inhibits liver cancer cell migration by repressing the protein expression and function of cAMP response element-binding protein. *J Biol Chem* 2013; **288**: 28893–9.
- 12 Pullamsetti SS, Banat GA, Schmall A *et al.* Phosphodiesterase-4 promotes proliferation and angiogenesis of lung cancer by crosstalk with HIF. *Oncogene* 2013; **32**: 1121–34.
- 13 Gao M, Ma Y, Bast RC Jr *et al.* Epac1 knockdown inhibits the proliferation of ovarian cancer cells by inactivating AKT/Cyclin D1/CDK4 pathway in vitro and in vivo. *Med Oncol* 2016; **33**: 73.
- 14 Lee TM, Lin SZ, Chang NC. Both PKA and Epac pathways mediate N-acetylcysteine-induced Connexin43 preservation in rats with myocardial infarction. *PLoS ONE* 2013; **8**: e71878.
- 15 Yi ZC, Wang H, Zhang GY, Xia B. Downregulation of connexin 43 in nasopharyngeal carcinoma cells is related to promoter methylation. *Oral Oncol* 2007; **43**: 898–904.
- 16 Tang B, Peng ZH, Yu PW, Yu G, Qian F. Expression and significance of Cx43 and E-cadherin in gastric cancer and metastatic lymph nodes. *Med Oncol* 2011; **28**: 502–8.
- 17 Sirnes S, Bruun J, Kolberg M *et al.* Connexin43 acts as a colorectal cancer tumor suppressor and predicts disease outcome. *Int J Cancer* 2012; **131**: 570–81.
- 18 Wang G, Zhao Y, Liu X *et al.* Allelic loss and gain, but not genomic instability, as the major somatic mutation in primary hepatocellular carcinoma. *Genes Chromosomes Cancer* 2001; **31**: 221–7.