ORIGINAL ARTICLE

p300 expression repression by hypermethylation associated with tumour invasion and metastasis in oesophageal squamous cell carcinoma

Changsong Zhang, Ke Li, Lixin Wei, Zhengyou Li, Ping Yu, Lijuan Teng, Kusheng Wu, Jin Zhu

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Correspondence to: Dr Ke Li, Department of Cancer Molecular Epidemiology, Shantou University Medical College, 22 Xinling Road, Shantou 515041, Guangdong, China; kli@stu.edu.cn

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Background: Aberrant promoter methylation is an important mechanism for gene silencing.

Aims: To evaluate the promoter methylation status of p300 gene in patients with oesophageal squamous cell carcinoma (OSCC).

Methods: The methylation status of p300 promoter was analysed by methylation-specific PCR (MSP) in 50 OSCC tissues and the matching non-cancerous tissues. Oesophageal cancer cell lines (ECa-109 and TE-10) were treated with the demethylation agent 5-aza-2'-deoxycytidine (5-Aza-CdR), and p300 mRNA expression was detected by RT-PCR.

Results: p300 methylation was found in 42% (21/50) of the OSCC tissues, but in only 20% (10/50) of the corresponding non-cancerous tissues ($p=0.017$). In OSCC samples, 65% of those with deep tumour invasion (adventitia) and 63% samples with metastasis revealed p300 promoter methylation ($p<0.05$). p300 mRNA expression was observed in 19.0% (4/21) of methylated tumours and 58.6% (17/29) of unmethylated tumours ($p=0.005$). In addition, p300 mRNA expression was observed in 40% (4/10) of methylated non-neoplastic tissues and 87.5% (35/40) of unmethylated non-tumours ($p=0.001$). The demethylation caused by 5-Aza-CdR increased the p300 mRNA expression levels in oesophageal cancer cell lines.

Conclusions: p300 transcription silenced by promoter hypermethylation could play a role in the pathogenesis of oesophageal squamous cell carcinoma.

Oesophageal squamous cell carcinoma (OSCC) is one of the most malignant gastrointestinal cancers, its incidence rate varies largely among regions. The high-risk areas are in Asia (including Southern China, Hong Kong, and other regions) and South America.^{1–4} The development of OSCC is a progressive process through a multi-step pathway of genetic and epigenetic changes. To date, the exact cellular and molecular mechanisms leading to neoplastic progression in OSCC are not clearly known. It is important to understand in detail the signals of different pathways interfering with cell-cycle regulation and proliferation.⁵

p300 is a transcriptional co-activator which was originally used in protein-interaction assays with the adenoviral E1A oncoprotein.⁶ It has been implicated in a number of diverse biological functions including proliferation, cell cycle regulation, apoptosis, differentiation, and DNA damage response.⁷ It is also a key cofactor in the proper function of other tumour-suppressor proteins. Critical pathways such as the TGF- β , p53, and RB-F2F pathways require p300 cofactor activation to mediate the transcription of target genes.^{8–11} p300 contributes to maintaining p53 stability through mdm2-dependent or independent mechanisms.^{12–13} It has been shown that phosphorylation of p300 protein in the activation domain could augment the physical interaction with p300, thereby enhancing the transcription of E2F target genes.¹⁴ The role of p300 in cell cycle regulation could be an important mechanism by which p300 contributes to cancer formation.¹⁵ In cycling epithelial cells, the Rb-E2F pathway serves as a focal point controlling G1- to S-phase transition.¹⁶ In early G1 phase, p300 acts to prevent RB hyperphosphorylation and delay premature S-phase entry, which suggests that p300 is required for orderly G1/S transition in human cancer cells.^{17–18} p300 is a transcriptional co-activator with intrinsic histone acetyltransferase activity by cooperating with E2F during the G1/S transition,¹⁹ and is known to acetylate p53 in response to DNA damage.⁹

p300 activity can be under aberrant control in human disease, particularly in tumorigenesis.¹⁵ It is known that the abnormal methylation of CpG islands associated with tumour suppressor genes can lead to transcriptional silencing, which provides an alternative mechanism for gene inactivation in cancer cells.^{20–21} However, there is no experimental evidence reported on CpG methylation in the promoter sequence of the p300 gene or the association between methylation events and p300 mRNA expression in OSCC. The aim of this study is to better understand the transcriptional regulation mechanisms under which p300 promoter activity is controlled, and to find out the association between p300 promoter methylation status and clinicopathological variables in OSCC.

MATERIALS AND METHODS

Patients and samples

Tumours and the corresponding non-neoplastic epithelial tissues were obtained from 50 patients with OSCC who underwent curative surgery without prior chemotherapy or radiotherapy between 2001 and 2003 at the Jieyang hospital, in the Chaoshan area of Southern China, a well-recognised high-risk area for OSCC. The present study was in accordance with the Ethics Standards of the Committee on Human Experimentation of Shantou University. All samples were taken during treatment procedures for curative intent. The samples were kept at -80°C until processing for RNA/DNA extraction. The matching normal oesophageal squamous epithelia were obtained from OSCC, avoiding contamination of tumour tissue. Each frozen specimen was histologically controlled for its tissue content. In all cases, H&E-stained slides were re-examined independently by three experienced pathologists without any

Abbreviations: MSP, methylation-specific PCR; 5-Aza-CdR, 5-aza-2'-deoxycytidine; OSCC, oesophageal squamous cell carcinoma

Table 1 Correlation between p300 methylation and clinicopathological parameters in patients with oesophageal squamous cell carcinoma

Variable	No	Methylation	Unmethylation	p-Value*
Sex				
Male	39	15	24	0.543
Female	11	6	5	
Location				
Upper	2	1	1	0.814
Middle	44	19	25	
Lower	4	1	3	
Tumour size				
<4 cm	22	7	15	0.196
≥4 cm	28	14	14	
Differentiation				
Poorly	8	4	4	0.718
Moderately	29	13	16	
Well	13	4	9	
Depth of invasion				
Submucosa	6	1	5	0.025
Muscularis	24	7	17	
Adventitia	20	13	7	
Nodal metastasis				
Absent	31	9	22	0.018
Present	19	12	7	
Age (y), mean (SD)		57.76 (9.73)	59.45 (9.57)	0.701†

*Statistical significance using Pearson's χ^2 test and Fisher's exact test.

†Statistical significance using the independent sample t test.

knowledge of clinical data. Slides were reviewed to analyse pathological parameters, including tumour size, histological grading, depth of invasion and the presence of nodal metastasis. Tumour stage and grade were determined on the basis of the surgical staging system of the International Union against Cancer.²² Six cases were in stage II, 24 were in stage III, and 20 were in stage IV. Age ranged from 39 to 76 years, with an average age of 58.7 years. Eleven patients were female and 39 were male. Thirteen cancers (26%) were well-differentiated, 29 (58%) were moderately differentiated, and 8 (16%) were poorly differentiated.

Cell treatments

The oesophageal cancer lines (ECa-109 and TE-10) were kept in our laboratory. The cells were cultured in RPMI medium plus 10% fetal bovine serum in a humidified 37°C incubator containing 5% CO₂. They were plated (3×10^5 cells/100 mm dish) and treated 24 hours later with 5×10^{-6} M 5-Aza-CdR (Sigma). The fresh medium was changed every 24 hours to maintain the 5-Aza-CdR concentration. RNA and DNA were isolated 6 days after treatment.²³

DNA extraction

DNA was prepared from 50 OSCCs, adjacent normal tissues, and cell lines. After microdissection using a MicroBeam system (PALM, Bernried, Germany), the tissue samples were placed

into Eppendorf tubes and were incubated overnight with proteinase K at 37°C. The tissue was extracted twice in phenol and twice in chloroform, followed by ethanol precipitation. DNA was quantified using a spectrophotometer (Shanghai FuRi Technology, Shanghai, China).

Sodium bisulphite modification and MSP

The genomic DNA (3 µg) of tumours, matching tissues, and cells was denatured with 0.3 M NaOH at 37°C for 10 min. Freshly prepared (208 µl) 3 M sodium bisulphate (pH 5.0) and 12 µl fresh 100 mM hydroquinone solutions were added. Sodium bisulphite treatment (during which methylated DNA is protected and unmethylated cytosine is converted to uracil) was carried out for 16 h at 52.5°C on denatured genomic DNA as previously reported.²⁴ DNA was purified using the Wizard DNA clean-up system (Promega) and eluted in 50 µl water. Purified DNA was treated with NaOH at a final concentration of 0.3 M at room temperature for 10 min, precipitated by ethanol and resuspended in TE solution (pH 8.0). Bisulphite-treated DNA was used for amplification of p300 promoter. PCR amplification with specific primers was performed to distinguish methylated from unmethylated DNA. Primers specific for unmethylated p300 (5'-TGT TGT TTG GTT TGG TTT TTT T-3' (sense), 5'-CAC AAA AAA CTC ACC CAA ACC A-3' (anti-sense)) or methylated p300 (5'-CGT TGT TCG GTT CGG TTT TTT C-3' (sense), 5'-CGC AAA AAA CTC GCC CGA ACC G-3' (anti-sense)).

Table 2 p300 mRNA expression by types of oesophagus tissue

	No cases	p300 mRNA (+)	p300 mRNA (-)	p-Value*
Different samples				
OSCC	50	21	29	<0.001
Non-tumour	50	39	11	
OSCC				
Unmethylation	29	17	12	0.005
Methylation	21	4	17	
Non-tumour				
Unmethylation	40	35	5	0.001
Methylation	10	4	6	

OSCC, oesophageal squamous cell carcinoma.

*Statistical significance using Pearson's χ^2 test and Fisher's exact test.

(antisense)) were used, which amplify a 138 bp product and 138 bp product, respectively.²⁵ MSP were performed in a 50 µl reaction volume containing 1×PCR buffer, 2.5 mM MgCl₂, 200 mM deoxynucleoside triphosphates, 0.5 µM of each PCR primer, 1.5 U of Ampli Taq polymerase, and approximately 25 ng of bisulphite-modified DNA, as described previously.²⁴ Reactions were hot-started at 95°C for 5 min, followed by 38 cycles at 95°C 45 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, then a 10-min extension at 72°C in DNA Thermocycler. Water blank was used as a negative control with PCR amplification; DNA methylated by SssI methylase (Sss DNA) was used as positive control. The amplification products were separated on a 2% agarose gel and visualised by ethidium bromide staining and UV transillumination.

RT-PCR analysis

RNA was isolated from 50 OSCCs, adjacent normal tissues, and cell lines. In brief, total RNA was extracted using TRIzol reagent, according to the protocol provided by the manufacturer. After washing twice with 70% ethanol, the RNA was dissolved in diethylpyrocarbonate-treated water. The quantity and quality of the RNA samples were measured carefully by spectrophotometer and electrophoresis. The first-strand cDNA

was synthesised from 2 µg of total RNA. Primer sequences of p300 for reverse transcription-PCR (RT-PCR) reaction were forward (5'-GGT CCA CTC CAA TCC AG-3') and reverse (5'-CTC AAG ATG TCT CGG AA-3'), which amplify a 186 bp product.²⁶ Primer sequences of β-actin for RT-PCR reaction were forward (5'-CTG GGA CGA ATG GAG AAA-3') and reverse (5'-AAG GAA GGC TGG AAG AGT GC-3'), which amplify a 564 bp product.²⁷ The cDNA was then used for PCR in a 50 µl reaction mixture with 5 pmol of each primer, 5 mM dNTP, 1.5 U Taq polymerase, 1×PCR buffer with MgCl₂ and sterile deionised water. RT-PCR for p300 mRNA expression was performed under the following conditions: 4 min at 94°C, 37 cycles of 30 seconds at 94°C, 45 seconds at 55°C, and 60 seconds at 72°C followed by final extension for 10 min at 72°C. As an internal control for RT-PCR, β-actin mRNA expression was amplified from the same cDNA samples for 35 cycles. PCR products were run on 2% agarose gel and visualised by ethidium bromide staining and UV transillumination.

Statistical analysis

The continuous variables were analysed by t-test. Categorical data were analysed by Pearson's χ² test or Fisher's exact test, depending on the absolute numbers included in the analysis, using SPSS V.13.0 (SPSS, Chicago, IL, USA). Results were considered statistically significant at p<0.05 by two-tailed test.

RESULTS

Methylation status of p300 promoter in OSCC and its adjacent tissue

The methylation status of p300 promoter region was analysed as one of the putative regulatory mechanisms of p300 mRNA expression in 50 OSCCs and their adjacent normal epithelial tissues. The hypermethylation contains only methylated PCR product, the partial methylation contains both methylated and unmethylated PCR products, and the unmethylation contains only unmethylated product. p300 promoter was methylated in 42.0% (21/50) of OSCCs, and in 20.0% (10/50) of non-tumour samples. The difference of p300 methylation between the tumour and non-tumour groups was statistically significant (p = 0.017) (fig 1).

Methylation status of p300 promoter with clinicopathological parameters in patients with OSCC

Table 1 summarises the correlations between p300 methylation and clinicopathological parameters in OSCC patients. We

Figure 1 Demonstration of p300 promoter methylation by MSP. (A) Representative patterns of p300 promoter methylation. U, reaction specific for unmethylated DNA; M, reaction specific for methylated DNA; 1, unmethylation control; 2, methylation control; 3, partial methylation; 4, blank. Distilled water was used as negative control (H₂O), DNA methylated by SssI methylase (Sss DNA) was used as positive control (Pos). (B) Methylation of p300 promoter in oesophageal squamous cell carcinoma (OSCC) and corresponding non-carcinoma tissues. "T" indicates OSCC sample, "N" indicates paired non-tumour tissue. (C) Methylation frequency was higher in OSCC than in non-tumour (42.0% and 20.0%, respectively). There was significant correlation between methylation status of p300 promoter with OSCC and non-tumours (p = 0.017). U, unmethylation; M, hypermethylation.

Figure 2 p300 promoter methylation related to tumour invasion and metastasis in oesophageal squamous cell carcinoma (OSCC). (A) Representative methylation status of p300 promoter in OSCC with deep tumour invasion (adventitia) and metastasis. (B) Representative methylation status of p300 promoter in OSCC with tumour invasion (muscularis) and without metastasis. U, reaction specific for unmethylated DNA; M, reaction specific for methylated DNA. "T" indicates OSCC sample.

observed that methylation status of p300 promoter was associated with tumour invasion and lymph nodal metastasis in OSCC (fig 2). The methylation of p300 promoter was more frequent in deeply invasive tumour tissues (adventitia, 13/20, 65%) than invasive tumour tissues (submucosa and muscularis, 8/30, 27%) ($p = 0.025$). In addition, the methylation frequency was higher in metastatic tumour tissues (12/19, 63%) than non-metastatic tumour tissues (9/31, 29%) ($p = 0.018$). The methylation status of p300 promoter was not associated with the remaining clinicopathological parameters evaluated, including sex, age of patients, tumour differentiation, and other features.

Association of p300 methylation with p300 mRNA expression in OSCC and corresponding normal tissues

To test whether p300 promoter methylation in OSCC might be correlated with repression of p300 mRNA transcription, RT-PCR was used for the expression of p300 transcripts in all tissue samples. Figure 3 shows representative results. Low levels of p300 mRNA expression were significantly more frequently found in OSCC samples (42%) than in matching non-tumour tissues (78%, $p < 0.001$). The frequency of p300 mRNA expression in OSCC samples was 19.0% (4/21) for methylated tissues and 58.6% (17/29) for unmethylated tissues. For non-tumour samples, p300 mRNA expression was present in 40% (4/10) of methylated tissues and 87.5% (35/40) of unmethylated tissues. The frequency of p300 mRNA expression in both OSCC and matching non-tumour tissues with unmethylation was significantly higher than in those with methylation ($p = 0.005$ and $p = 0.001$, respectively; table 2).

Demethylation by 5-Aza-CdR

The oesophageal cancer lines (ECA-109 and TE-10) showed methylation of p300 promoter (fig 4A). To examine the ability of 5-Aza-CdR to reactivate gene expression, ECA-109 and TE-10 cells were grown in low density for 24 hours in six-well plates and then treated with 5 μ mol/l 5-Aza-CdR or mock treated with phosphate buffered saline for 6 days. However, after treatment with the demethylation reagent, p300 mRNA expression was up-regulated (fig 4B).

DISCUSSION

Oesophageal cancer is a common malignancy. The pathogenesis of OSCC implicates a multi-step progression from injury of normal oesophageal mucosa, neoplastic mucosa to OSCC.^{3 28–31} Within this complex process, the accumulation of genetic and epigenetic alterations involves the inactivation of tumour suppressor genes and the activation of oncogenes, required for the emergence of a fully malignant tumour.³² The molecular aspects of the carcinogenesis in OSCC have been studied recently, especially in epigenetic alteration.^{32–35} The CpG islands of genes in normal tissues are generally protected from aberrant

Figure 3 p300 mRNA expression in oesophageal squamous cell carcinoma (OSCC) and non-cancerous tumours. U, reaction specific for unmethylated DNA; M, reaction specific for methylated DNA. “T” indicates OSCC sample, “N” indicates paired non-tumour tissue.

Figure 4 Treatment of oesophageal cancer cell lines (ECA-109 and TE-10) with 5-Aza-CdR. (A) Methylation status of p300 promoter in oesophageal cancer cell lines treated and untreated with 5-Aza-CdR. U, reaction specific for unmethylated DNA; M, reaction specific for methylated DNA. (B) Reactivation of p300 mRNA expression in oesophageal cancer cell lines after treatment with 5-Aza-CdR.

methylation, but this protection may be lost in early tumour-igenesis. This may led to methylation of tumour suppressor genes.^{36 37}

p300 transcriptional co-activator protein plays a central role in coordinating and integrating multiple signal-dependent events with the transcription apparatus, allowing the appropriate level of gene activity to occur in response to diverse physiological cases.³⁸ In the present study, we found that the methylation frequency of p300 promoter was significantly higher in OSCC than in corresponding non-cancerous tissues (42.0% and 20.0%, respectively). In addition, there was a significant association between methylation status of p300 promoter and its mRNA expression. The samples with p300 promoter methylation had a lower frequency of p300 mRNA expression, whereas those samples with unmethylation had a higher p300 mRNA expression frequency. Our results showed that promoter methylation of p300 gene is a frequent event in OSCC, and OSCC samples have a clear decrease of p300 mRNA expression, suggesting that the defective p300 expression in OSCC very likely stemmed from the altered transcription of the gene. These findings indicate that methylation of CpG islands is a possible mechanism to decrease p300 expression and to dysregulate tumour suppression gene function in OSCC.

Promoter methylation and transcriptional repression of functionally important cancer-related genes may play an important role for the poor prognosis in oesophageal adenocarcinoma.³⁹ Here, we showed that p300 promoter methylation is correlated with tumour invasion and metastasis in patients with OSCC ($p < 0.05$). These findings suggest that p300 promoter methylation may be a potential marker in relation to the poor clinical outcome of patients with OSCC. In this study, p300 promoter methylation was detected in 10/50 (20.0%) corresponding non-neoplastic epithelial tissues. We speculate that the presence of p300 gene promoter methylation in matching non-cancerous tissues may represent the appearance of pre-malignant lesions.

Take-home messages

- This is the first report that p300 mRNA is silenced by promoter methylation in oesophageal cancer.
- p300 methylation is correlated with tumour invasion and metastasis in oesophageal squamous cell carcinoma.
- 5-Aza-CdR leads to an increase of p300 mRNA expression levels in oesophageal cancer cell lines.

Epigenetic as well as genetic alterations are essential for not only the maintenance but also the initiation of many human tumour types. Understanding the molecular events that initiate and maintain epigenetic gene silencing could lead to the development of clinical strategies for cancer prevention and therapy that reverse the silencing process.^{20–21, 40} Previous studies have shown that 5-Aza-CdR is able to reduce DNA methylation and reactivate gene expression in cancer cell lines.^{23–41} DNA methyltransferase inhibitors such as 5-aza-2'-deoxycytidine could be used to treat human cancer in the future.⁴² The present study has shown that the methylation inhibitor 5-Aza-CdR induced p300 mRNA expression in the oesophageal cancer cell line, indicating a potential therapy for OSCC patients.

Taken together, p300 promoter methylation is frequently observed in OSCC and is associated with loss of mRNA expression in many tissues. These results indicate that aberrant 5' CpG methylation of the p300 gene is the likeliest mechanism for the down-regulation of this gene at a transcriptional level, which may play an important role in the pathogenesis of OSCC.

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Authors' affiliations

Changsong Zhang, Ke Li, Ping Yu, Lijuan Teng, Kusheng Wu, Jin Zhu, Department of Cancer Molecular Epidemiology, Shantou University Medical College, Shantou, Guangdong, China

Lixin Wei, Tumour Immunology and Gene Therapy Center, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University, Shanghai, China

Zhengyou Li, Biology College, Shandong Normal University, Jinan, Shandong, China

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