

Association between TAp73, p53 and VASH1 expression in lung adenocarcinoma

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Abstract. TAp73 and p53 are involved in regulating tumor angiogenesis and vasohibin-1 (VASH1) is an anti-angiogenic factor. Whether TAp73 regulates angiogenesis positively or negatively is controversial. The status of P53 may determine the effect of TAp73 on angiogenesis. To the best of our knowledge it has not been previously reported whether TAp73, p53 and VASH1 are coexpressed in lung cancer. We profiled the association between TAp73 and p53 and VASH1 expression in lung adenocarcinoma (LAC) and investigated the function of TAp73 in regulating tumor angiogenesis. TAp73, p53 and VASH1 expression in 53 human LAC tissues and the adjacent normal tissues were evaluated using immunohistochemistry. The positive expression rates of p53, TAp73 and VASH1 were significantly higher (92.6, 97.7 and 67.4%, respectively) in LAC tissue compared with paraneoplastic lung tissue (7.4, 2.3 and 32.6%, respectively, $P < 0.01$). Pearson's correlation coefficient showed a significant positive correlation between p53 and TAp73 ($r = 0.474$, $P < 0.01$) and TAp73 α and VASH1 ($r = 0.367$, $P < 0.01$). The positive expression rate of p53 and VASH1 was almost significantly correlated ($r = 0.187$, $P = 0.055$). Similarly, p53 expression intensity had a significant positive correlation with TAp73 α ($r = 0.517$, $P < 0.01$) and with VASH1 ($r = 0.277$, $P < 0.01$), as did TAp73 α with VASH1 ($r = 0.351$, $P < 0.01$). TAp73, p53 (mutant) and VASH1 expression was significantly higher in LAC tissue compared with paraneoplastic lung tissue. The

expression trends of the three proteins were significantly positively correlated with each other in LAC. These results suggest that TAp73 may suppress tumor angiogenesis in LAC.

Introduction

Lung cancer is one of the five leading causes of cancer death worldwide. The incidence of pulmonary cancer has been increasing steadily in both men and women in the developing countries, including China, by the year. Non-small cell lung cancer (NSCLC), which is aggressive and has a poor prognosis, accounts for approximately 75-85% of lung cancers (1). The proportion of lung adenocarcinoma (LAC), a common form of NSCLC, is also increasing. Therefore, in-depth research of the pathogenesis of LAC would be of significance.

Tumor angiogenesis is one of the most important hallmarks in the development of lung cancer. An extensive microvascular system is closely related to the malignant behaviors of LAC, and is also an important target in LAC treatment (2). p73, a homologue of the tumor suppressor p53, regulates vascular development and cell proliferation, migration, and differentiation. There are two isoforms of p73: TAp73, which contains full-length N-terminal transactivating domains; it is also termed TAp73 α . The other is the Δ Np73 isoform, which has a truncated N-terminal and promotes cancer gene programs (3). P73-knockout (TAp73 $^{-/-}$) mice develop gastrointestinal and cranial hemorrhage (4), indicating vascular fragility. In addition, TAp73 regulates GATA-1 (5), which is critical for endothelial and hematopoietic cell differentiation. Accordingly, we conjectured that p73 might be related to vasculogenesis/angiogenesis. However, the function of TAp73 in regulating angiogenesis in cancer is hotly debated (6,7). Some suggest that TAp73 suppresses angiogenesis (8,9). Conversely, others suggest that both the TAp73 and Δ Np73 forms are proangiogenic (10-12). Interestingly, p53 status might be a determinant of the effect of TAp73 on angiogenesis. TAp73 regulated vascular endothelial growth factor A (VEGFA), where wild-type p53 downregulated angiogenesis and mutant p53 promoted it (13). Altogether, whether TAp73 acts as a positive or negative regulator of angiogenesis is unclear.

The tumor suppressor gene *TP53* has been described as a critical determinant of the angiogenic potential of tumor cells.

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Indeed, the *TP53* gene is mutated in many human tumors. Mutated *TP53* genes promote the invasion, metastasis, proliferation, and angiogenesis of malignant cells (14). In addition, mutant p53 protein upregulates VEGF and promotes angiogenesis (15). In contrast to p53, analysis of TAp73 in different human tumors did not detect any significant mutation. Rather, wild-type TAp73 is overexpressed in many human tumors, suggesting that TAp73, as well as p53, is involved in regulating tumor angiogenesis. However, whether TAp73 and P53 are coexpressed in lung cancer, especially LAC, has not been reported.

Numerous endogenous regulators participate in cancer angiogenesis. Vasohibin-1 (VASH1) is a VASH family member that has been identified as a novel negative feedback regulator of angiogenesis (16). It is expressed and secreted by the termination zone of endothelial cells. Consequently, VASH1 halts angiogenesis and exerts some anti-angiogenic activity in endothelial cells (17). High VASH1 expression has also been detected in some tumor tissues (colorectal cancer, NSCLC) and correlates positively with clinicopathological features, including lymph node status and tumor-node-metastasis (TNM) stage, and is an independent prognostic factor (18). It is well-established that VASH1 acts as an anti-angiogenic factor. Its overexpression in several tumors is due to VEGF induction. Reduced VASH1 expression has been reported in renal cell carcinoma (RCC) (19). Lu *et al* also reported that EZH2 silenced VASH1 expression in ovarian cancer, worsening prognosis (20). Delivery recombinant adenovirus encoding VASH1 prevents tumor angiogenesis and tumor growth (21). Although TAp73, p53, and VASH1 have been studied individually in tumors, little is known about their coexpression status in lung cancer. In the present study, we profile the association between TAp73, p53, and VASH1 expression in LAC by immunohistochemical (IHC) staining and statistical analysis. The study will be of benefit in that it provides clues to the function of TAp73 in regulating angiogenesis in LAC.

Materials and methods

Patients and tissue microarray. Microarray sections of human LAC tissues and adjacent normal tissues with pathological information were purchased from Shanghai Biochip (OD-CT-RsLug04-003; Shanghai, China). Table I lists the clinicopathological characteristics of the patients. TAp73, p53, and VASH1 protein expression was analyzed in the 53 LAC tissue samples and the paired adjacent normal tissues.

Immunohistochemistry. Tissue sections (4- μ m thick) were cut from each tissue microarray block and the tissue microarrays were performed according to the streptavidin-peroxidase IHC method. The tissue sections were conventionally dewaxed, and antigen retrieval was performed by submerging the slides in sodium citrate buffer (pH 6.0) under high-pressure and high-temperature conditions, 3-minute boiling, followed by 30-min cooling at room temperature. Then, the tissue sections were incubated in hydrogen peroxide (3%) in phosphate-buffered saline containing 1% Triton X-100 (PBS-T) for 10 min to block endogenous peroxidase activity. The sections were washed repeatedly in 5% bovine serum albumin in PBS-T and incubated with mouse polyclonal anti-VASH1 antibody

(1:100; ab67423; Abcam, Hong Kong, China), rabbit polyclonal anti-P73 antibody (1:1,000; ab14430; Abcam), and monoclonal anti-p53 antibody (1:1,000; p5813; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 5% bovine serum albumin at 4°C overnight. The next day, the sites of immunoreactivity were detected using an EnVision Detection kit/DAB staining kit (GK500705; Dako A/S, Glostrup, Denmark) according to the manufacturer's protocol. The slides were counterstained with hematoxylin (ZLI-9609; ZSGB-Bio, Beijing, China) for 1 min at room temperature, and then dehydrated and sealed using crystal mounting solution (ZLI-9516; ZSGB-Bio).

Image analysis and scoring. Two pathologists evaluated the immunostaining independently using a double-blind method. Staining intensity was evaluated according to the following scale: 0 points, unstained; 1 point, faint yellow; 2 points, brownish yellow; 3 points, brown. Staining was also assessed according to the percentage of positive cells (0-100%). In the tissue slides, negative and positive expression was determined by the percentage of positively stained cells and the staining intensity; score ≤ 3 was defined as negative expression; score > 3 was defined as positive expression (Table II). By multiplying the positively stained cells by the staining intensity, the protein expression intensity was classified on a subjective spectrum as follows: -, 0-1 points; +, 2-4 points; ++, 5-8 points; +++, > 9 points (Table III).

Statistical analysis. All statistical analyses were performed using SPSS (version 21.0; SPSS, Inc., Chicago, IL, USA). The clinical characteristics of the patients according to negative and positive expression of p53, TAp73, and VASH1 were assessed using the chi-square test and Student's t-test. Spearman's correlation was used to determine the relationship between positive and negative protein expression and with expression intensity. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Study population. Table I summarizes the demographic and clinicopathological features of the samples, which included 53 LAC tissue samples and 53 paraneoplastic tissue samples. The mean patient age was 64.0 years, and 52.8% of the patients were male. Approximately 43.4% of the patients were smokers. Most patients had stage II LAC (75.5%); the remaining patients had stage I (1.9%), stage I-II (1.9%), stage II-III (13.2%), and stage III disease (7.5%).

P53, TAp73, and VASH1 expression in LAC. P53, TAp73, and VASH1 expression in the LAC and paraneoplastic lung tissue was analyzed by immunohistochemistry. The expression of the proteins was classified into two groups: Negative and positive expression. P53 and TAp73 were mainly localized to the pulmonary alveoli nuclei and expressed in LAC. VASH1 staining was observed mainly in the cytoplasm of LAC cells and in the alveolar epithelium. Different staining intensities were observed for the three proteins in the LAC or paraneoplastic lung tissues (Fig. 1). The positive expression rates of p53, TAp73, and VASH1 were significantly higher in LAC tissue (92.6, 97.7 and 67.4%, respectively) than in

Table I. General patient characteristics (n=106).

Feature	% (n)
Mean age	64.0 (9.9)
Median age (p25-p75)	65 (56-72)
Median age (years)	
≤60	35.8 (38/106)
>60	64.2 (68/106)
Sex	
Male	52.8 (56/106)
Female	47.2 (50/106)
Smoking exposure	
Non-smoker	56.6 (60/106)
Smoker	43.4 (46/106)
Histological type	
Adenocarcinoma	50.0 (53/106)
Paraneoplastic	50.0 (53/106)
Disease stage	
I	1.9 (1/53)
I-II	1.9 (1/53)
II	75.5 (40/53)
II-III	13.2 (7/53)
III	7.5 (4/53)

I, well-differentiated adenocarcinoma; II, moderately-differentiated adenocarcinoma; III, poorly-differentiated adenocarcinoma.

paraneoplastic lung tissue (7.4, 2.3 and 32.6%, respectively, $P<0.01$) (Tables II and III). There was a clear trend for upregulated p53, TAp73, and VASH1 expression in LAC tissue as compared to paraneoplastic lung tissue. However, there was no significant difference between p53, TAp73, and VASH1 expression and age, sex, smoking exposure, or disease stage ($P>0.05$) (Table II).

Correlation between positive rate of p53, TAp73, and VASH1 expression in LAC. Immunohistochemistry analysis showed that the positive rate and intensity of p53, TAp73, and VASH1 expression were higher in LAC tissue than in paraneoplastic lung tissue. We evaluated the correlation between the three using Pearson's correlation coefficient. There was extremely significant positive correlation for the rate of positive expression between p53 and TAp73 ($r=0.474$, $P<0.01$) and between TAp73 and VASH1 ($r=0.367$, $P<0.01$; Table IV). There was a marginal significant correlation for the rate of positive expression between p53 and VASH1 ($r=0.187$, $P=0.055$).

Correlation between p53, TAp73, and VASH1 expression intensity in LAC. To further estimate the correlation between p53, TAp73, and VASH1 expression in LAC, we analyzed their expression intensity in LAC and paraneoplastic lung tissues. Similar to the rate of positive expression, p53 expression intensity had extremely significant positive correlation with TAp73 ($r=0.517$, $P<0.01$) and with VASH1 ($r=0.277$, $P<0.01$). There was also extremely significant positive correlation the TAp73

and VASH1 expression intensities ($r=0.351$, $P<0.01$; Table V). Therefore, the statistical analysis suggested a positive association between p53, TAp73, and VASH1 expression abundance in LAC.

Discussion

Approximately 140 million people die from lung cancer, particularly NSCLC, each year, and lung cancer is the most common cause of cancer-related death around the world. The molecular basis of lung cancer is complex and warrants further investigation.

The members of the p53 family include p53, p73, and p63. Wild-type p53 suppresses tumor formation. The normal protein has a very short half-life and is present in only minute amounts in normal tissues and cells. However, *TP53* is the most frequently mutated gene in numerous human cancers, as recent whole-exome sequencing studies have shown (22). *TP53* mutations gain new activity to promote tumor progression and angiogenesis (23). In contrast to the wild type, mutant p53 protein produced by malignant cells is usually a product of a point mutation in the *TP53* gene, leading to the substitution of a single amino acid that significantly prolongs the half-life of the protein. The accumulation of high levels of p53 is a potential novel marker of malignancy. As the mutation is found in more than 60% of NSCLC cases, and the mutant protein has a longer half-life than the wild-type protein in the nucleus, the nuclear p53 protein accumulation in LAC tissues we detected by IHC is likely the mutant type. Ultimately, direct sequencing of the LAC samples would confirm the *TP53* mutations.

Another member of the p53 family is TAp73. TAp73 has two main isoforms (24). One is the full-length TAp73 yielded by the P1 promoter, and contains the N-terminal transactivation domain. Cell-based assays have shown that it is functionally analogous to p53. The other isoform is Δ Np73 which lacks the N-terminal transactivation domain and acts as a repressor of p53 and TAp73 (25). Recently, it was shown that TAp73 as well as p53 are involved in regulating tumor angiogenesis. However, the role of TAp73 in vasculogenesis remains contradictory and unclear. Some reports have suggested that TAp73 has a suppressive effect on angiogenesis (8,9). Conversely, other reports suggest that TAp73 is a proangiogenic factor. Some researchers have proposed the explicit possibility that TAp73 both promotes and inhibits angiogenesis in cancer depending on the cellular context. Transient overexpression of TAp73 leads to pro-angiogenesis. In contrast, long-term induction of TAp73 leads to angiogenic suppression (26). Similarly, we also found TAp73 could inhibit the ability of tube formation in human umbilical vein endothelial cells (HUVEC) when HUVEC co-cultured with H1299 LAC cells with TAp73 stable overexpressed not for transient transfection (data not shown). Based on this point of view, we speculate that TAp73 suppresses angiogenesis in the long-term development of cancer.

In addition, a different p53 status might affect the role of TAp73 in angiogenesis (6). Therefore, profiling the coexpression status of TAp73 and p53 in tumor cells is crucial. We observed significantly higher expression of TAp73 and mutant p53 in LAC tissues compared to paraneoplastic lung tissue. Moreover, both proteins exhibited extremely significant positive correlation expression trends. In addition to hypoxia, DNA damage

Table II. Clinical patient characteristics according to positive/negative p53, Tap73 and VASH1 expression.

Feature	p53			Tap73			VASH1		
	Negative N=79% (n)	Positive N=27% (n)	P-value	Negative N=62% (n)	Positive N=44% (n)	P-value	Negative N=60% (n)	Positive N=46% (n)	P-value
Mean (±SD)	64.8 (9.1)	61.6 (11.2)	0.140 ^b	63.5 (9.6)	64.0 (10.0)	0.594 ^b	65.1 (9.4)	62.4 (10.3)	0.161 ^b
Median age									
≤60	32.9 (26/79)	44.4 (12/27)		37.1 (23/62)	34.1 (15/44)		30.0 (18/60)	43.5 (20/46)	
>60	67.1 (53/79)	55.6 (15/27)	0.281 ^a	62.9 (39/62)	65.9 (29/44)	0.751 ^a	70.0 (42/60)	56.5 (26/46)	0.152 ^a
Sex									
Male	50.6 (40/79)	59.3 (16/27)		51.6 (32/62)	54.5 (24/44)		51.7 (31/60)	54.3 (25/46)	
Female	49.4 (39/79)	40.7 (11/27)	0.438 ^a	48.4 (30/62)	45.5 (20/44)	0.766 ^a	48.3 (29/60)	45.7 (21/46)	0.784 ^a
Smoking									
Non-smoker	53.2 (42/79)	66.7 (18/27)		56.5 (35/62)	56.8 (25/44)		61.7 (37/60)	50.0 (23/46)	
Smoker	46.8 (37/79)	33.3 (9/27)	0.222 ^a	43.5 (27/62)	43.2 (19/44)	0.970 ^a	38.3 (23/60)	50.0 (23/46)	0.230 ^a
Histological type									
Adenocarcinoma	35.4 (28/79)	92.6 (25/27)		16.1 (10/62)	97.7 (43/44)		36.7 (22/60)	67.4 (31/46)	
Paraneoplastic	64.6 (51/79)	7.4 (2/27)	<0.001 ^a	83.9 (52/62)	2.3 (1/44)	<0.001 ^a	63.3 (38/60)	32.6 (15/46)	0.002 ^a
Disease stage									
I	0 (0/28)	4.0 (1/25)	0.339 ^a	0 (0/10)	2.3 (1/43)	0.431 ^a	0 (0/22)	3.2 (1/31)	0.695 ^a
I-II	3.6 (1/28)	0 (0/25)		0 (0/10)	2.3 (1/43)		0 (0/22)	3.2 (1/31)	
II	78.6 (22/28)	72.8 (18/25)		6.0 (6/10)	79.1 (34/43)		81.8 (18/22)	71.0 (22/31)	
II-III	7.1 (2/28)	20.0 (5/25)		2.0 (2/10)	11.6 (5/43)		9.1 (2/22)	16.1 (5/31)	
III	10.7 (3/28)	4.0 (1/25)		2.0 (2/10)	4.7 (2/43)		9.1 (2/22)	6.5 (2/31)	

^aChi-squared P-value. ^bStudent's t-test P-value. Score ≤3 indicates negative expression; score >3 indicates positive expression; SD, standard deviation; I, well-differentiated adenocarcinoma; II, moderately-differentiated adenocarcinoma; III, poorly-differentiated adenocarcinoma; VASH1, vasohibin-1.

Table III. Expression intensity of p53, TAp73 and VASH1 (n=106).

	p53			TAp73			VASH1		
	LAC	Paraneoplastic	Total	LAC	Paraneoplastic	Total	LAC	Paraneoplastic	Total
-	21	49	70	7	43	50	9	19	28
+	12	4	16	22	10	32	26	30	56
++	14	0	14	21	0	21	13	4	17
+++	6	0	6	3	0	3	5	0	5
Total	53	53	106	53	53	106	53	53	106

Protein expression intensity was classified on a subjective spectrum: -, 0-1 points; +, 2-4 points; ++, 5-8 points; +++, >9 points; VASH1, vasohibin-1; LAC, lung adenocarcinoma.

Table IV. Correlation between the positive expression rate of p53, TAp73 and VASH1 (n=106).

	p53	TAp73	VASH1
p53			
r	1.000	0.474	0.187
P-value	-	<0.001	0.050
TAp73			
r	0.474	1.000	0.367
P-value	<0.001	-	0.006
VASH1			
r	0.187	0.367	1.000
P-value	0.050	0.006	-

r, Spearman correlation; VASH1, vasohibin-1.

Table V. Correlation between p53, TAp73 α , and VASH1 expression intensity (n=106).

	p53	TAp73 α	VASH1
p53			
r	1.000	0.517	0.277
P-value	-	0.004	<0.001
TAp73 α			
r	0.517	1.000	0.351
P-value	0.004	-	<0.001
VASH1			
r	0.277	0.351	1.000
P-value	<0.001	<0.001	-

r, Spearman correlation; VASH1, vasohibin-1.

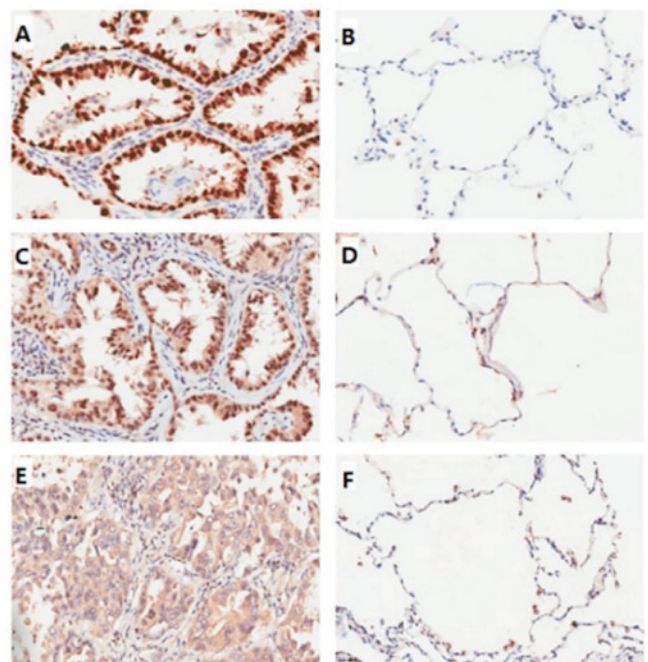


Figure 1. IHC analysis of p53, TAp73, and VASH1 expression in LAC tissues and paraneoplastic lung tissues. Micrographs show immunostaining in LAC tissues and paraneoplastic lung tissues for (A and B) p53, (C and D) TAp73, and (E and F) VASH1 expression, respectively (x200 magnification).

affecting angiogenesis; however, the specific mechanism still requires further study. Our results imply that TAp73 might function as an anti-angiogenic in LAC with mutant p53 expression. Our assumption remains to be verified in future studies.

How does TAp73 inhibit angiogenesis in LAC? It has been reported that VASH1 is a negative regulator of angiogenesis in normal tissue differentiation or tumor development. A secretory protein, VASH1 can be induced as a negative feedback regulator in endothelial cells by stimulators of angiogenesis such as VEGF and fibroblast growth factor 2 (FGF-2) (27). Many reports have indicated that VASH1 expression is high in malignant solid tumors, and that its expression is closely related to tumor angiogenesis, such as in breast (28), liver (29), renal (19), ovarian (30), colorectal (18), and lung cancer (31). Similar to these reports, we found the higher VASH1 expression in LAC tissue than in the adjacent normal

and factors such as p53 status can be p73-mediated angiogenesis response modulators. The mechanism may be related to TAp73 regulation of VEGF in the presence of wild-type p53 conditions, where TAp73 can upregulate VEGF expression (6), thereby

lung tissues. More interestingly, there was a significant positive correlation between VASH1 and TAp73 expression levels similar to that between VASH1 and mutant p53. To our knowledge, there are few reports on the mechanism of TAp73 regulation of VASH1, until now. Considering TAp73 functions as a transcription factor, we used the MatInspector Database to analyze the promoter/enhancer region of VASH1, and found several potential p73 binding sites (data not shown). Whether TAp73 regulates VASH1 expression remains to be elucidated.

Collectively, our results show that TAp73, mutant p53, and VASH1 expression is significantly higher in LAC tissue compared to paraneoplastic lung tissue. Moreover, the expression trends of TAp73, mutant p53, and VASH1 are significantly positively correlated. The results imply the function of TAp73 in regulating tumor angiogenesis.

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