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## High expression of TAZ/YAP promotes the progression of malignant melanoma and affects the postoperative survival of patients

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**Background:** YAP/TAZ have been reported to be highly expressed in malignant tumors. This research was aimed to study the expression of TAZ/YAP protein in malignant melanoma and to investigate its relationship to the clinicopathology and prognosis of patients with malignant melanoma. **Methods:** A total of 156 pairs of malignant melanoma tumor tissues and melanocytic nevi tissues were collected. The expression of TAZ and YAP proteins were detected by immunohistochemistry and western blotting. Relationship between TAZ/YAP and clinical data of malignant melanoma were analyzed. **Results:** The number of positive cells of TAZ and YAP in malignant melanoma tumor tissues was significantly higher than that in melanocytic nevi tissues, and the expression of both proteins was positively correlated with malignant melanoma ( $r = 0.687$ ,  $P < 0.01$ ); TAZ and YAP protein expression in malignant melanoma tissue was significantly correlated with tumor thickness, invasive depth grade, lymph node metastasis and TNM stages ( $P < 0.05$ ), and increased with TNM staging. The expression of TAZ and YAP in patients with lymph node metastasis was significantly higher than that in non-metastatic patients ( $P < 0.05$ ). High expression of TAZ/YAP significantly reduced the postoperative survival rate in patients with malignant melanoma. **Conclusion:** TAZ/YAP is highly expressed in malignant melanoma tumor tissues. The high expression of TAZ/YAP promotes the progression of malignant melanoma and affects the postoperative survival of patients.

### 1. Introduction

Malignant melanoma (MM) is a kind of tumor caused by cancerous lesions of melanocytes of the skin or other tissues and organs. Although the incidence of malignant melanoma in China is still low, the incidence is rising. Melanoma patients often have metastases at the time of diagnosis, and their malignancy and mortality are very high (Chen et al. 2016; Chen et al. 2017).

The Hippo signaling pathway is a highly conserved tumor suppressive signal pathway in mammalian cells. It plays an important regulatory role in cell proliferation and growth, DNA damage repair and carcinogenesis (Johnson and Halder 2014; Zheng et al. 2011). Tumors may happen once this pathway got any deficiency. And this pathway may promote invasion and migration by affecting the epithelial-mesenchymal transition of tumor cells (Wang et al. 2016; Zhao et al. 2011). YAP and TAZ proteins are a group of homologues, all of which are important effector proteins in the downstream of the Hippo signaling pathway. In recent years, YAP/TAZ have been reported to be highly expressed in malignant tumors such as esophageal cancer (Muramatsu et al. 2011), non-small cell lung cancer (Noguchi et al. 2014), ovarian cancer (Jeong et al. 2013) and colorectal cancer (Yuen et al. 2013). Its high expression is closely related to tumor invasion and migration and the patient's clinical prognosis. In this study, TAZ/YAP expression was examined in 156 pairs of malignant melanoma tumor tissues and melanocytic nevi tissues, to explore the clinical significance of TAZ/YAP expression in malignant melanoma.

### 2. Investigations and results

#### 2.1. Expression of TAZ/YAP protein in malignant melanoma tissue

Western blot analysis showed that YAP and TAZ protein expression in malignant melanoma were significantly higher than in

melanocytic nevi tissues, as shown in Fig. 1A. Immunohistochemistry was used to detect the expression of YAP and TAZ in 156 malignant melanoma tissue samples. The results showed that the percentage of YAP and TAZ positive cells in malignant melanoma tissues were  $44.30 \pm 11.42\%$  and  $42.61 \pm 12.60\%$ , respectively, both were significantly higher than positive cells in 23 melanocytic nevi tissues ( $8.92 \pm 0.58\%$  and  $9.57 \pm 0.62\%$ ), as shown in Fig. 1B. There was a positive correlation between TAZ and YAP expression in malignant melanoma ( $r = 0.687$ ,  $P < 0.01$ ) as shown in Fig. 1C.

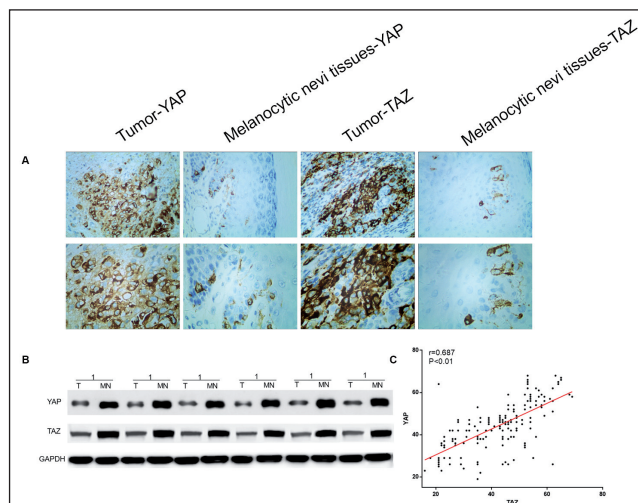


Fig. 1: TAZ/YAP is highly expressed in malignant melanoma tissue. A: immunohistochemistry of YAP/TAZ in tumor and MN tissues in the same patients. B: western blot results of YAP/TAZ in tumor and MN tissues in the same patients. C: the correlation of YAP and TAZ in malignant melanoma tissues. MN: melanocytic nevi.

**Table: TAZ/YAP expression and malignant melanoma clinical data (n)**

items	cases	TAZ		P	YAP		P
		High expression	Low expression		High expression	Low expression	
<b>Age (year)</b>							
≤45	54	31	23	0.772	24	30	0.111
>45	102	61	41		59	43	
<b>Gender</b>							
Male	71	42	29	0.674	35	36	0.371
Female	85	50	30		48	37	
<b>Tumor thickness (mm)</b>							
≤2	86	43	43	0.012	37	49	0.005
>2	70	49	21		46	24	
<b>Invasion depth classification</b>							
I	54	15	39	0.000	17	37	0.000
II	31	19	12		16	15	
III	40	28	12		29	11	
IV	27	26	1		20	7	
V	4	4	0		3	1	
<b>Lymph node metastasis</b>							
metastasis	115	76	39	0.002	69	46	0.004
Non-metastasis	41	16	25		14	27	
<b>Histological classification</b>							
Freckles	13	8	5	0.996			0.996
Superficial diffusion	20	12	8				
Acromegaly freckles	109	64	45				
Nodular	14	8	6				
<b>TNM stage</b>							
I+II	46	33	13	0.036	31	15	0.22
III+IV	110	59	51		52	58	

### 2.2. Relationship between TAZ/YAP expression and clinical data of malignant melanoma

In this study, the proportion of TAZ positive cells  $\geq 42\%$  was identified as high expression, and the ratio of YAP positive cells  $\geq 44\%$  was identified as high expression. The relationship between TAZ/YAP expression and clinical data of malignant melanoma was analyzed. The results showed that TAZ and YAP were highly expressed in 92 and 83 cases, respectively, in 156 malignant melanoma tissues. Their expressions were significantly correlated with tumor thickness, invasion depth grade, lymph node metastasis and TNM stage ( $P < 0.05$ ), but not with age, gender and histological type ( $P > 0.05$ ), as shown in the Table.

### 2.3. Relationship between TAZ/YAP expression and clinical stage of malignant melanoma

Of the 156 patients with malignant melanoma, there were 26 cases of TNM stage I, 20 cases of stage II, 96 cases of stage III and 14 cases of stage IV. The levels of TAZ and YAP in different TNM staged malignant melanoma tumor tissues were detected by western blotting and immunohistochemistry. The results showed that the protein expression of TAZ/YAP protein in malignant melanoma tumor tissue expression increased with TNM staging, as shown in Fig. 2.

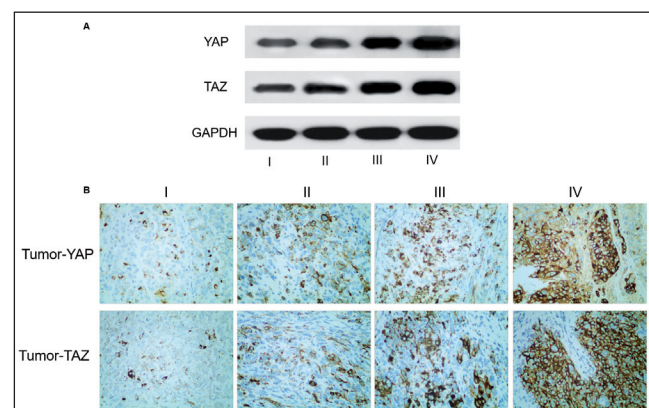


Fig. 2: Relationship between TAZ / YAP expression and the clinical stage of malignant melanoma. A: the western blot results of TAZ/YAP in the clinical stage I/II/III/IV of malignant melanoma. B: the immunohistochemistry of TAZ/YAP in the clinical stage I/II/III/IV of malignant melanoma.

### 2.4. Relationship between TAZ/YAP expression and lymph node metastasis in malignant melanoma

In 156 cases of malignant melanoma, there were 115 cases with lymph node metastases and 41 cases without lymph metastases.

The expression level of TAZ/YAP in malignant melanoma with lymph node metastases was significantly higher than that in non-metastatic tissue, as shown in Fig. 3.

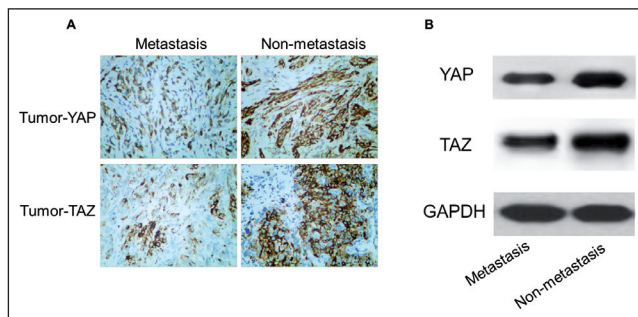


Fig. 3: Relationship between TAZ / YAP expression and lymph node metastasis of malignant melanoma. A: immunohistochemistry of YAP/TAZ in metastasis and non-metastasis tumor tissue. B: the western blot results of YAP/TAZ in metastasis and non-metastasis tumor tissue.

### 2.5. Relationship between TAZ/YAP expression and the prognosis of malignant melanoma

A total of 156 patients with malignant melanoma were followed up for 4 years (48 months). The results showed that the 1-year, 2-year, 3-year and 4-year survival rates of patients with low expression of TAZ protein in malignant melanoma were 87.50 %, 81.25%, 75.00 % and 75.00 %, respectively, while the surviving rates of patients with high expression of TAZ were 73.91 %, 66.30 %, 47.83 % and 39.13 %, respectively. Patients with low expression of YAP protein in malignant melanoma tumor tissue had 1-year, 2-year, 3-year and 4-year survival rates of 89.04 %, 89.04 %, 79.45 % and 71.23 %, respectively, while the corresponding patients with high expression of YAP had 71.08 %, 57.83 %, 40.96 % and 39.76 % respectively. The expression of TAZ or YAP in malignant melanoma tissue reduces the postoperative survival rate of patients, as shown in Fig. 4.

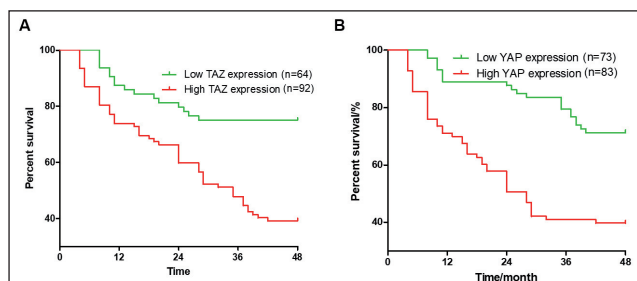


Fig. 4: High expression of TAZ / YAP reduces the postoperative survival rate of malignant melanoma patients. A: low and high TAZ expressions in 1-year, 2-year, 3-year and 4-year survival rates. B: low and high YAP expressions in 1-year, 2-year, 3-year and 4-year survival rates.

## 3. Discussion

In this study, we detected the expression of TAZ/YAP protein in 156 cases of malignant melanoma and 23 cases of melanocytic nevi tissues by immunohistochemistry and western blot. The results showed that the number of TAZ and YAP protein positive cells in malignant melanoma tumor were significantly higher than that in melanocytic nevi tissues. And the expression of two proteins in malignant melanoma tissue was positively correlated ( $r = 0.687$ ,  $P < 0.01$ ). TAZ/YAP are important components of the Hippo signaling pathway. When cells growth reaches a certain density, Hippo signaling pathway is activated and then causes Lats1/2 gene expression and phosphorylation, while TAZ/YAP transcriptional function will be inhibited, and then the cells are arrested in the G1/S and G2/M phases and cell proliferation is inhibited (Cordenonsi et al. 2011; Hau et al. 2013). However,

when the Hippo signaling pathway is blocked or inactivated, TAZ/YAP will be transcribed without transcriptional repression, and then entered the nucleus and bind to the TEA transcription factor family to enhance its transcriptional function, inducing cell mitosis and proliferation, and further promote the epithelial-mesenchymal transition of cells which eventually increases the invasion and migration and proliferation of cells (Cordenonsi et al. 2011; Hau et al. 2013). The Hippo signaling pathway is a tumor suppression pathway and is involved in the invasion and metastasis of many malignant tumors. The signal transduction of the Hippo signaling pathway is often blocked or inactivated in malignant tumors. As an important effector protein of Hippo signaling pathway, TAZ/YAP often get highly expressed due to inhibition of the Hippo pathway inactivation or block of conduction (Wang et al. 2016).

The occurrence and development of malignant tumors is a multi-step process which is caused by multiple factors both inside and outside. The characteristic that tumor cells continue to grow from the primary site to the other sites is not only the criterion for differentiating the benign and malignant tumors, but also the cause of treatment failure or even death of malignant tumor patient. The process of epithelial-mesenchymal transition is one of the key steps for tumor cell invasion and migration and is one of the most important causes of death in patients with malignant tumors (Jeong et al. 2013; Setonrogers 2016; Sung et al. 2016).

In this paper, the relationship between TAZ/YAP expression and clinical data of patients with malignant melanoma was analyzed. It was found that the expression of TAZ/YAP in malignant melanoma tumor tissue was significantly correlated with tumor thickness, invasion depth grade, lymph node metastasis and TNM stage. Their expression increased with the TNM staging, and their expression in patients with lymph node metastasis was significantly higher than that in patients without metastasis.

Related studies have confirmed that when TAZ/YAP protein expressed in large quantities and exerts their transcriptional function, they promote the epithelial-mesenchymal transition of tumor cells and enhances the invasion and metastasis of tumor cells (Lei et al. 2008; Shao et al. 2014). In their study of malignant melanoma, Feng et al. (2017) detected the expression levels of MAPK and Hippo signaling pathway effector proteins in four melanoma cell lines, C32, HS695T, SK-MEL and A375 by western blotting. RAF-1 in melanoma cells can significantly inhibit the expression of TAZ/YAP, suggesting that there may be some connection between the MAPK and the Hippo pathway. The synergistic effect of MAPK and PI3K/Akt signaling pathway on epithelial-mesenchymal transition of tumor cells has been confirmed by several studies (Bai et al. 2017; Jin et al. 2012; Zhou et al. 2017). Combined with the above, we suggest that TAZ/YAP enhance the invasion and migration of malignant melanoma cells not only related to their function in the Hippo signaling pathway, but also may play a role in promoting epithelial-mesenchymal transition of tumor cells independent of the Hippo signaling pathway.

In addition, this study also found that patients with malignant melanoma that highly express TAZ or YAP protein had significantly lower postoperative survival rates than those with low expression. Epidemiological studies found that the clinical stage and prognosis of malignant melanoma were significantly correlated. The 5-year survival rates of stage I, II, III and IV were 94 %, 44 %, 38 % and 4.6 %, respectively. However, patients with lymph node metastasis have a 5-year survival of less than 60 % and patients with distant organ metastases have a 5-year survival of less than 15 % (Olszanski 2014).

Taken together, TAZ/YAP is overexpressed in malignant melanoma and its high expression promotes the progression of malignant melanoma and affects the survival of patients. However, it should be noted that there are still some shortcomings of this study. First, the number of cases involved is limited and has geographical limitations. Second, the specific molecular mechanism by which TAZ/YAP is highly expressed in malignant melanoma tissue to promote disease progression has not yet been revealed.

## 4. Experimental

### 4.1. Research objects

A total number of 156 malignant melanoma tumor tissues surgically excised from The Fourth Hospital of Hebei Medical University from January 2010 to December 2013 were collected. There were 89 males and 67 females between 32 and 69 years old, the median age was 47 years. The clinical data of study subjects are shown in the Table. A total number of 23 melanocytic nevi tissues (12 males and 11 females with age of 32 - 69 years old) were surgically excised from the same time and same hospital as malignant melanoma tumor tissues. The study was approved by the ethics committee of The Fourth Hospital of Hebei Medical University. Inclusion criteria: First, postoperative diagnosis of malignant melanoma by pathological diagnosis. Second, clinical data such as age, gender, tumor diameter, cancer metastasis and clinical stage were complete. Third, no preoperative radiotherapy and chemotherapy, immunotherapy or molecular targeted therapy treatment. Fourth, regular follow-up or review after surgery, the cause of death or time is clear. Fifth, patients or their families were informed of this study, and signed informed consent. Exclusion criteria: First, the merger of other malignancies, or malignant melanoma is metastable. Second, postoperative non-disease or death due to other sudden illness. Third, the merger of chronic infectious diseases, liver and kidney or other organ dysfunction, or other serious cardiovascular and cerebrovascular diseases. Fourth, poor physical fitness, cannot tolerate surgery or other related tests, postoperative mental state is poor, because of their own basic conditions and prognosis affect. Fifth, postoperative follow-up loss or death is not clear.

### 4.2. Western Blot to detect the protein expression

Tissue samples cryopreserved in liquid nitrogen were promptly added to a liquid nitrogen mill (Haizhou Special Gas, China), and the tissue samples were repeatedly pulverized by adding liquid nitrogen repeatedly. After referring to the instruction manual of ProteoPrep Total Extraction Sample Kit (Sigma-Aldrich, Germany), the protein in malignant melanoma tumor tissues and healthy tissues was extracted and the protein concentration was determined by BCA Protein Quantification kit (Yeasen Biotechnology, China). Then the protein was separated by SDS-PAGE (90 V for 30 min and 120 V for 60 min), transferred to PVDF membrane (400 mA for 90 min), fixed in anhydrous methanol for 1 min, washed in TBST three times (5 min) 5% skim milk was blocked for 2 h at room temperature. Rabbit monoclonal antibody GAPDH or YAP1 or TAZ (Abcam, UK) was incubated overnight at 4 °C, washed three times with TBST (10 min) and incubated with Goat Anti-Rabbit IgG H & L (HRP) for 1-2 h, then washed with TBST three times (10 min / times) and then detected the protein expression with ECL kit. ImageJ software was used to analyze the grayscale of protein bands. The relative protein expression level was calculated by the ratio of the grayscale of the target protein and the gray value of GAPDH bands.

### 4.3. Immunohistochemistry to detect TAZ/YAP expression

All tissue samples obtained from operation were taken appropriate part to make paraffin sections. The paraffin sections are deparaffinized to water, sealed with H<sub>2</sub>O<sub>2</sub>, and washed with double distilled water. After antigen retrieval immunohistochemistry was used to detect the expression of the target proteins. The specific detection steps are performed according to the instructions of the VECTASTAIN® Elite® ABC Kit (Vector Laboratories, USA), as follows: antigen-fixed paraffin sections were washed with PBS 2-3 times (5 min / times), blocked with 5% goat serum (TransGen Biotech, China) at 37 degrees for 20min, the filter paper was used to remove the serum and YAP or TAZ Rabbit polyclonal antibody (Abcam, UK) was added dropwise and incubated overnight at 4 °C, washed three times with PBS (5 min), and incubated with Goat Anti-Rabbit IgG H & L (HRP) at 37 °C for 2 h, and use DAB for color development (PBS instead of primary antibody was used as a negative control). Each paraffin section was photographed at five fields and counted. YAP/TAZ was located in the nucleus and cytoplasm, both of which were positive in yellow-brown, and the positive cell rate was used as the measurement of target protein expression.

### 4.4. Statistical analysis

All the results were statistically analysed by SPSS 20.0 (IBM, USA). Chi-square test was used to analyze the relationship between TAZ/YAP protein expression and clinical pathology. Paired-samples t-test was used to compare the difference of

measurement data. Spearman analysis of expression correlation of TAZ and YAP in malignant melanoma tissue; Survival analysis by Kaplan-Meier curve and log-rank test; Multivariate analysis by Cox regression model; all statistical analyzes showed significant difference with P < 0.05.

Conflicts of interest: None declared.

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