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Research Article

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EZH2 Expression and its Correlation with Clinicopathological Features in Patients with Colorectal Carcinoma

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Abstract: *Objective*: To explore the correlation between the enhancer of zeste homolog 2 (EZH2) expression and clinicopathological features in colorectal cancer patients. *Methods*: A total of sixty-six patients with colorectal carcinoma were admitted to our general surgery department from January 2011 to December 2014. The EZH2 expression levels in the cancer tissues (CTs) from the 66 patients with colorectal cancer and those in distant normal colorectal tissues from 30 cases were examined through immunohistochemistry and western blotting assays. The relationship between the expression of EZH2 and the clinicopathological features and prognosis of the patients was analyzed. *Results*: EZH2 in colorectal carcinoma tissues is granularly brown, predominantly expressed and diffused in the nuclei of tumor cells. Positive rates of EZH2 in intestinal CTs and in distant normal intestinal tissues are 62.12% (41/66) and 6.67% (2/30), respectively with significant difference ($P < 0.05$). Western blotting also confirmed its elevated expression in colorectal CTs. EZH2-positive expression in CTs was related to degree of differentiation, Duke staging, and tumor size ($P < 0.05$) but was unrelated to the patient's gender, age or tumor site ($P > 0.05$). The 3-year progression-free survival (PFS) rates of the EZH2-positive group and the EZH2-negative group were 43.8% and 67.5%, respectively. The risk of disease progression of the EZH2-positive patients in the follow-up period was significantly higher than that of the EZH2-negative patients (HR = 2.49, 95% CI = 1.04–4.80, $P < 0.05$). *Conclusion:* EZH2 is closely related to colorectal carcinoma development and disease progression, and thus could be used as a tumor biomarker that may indicate prognosis.

Keywords: EZH2; colorectal carcinoma; immunohistochemistry; clinicopathological features; prognosis

1 Introduction

Colorectal carcinoma is a common digestive tumor. According to North American cancer epidemiology data, the incidence of colorectal cancer ranks fourth among all solid tumors [1]. In 2012, the number of patients newly diagnosed with colorectal cancer in the United States was 143,460, and the number of deaths among them was 51,690 [2]. In recent years, the incidence of colorectal carcinoma in China has increased [3], but the molecular mechanisms of the occurrence and the development, invasion, and metastasis of colorectal carcinoma remain unclear [4]. With continuous advancements in molecular biology technique, the regulatory mechanism of the malignant biological behaviors of colorectal cancer has been elucidated. This mechanism is a multi-step regulation that involves various genes and protein factors. For instance, the enhancer of zeste homolog 2 (EZH2) plays an important role in the development of various tumors [5]. The abnormal expression of the related gene is likely associated with the malignant phenotypes of various solid tumors [6]. In our study, EZH2 expression in colorectal carcinoma tissues and distant normal intestinal tissues was examined through immunohistochemistry and western blot to establish the relationship between the expression of EZH2 and the clinicopathological features and prognosis of the disease, to explore the role of EZH2 in colorectal carcinoma development.

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2 Materials and methods

2.1 Subjects

A total of 66 colorectal carcinoma patients admitted to our general surgery department from January 2011 to December 2014 were enrolled as subjects in this study. EZH2 expression levels in cancer tissues (CTs) from the 66 patients and in distant normal colorectal tissues from 30 cases were examined through immunohistochemistry or western blot assays. The relationship between the expression of EZH2 and the clinicopathological features and prognosis of the patients was analyzed. Of the 66 patients, 36 were males and 30 were females, with an average age of 62.1 ± 15.6 years. Of these patients, 26 were diagnosed with colon carcinoma and 40 were diagnosed with rectal carcinoma. Of the 66 cases, 14 were highly differentiated and 52 were poorly differentiated or undifferentiated. In terms of Duke staging, 38 cases were in the A/B stage and 26 cases were in the C/D stage.

2.2 Methods

2.2.1 Instruments and equipment

The following instruments were used in this study: (1) paraffin slicer (Leica Camera AG; Model: SHANDONAS-325); (2) OLYMPUSBX-40 microscope (Olympus); (3) constant-temperature bath (Grant Instruments); (4) water purification system (Millipore Corporation); and (5) low-speed tabletop centrifuge machine (B160A; Beijing Bai Yang Centrifuges Co., Ltd.).

2.2.2 Immunohistochemistry

2.2.2.1 De-waxing and dehydration

Tissue slices were soaked initially in dimethylbenzene for 10 min and then in absolute ethyl alcohol for 5 min. Afterwards, the slices were successively soaked in 95%, 80%, and 79% ethanol for 5 min.

2.2.2.2 Antigen retrieval

The tissues slices were subsequently soaked in a water bath containing 0.01% mol/L sodium citrate solution at 95 °C 15 min.

2.2.2.3 Immunohistochemical staining

The tissues were de-waxed and dehydrated step-by-step in accordance with the manufacturer's instructions. Primary and secondary antibodies were added by dripping after the antigen was retrieved. The tissues were developed and mounted for microscopic examination.

2.2.3 Determination of positive results

Staining results were determined via a double-blind test. Under a 400× microscope with five fields, the total number of cells per field and the population of EZH2-positive cells were counted on each slide. The following criteria were considered to determine the positive results in terms of staining intensity: 0, not stained; 1, lightly stained; 2, moderately stained; and 3, strongly stained. The following criteria were considered to classify the percentage of positive cells: 0, not stained; 1, < 25%; 2, 25%–50%; and 3, > 50%. Results were semi-quantitatively categorized on the basis of the sum of the two integers: 0, "–"; 1–2, "+"; 3–4, "++"; and, 5–6, "+++". Results were qualitatively described as follows: (1) positive if >2 (++ and +++) and (2) negative if <2 (– and +).

2.2.4 Western blot

Protein was isolated by lysis buffer from fresh samples under the standard protocol. After protein was quantified, equal amount of protein was provided for SDS-PAGE running and transferring to the PVDF membrane, followed by incubation with primary antibodies and the appropriate HRP-conjugated secondary antibodies. Finally, all blots were visualized with ECL and analyzed by ImageJ software.

2.3 Statistical analysis

Data were statistically analyzed using SPSS17.0 (http://www-01.ibm.com/software/analytics/spss/) and expressed as $\bar{x} \pm s$. Between-group comparison was conducted via *t*-test, and the counted data were expressed in terms of rate. Between-group comparison was performed via χ^2-test or Fisher's test for exact test. A proportional hazard model was selected for survival analysis through a log-rank test. Differences were considered significant at $P < 0.05$.

3 Results

3.1 EZH2 expression in CTs and normal intestinal tissues

We firstly analyzed EZH2 gene expression level in various kinds of human malignancies using the online database (Metabolic gEne RApid Visualizer), and found that the EZH2 gene might be upregulated in colon primary tumors in comparison with normal colon tissues (Figure 1). Our western blotting results further supported this prediction (Figure 2). IHC staining method showed that EZH2 in colorectal carcinoma tissues is a granular brown substance predominantly expressed and diffused in the nuclei of tumor cells. In our study, the positive rates of EZH2 in CTs and distant normal intestinal tissues were 62.12% (41/66) and 6.67% (2/30), respectively with statistical difference ($P < 0.05$).

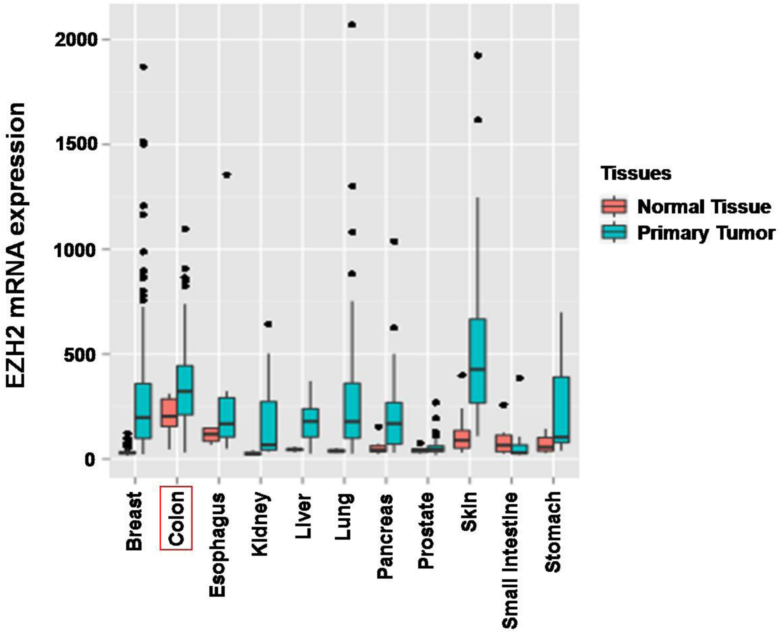

Figure 1. EZH2 gene expression in different kinds of human cancers. EZH2 gene was selected for mRNA expression analysis on the online database (Metabolic gEne RApid Visualizer). Different primary tumors and matched normal tissues were selected, and the results were given as a boxplot curve.

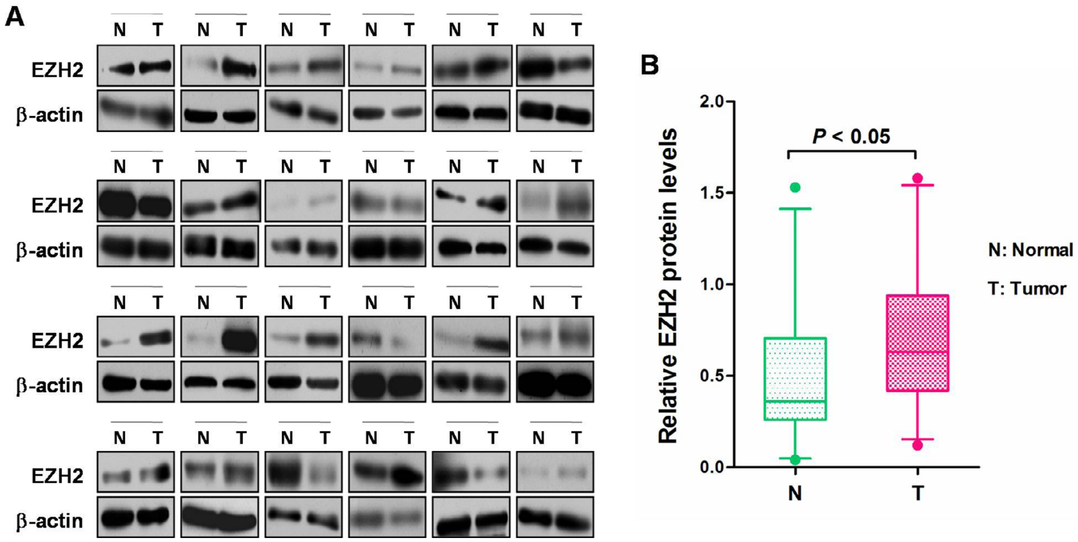

Figure 2. EZH2 protein expression in selected pairs of colorectal carcinomas. **(A)** 24 pairs of colorectal carcinomas were for protein extracting and western blotting for detecting the protein levels of EZH2. B-actin was as the loading control. **(B)** Quantified data was given according to the results of **(A)**. Between-group comparison was conducted via *t*-test. N, normal; T, tumor.

3.2 Relationship and pathological features

EZH2-positive expression in CTs was related to differentiation degree, Duke staging, and tumor size ($P < 0.05$) but was unrelated to gender, age or tumor site ($P > 0.05$; Table 1).

3.3 Correlation between EZH2 expression and prognosis

The 3-year progression-free survival (PFS) rates of the EZH2-positive group and the EZH2-negative group were 43.8% and 67.5%, respectively. The risk of disease progression of the EZH2-positive patients in the follow-up period was significantly higher than that of the EZH2-negative patients (HR = 2.49, 95% CI = 1.04–4.80, $P < 0.05$).

4 Discussion

EZH2, also known as ENX-1, was discovered by Hobert et al. using a yeast two-hybrid system in 1996 [7]. It is a human homolog of the *Drosophila* enhancer of zeste gene [E(z)] acting with Vav in vitro or in vitro. Human EZH2, located in Region-5 in Band-3 of Chr-20, is an important member of the polycomb group. The EZH2 product can promote the proliferation and diffusion of tumor cells; EZH2 is also abnormally expressed in a broad range of malignant tumors, such as prostate cancer, bladder carcinoma, breast carcinoma, liver cancer, and gastric cancer [8]. However, the relationship between EZH2 and colorectal carcinoma has been rarely investigated; the relationship of EZH2 with biological behaviors, including occurrence, development, invasion, and metastasis of colorectal carcinoma, is yet to be fully elucidated. EZH2 may be involved in the proliferation and apoptosis of human colorectal carcinoma cells. EZH2 is also a typical oncogene highly expressed in various malignant cells, including breast carcinoma, prostate cancer, and lung cancer [9,10].

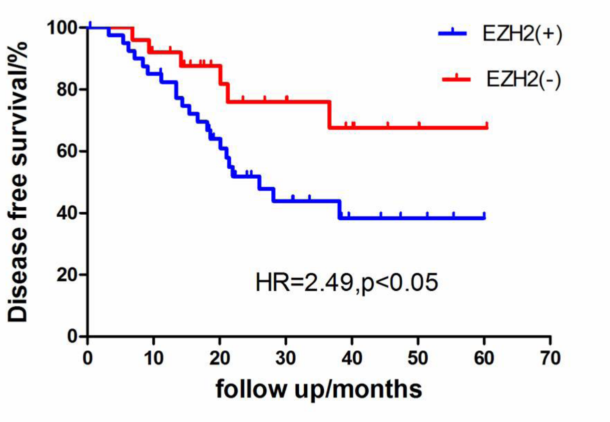

Figure 3. EZH2 Prognosis Survival Curve.

66 cases of colorectal carcinomas were classified into EZH2-positive and –negative groups. Survival curve was plotted by utilizing their survive time, and a proportional hazard model was selected for survival analysis through a log-rank test.

Table 1. Correlation between EZH2 expression and characteristics of colorectal cancer.

Characteristics	**n = 66**	**EZH2**		**P**
		Positive (n = 41)	**Negative (n = 25)**	
Gender				> 0.05
Male	36	22	14	
Female	30	19	11	
Age (year)				> 0.05
< 50	16	12	8	
50-60	26	17	10	
> 60	24	12	7	
Tumor site				> 0.05
colon	26	16	10	
rectum	40	25	15	
Tumor diameter (cm)				< 0.05
> 5	31	25	6	
≤ 5	35	16	19	
Differentiation				< 0.05
High / medium	14	5	15	
Low / undifferentiated	52	36	10	
Duke stage				< 0.05
A/B	38	18	20	
C/D	28	23	5	

However, the relationships between the expression of EZH2 and the clinicopathological features and prognosis of colorectal carcinoma have been rarely reported. In our study, specimens from colorectal carcinoma patients undergoing surgical resections in our hospital were analyzed. The expression of EZH2 gene in different kinds of human cancers was initially predicted and then its protein expression in intestinal CTs and distant normal intestinal tissues was detected through immunohistochemical as well as western blotting. The correlation between the expression of EZH2 and the clinicopathological features of colorectal carcinoma was analyzed. Our results revealed that higher EZH2 expression was related to differentiation degree, Duke staging, and tumor size ($P < 0.05$) but was unrelated to gender, age, tumor site, and general type ($P > 0.05$). Among the poorly differentiated patients with large tumor sizes and advanced Duke staging, the positive rate of EZH2 was high. This finding indicated that EZH2 expression was significantly correlated with malignant biological behaviors of colorectal carcinoma. The malignant biological behaviors of the EZH2-positive patients were more evident than those of the EZH2-negative patients. The patients enrolled in our study were divided into two groups, namely, the EZH2-positive group and the EZH2-negative group. Using a proportional hazard model, we plotted a survival curve to investigate the relationship between EZH2 and prognosis. The 3-year PFS rates of the EZH2-positive group and the EZH2-negative group were 43.8% and 67.5%, respectively. The risk of disease progression of the EZH2-positive patients in the follow-up period was significantly higher than that of the EZH2-negative patients ($P < 0.05$).

Although we had characterized the expression and clinical significance of EZH2 in colorectal carcinoma, we still have no knowledge about the mechanisms that drive the upregulation of this protein, and whether transcription/ epigenetic activation is involved. This is an interesting questions that deserves more attention.

To summarize, in this study, the EZH2 expression in intestinal cancer was detected through immunohistochemistry assay and western blot. The relationship between the expression of EZH2 and the clinical features of colorectal carcinoma was investigated. Our results confirmed that EZH2 is closely related to colorectal carcinoma development and disease progression. However, the precise molecular mechanisms by which EZH2 regulates the occurrence, development, and metastasis of colorectal carcinoma are yet to be described. We next will concentrate more on the biological roles and internal mechanisms of EZH2 in oncogenesis of colorectal carcinoma.

Conflict of interest: Authors declare nothing to disclose.

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