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Intratumoral heterogeneity of esophageal squamous cell carcinoma and its clinical significance

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ABSTRACT

Recent studies have shown that intratumoral heterogeneity is prevalent in esophageal squamous cell cancer (ESCC) based on DNA sequencing and chromosome analysis in multiple regions from the same tumor. This study aimed to investigate the expression of ZNF750, EP300, MTOR and KMT2D and their intratumoral heterogeneity (ITH) in patients with ESCC. A total of 106 cases, who underwent esophagectomy from 2008 to 2010, with two foci from each case, were tested by immunohistochemistry(IHC) as well as 12 cases were tested by RNAscope in this study.We found that 58/106 (54.72%), 66/106 (62.26%), 75/106 (70.75%%) of ESCC showed high expression of ZNF750, EP300, MTOR, respectively by IHC, and 8/12 (66.67%), 10/12 (83.33%), 4/12 (33.33%) and 6/12 (50%) showed high expression of ZNF750, EP300, MTOR and KMT2D, respectively by RNAscope. Multivariate analysis showed that MTOR expression was an independent infavorable prognostic factor of overall survival (OS) (HR = 1.921; P = 0.000). This study also found that 44/106(4151%), 37/106 (34.91%), 39/106(36.79%) of ESCC showed heterogeneous expression of ZNF750, EP300 and MTOR respectively by IHC, 8/12(66.67%), 8/12(66.67%), 4/12(33.33%), 4/12(33.33%) of ZNF750, EP300, MTOR and KMT2D respectively by RNAscope, IHC and RNAscope could successfully detect a high prevalence of ITH. In conclusion, findings of this study showed that ZNF750, EP300, MTOR and KMT2D heterogeneously expressed in ESCC. High expression of ZNF750 related to a better outcome, while EP300 and MTOR related to a poor prognosis.

1. Introduction

Esophageal cancer is one of the most lethal cancers and a public health concern worldwide [1]. China is of the highest incidence and mortality [2]. The most prevalent type is esophageal squamous cell carcinoma (ESCC), the annual incidence of new cases account for 50%. Intratumoral heterogeneity (ITH) has been found in leukemia, glioblastoma, as well as pancreas, breast, ovary, renal clear cell carcinoma, esophageal cancer [3–9]. In view of the evidence that ITH is the major cause of drug resistance and treatment failure, the 5-year survival rate is relatively and high risk of recurrence after definitive therapy [10,11].

To date, ITH in ESCC has been mostly studied using Next GenSequencing,which revealed DNA mutations in exomes. A recent study [9] about whole-exome sequencing on 51 tumor regions from 13 ESCC cases found MTOR, KMT2D, EP300 and ZNF750 are four of the geins of heterogeneity with few studies, and they represent important players in ESCC. Since immunohistochemistry (IHC) is a mature technology that can be applied to human sample relatively inexpensively, RNAscope technology makes it possible to study multiple transcripts in the same tissue at high resolution [12], which has a high degree of specificity and sensitivity and has been used in heterogeneity studies of multiple tumors such as breast cancer [13]. In this study,we decided to stain for ZNF750, EP300, MTOR and KMT2D both by IHC and RNAscope.

Deciphering the genomic diversity of ESCC tumors, identifying new targets, screening early diagnostic markers, and design individualized treatment strategies are hot and difficult to study today. Usually we perform a single biopsy to determine the patient's mutation signatures. If the tumor has regional heterogeneity, a single site assessment may miss the DNA mutations in other regions of the same tumor and will also fail to select which mutation is most important and whether it should be targeted, the best choice of treatment, patients may miss the best treatment.

2. Results

2.1. ZNF750, EP300, MTOR and KMT2D protein expression in ESCC

Among the 106 ESCC cases, high expression of ZNF750, EP300, MTOR were detected in 58 of 106(54.72%), 66 of 106(62.26%), 75 of

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Table 1
ZNF750, EP300, MTOR protein expression and clinicopathologic variables.

Variable		Cases N = 106	ZNF750 High	*P*	EP300 High	*P*	MTOR High	*P*
Age(years)	≤60	62	36	0.246	40	0.145	42	0.419
	> 60	44	22		26		33	
Gender	Male	80	44	0.885	53	0.694	56	0.844
	Femal	26	14		13		18	
Tumor location	Upper Middle	15	9	0.111	9	0.747	10	0.600
	Lower	62	36		40		44	
		29	13		17		21	
Pathological grade	G1	5	4	0.003	2	0.011	1	0.000
	G2	73	42		43		49	
	G3	28	12		21		23	
ClinStage	I	7	6	0.002	2	0.005	3	0.000
	II	39	26		25		24	
	III	60	26		39		48	
Lymph node metastasis	Negative	47	26	0.000	21	0.000	21	0.000
	Positive	59	49		45		10	
T status	T1	9	1	0.000	4	0.778	8	0.000
	T2	27	18		20		9	
	T3	70	54		42		16	
Tumor thrombus	Negative	67	42	0.001	36	0.001	25	0.001
	Positive	39	33		30		6	

ZNF750, Zinc finger protein 750; EP300, E1A-associated protein p300; MTOR, the mammalian target of rapamycin.

106(70.75%) respectively, low expression were detected in 48 of 106 (45.28%), 40 of 106(37.72%), 31 of 106 (29.25%) respectively. KMT2D was not detected by immunohistochemical of the 106 cases with ESCC.

ZNF750, EP300 and MTOR were closely related to histological grade, clinical stage, vascular thrombosis and lymph node metastasis. ZNF750 also was closely related to depth of invasion (Table 1). ZNF750 protein was positively correlated with the prognosis of ESCC patients (Fig. 1A). The expression of EP300 and MTOR protein was negatively correlated with the prognosis of ESCC patients (Fig. 1B and C). MTOR was an independent prognostic factor of multivariate analysis (Table 2).

2.2. Summary of protein expression in tumors and foci

We found that 54% of the 106 tumors were of high expression of ZNF750, 62% of EP300, 71% of MTOR (Fig. 2A). Because not all tow foci showed high expression of a specific marker (Fig. 3), the percentage of all foci that show high of expression was lower than for all cases. Thus, for foci, high protein expression was lower: 34%, 45%, 52% of foci high expression of ZNF750, EP300 and MTOR, respectively.

We report the percentage of tumors with protein high expression followed by percentage of foci with protein high expression in parenthesis. The high expression rates of ZNF750, EP300 and MTOR in stage I–III were 85.71% (42.86%), 66.67% (38.46%), 43.33% (25.00%)

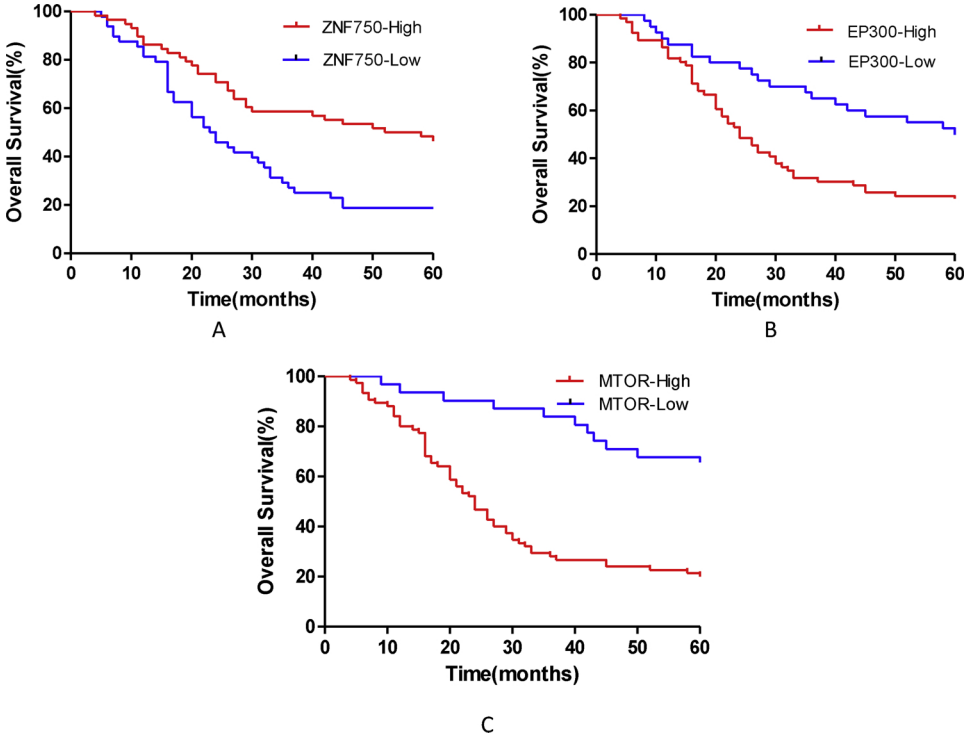

Fig. 1. Kaplan-Meier survival analysis of expression of each maker in106 ESCC patients: ZNF750 protein expression was positively correlated with the prognosis of ESCC patients, P = 0.049 (A), EP300 and MTOR protein expression were negatively correlated with the prognosis of ESCC patients, P = 0.032, P = 0.000 (B&C).

Table 2
Multivariate analysis of overall survival.

Factors	B	*P* Value	HR	95.0% CI for Exp(B)	
				Lower	Upper
cStage	.488	0.030	1.630	1.049	2.532
Pathological grade	.277	.102	1.319	.946	1.838
Tumor thrombus	.202	.306	1.224	.831	1.803
Lymph node metastasis	.108	.694	1.114	.649	1.914
ZNF750	−.091	.561	.913	.673	1.240
EP300	.258	.080	1.295	.969	1.729
MTOR	.653	.000	1.921	1.331	2.773

ZNF750, Zinc finger protein 750; EP300, E1A-associated protein p300; MTOR, the mammalian target of rapamycin.

vs 28.57% (21.43%), 62.10% (44.87%), 66.67% (57.50%) vs 42.85% (28.57%), 61.54% (45.69%), 80.00% (56.67%) respectively. ZNF750 expression decrease with rising tumor stage (Fig. 2B). EP300 expression and MTOR expression increase with rising tumor stage (Fig. 2C, D).

2.3. ZNF750, EP300, MTOR and KMT2D mRNA expression in ESCC

Of the 12 cases of esophageal squamous cell carcinoma, the high expression of ZNF750, EP300, MTOR and KMT2D mRNA were 66.67%, 83.33%, 33.33% and 50% respectively.

ZNF750 was closely related to histological grade, clinical stage, vascular thrombosis, lymph node metastasis and age. EP300 was closely related to histological grade, clinical stage, lymph node metastasis, sex and tumor location. MTOR was closely related to histological grade, clinical stage, vascular thrombosis, depth of invasion, lymph node metastasis and age (Table 3). There was no significant correlation between KMT2D expression and characteristics. Eight patients showed heterogeneous expression of ZNF750 and EP300 mRNA (Fig. 4A and B), four cases of MTOR and KMT2D mRNA showed heterogeneous expression (Fig. 4C and D).

3. Discussion

At present, targeted therapy for clinical treatment of ESCC has not yet been established, at the same time, subgroups of drug-resistant cells are present in most tumors, ITH will mean that some of the cancer cells also have other genes or epigenetic mutations that will lead to cell resistance to targeted therapy [14] which presents an important challenge to the field of precision medicine. Our study showed ZNF750, EP300, MTOR and KMT2D heterogeneous expression in ESCC, analysis of ESCC patients with genomic diversity and clone formation time will identify new targets and design individualized therapy strategy to provide a theoretical basis.

In recent years, several large-scale sequencing [13,15–17] studies have characterized the ESCC genome with hundreds of somatic mutations and copy number changes and have identified significantly mutated genes including ZNF750, KMT2D, EP300 and MTOR, among others. A study about spatial intratumor heterogeneity and temporal clonal evolutionary processes of ESCC also revealed that majority of driver mutations occurred in tumor-suppressor genes, including KMT2D and ZNF750, which were relatively late events during tumor evolution while PI3K- mTOR pathway (PIK3CA and MTOR). At present, ITH research of ESCC is mainly based on whole gene exon sequencing (WES) or second generation sequencing (NES) which provides an accurate representation of ITH in tumors, but the cost tends to be expensive and labor intensive [18]. In our study, we selected RNAscope technology and immunohistochemistry (IHC) to carry out experiments. RNAscope

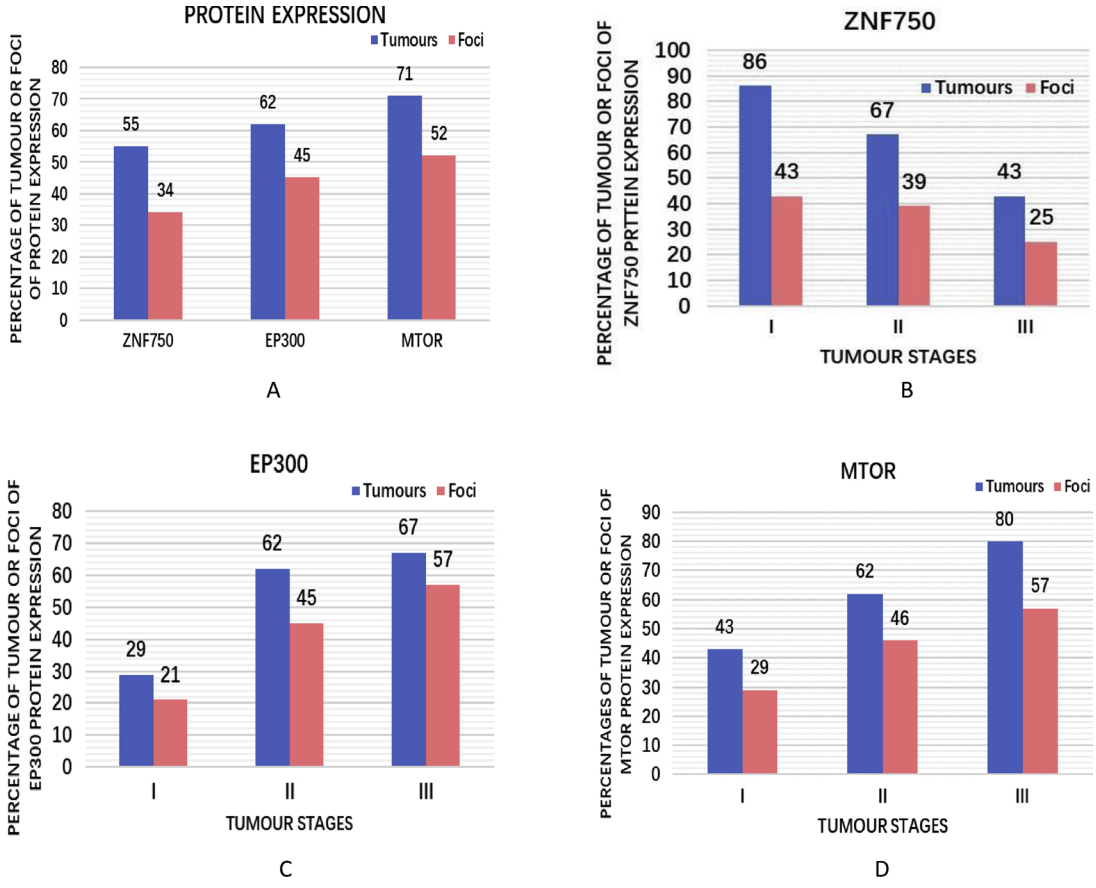

Fig. 2. Summary of high expression of protein markers in ESCC tumors and foci. (A)Overall high expression of each marker in tumor and foci. High expression of individual marker in different tumor stages: ZNF750, $P = 0.002$ (B), EP300, $P = 0.0025$(C), MTOR, $P = 0.000$ (D).

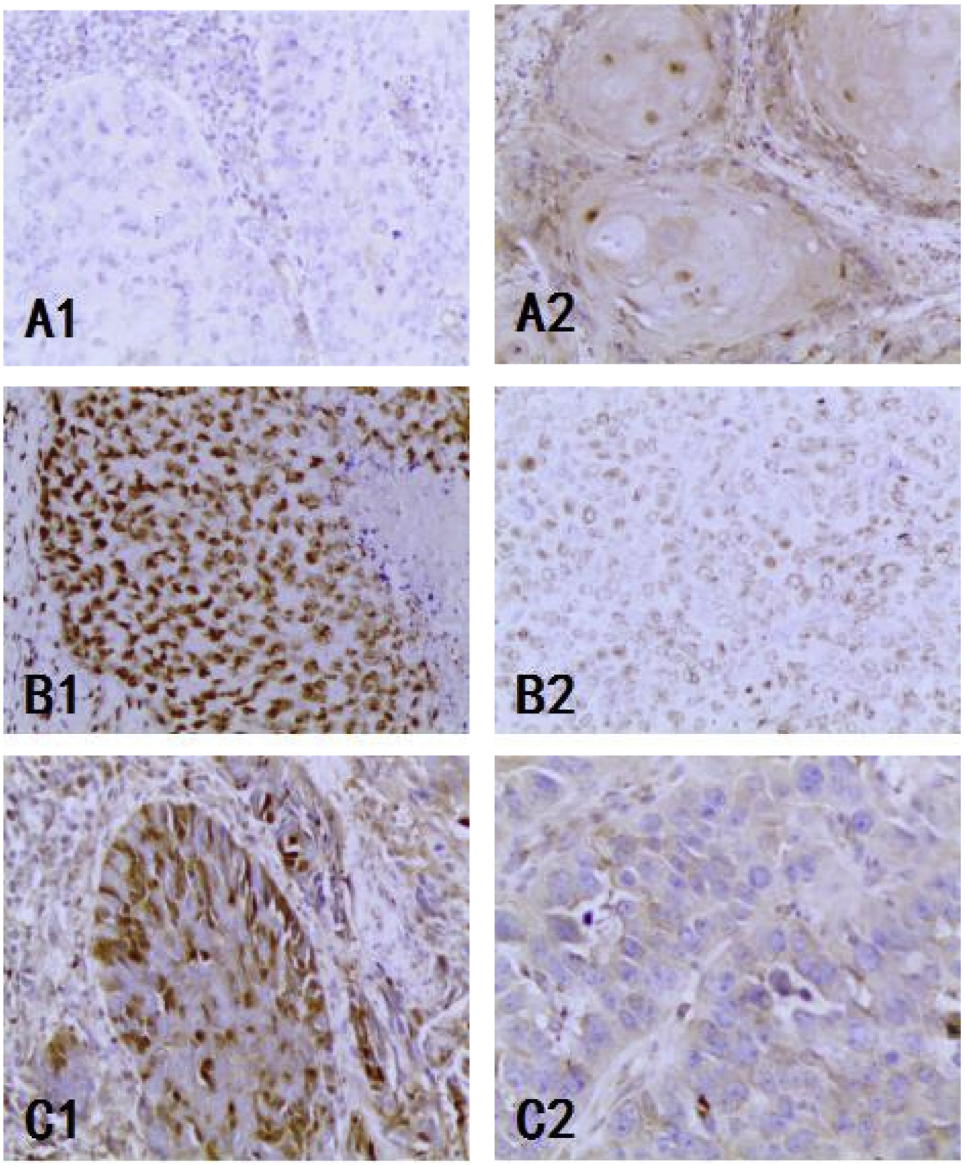

Fig. 3. Immunohistochemical analysis of ERCC foci from the same tumor.
Representative foci stained for different markers showing heterogeneity, two heterogeneous lesions of the same tumor showed different expression(20X). A1&A2:ZNF750; B1&B2: EP300; C1&C2: MTOR.

technology makes it possible to study multiple transcripts in the same tissue at high resolution, which has a high degree of specificity and sensitivity and has been used in heterogeneity studies of multiple tumors such as breast cancer, but it is very expensive. IHC is a mature technology that can be applied to human sample relatively inexpensively.

In our study, we found that ZNF750, EP300, MTOR are high expression by IHC. Previous studies [19–21] have mentioned that ZNF750, EP300 and mTOR expression were higher in ESCC tissues as the same with our study. Also, patients with ZNF750 high expression group had better overall survival than low expression group, in consistent with recent studies that showed cell growth, migration, and invasion were significantly increased by downregulation of ZNF750 [22]. While patients of EP300 or MTOR high expression experienced high mortality, Gao et al. revealed that high p300 expression had poor overall survival for resected small cell lung carcinoma(SCLC), MTOR, a oncogene,is well-known to us, which was similar to previous studies [23,24].

Furthermore, KMT2D expression can be detected by RNAscope while IHC can not. ZNF750, EP300, MTOR and KMT2D RNA expression were all detected by RNAscope, our results also suggested that ZNF750, EP300, MTOR overexpression were associated with the status of lymph node metastasis, pathological differentiation, tumor thrombus and clinical stage. Few studies had been reported about the four markers by RNAscope before. And RNAscope results matched well with the data of IHC and revealed that there was variability of the four marker expression in ESCC.

Our analyses also revealed that ITH is prevalent in ESCC both by IHC and RNAscope. In most cases, high expression of any given protein did not happen in two foci from the same tumor. We found that 44/106(4151%), 37/106 (34.91%), 39/106(36.79%) of ESCC showed heterogeneous expression of ZNF750, EP300 and MTOR respectively. RNAscope results also revealed that ITH is prevalent in ESCC.8/12(66.67%), 8/12(66.67%), 4/12(33.33%), 4/12(33.33%) of ZNF750, EP300, MTOR and KMT2D showed heterogeneous expression respectively. Our study revealed ITH is prevalent in ESCC in RNA and protein level, which were consistent with studied by WGS [12,13,16,17].RNA scope technology can not only show heterogeneity in the tumor in situ,

Table 3
ZNF750, EP300, MTOR mRNA Expression and Clinicopathologic Variables.

Variable		Cases N = 12	ZNF750 High	*P*	EP300 High	*P*	MTOR High	*P*
Age(years)	≤60	10	8	0.028	8	0.488	2	0.028
	> 60	2	0		2		2	
Gender	Male	8	4	0.273	8	0.001	4	0.273
	Femal	4	4		2		0	
Tumor location	Upper Middle	2	2	0.584	0	0.006	0	0.584
	Lower	4	4		4		2	
		6	2		6		2	
Pathological grade	G1	0	0	0.014	0	0.121	0	0.014
	G2	6	6		4		0	
	G3	6	2		6		4	
ClinStage	I	2	2	0.028	0	0.006	0	0.028
	II	4	4		4		0	
	III	6	2		6		4	
Lymph node metastasis	Negative	6	6	0.014	4	0.121	0	0.014
	Positive	6	2		6		4	
T status	T1	4	4	0.014	2	0.003	0	6.000
	T2	4	4		4		0	
	T3	4	0		4		4	
Tumor thrombus	Negative	6	6	0.014	4	0.121	4	0.014
	Positive	6	2		6		0	

ZNF750, Zinc finger protein 750; EP300, E1A-associated protein p300; MTOR, the mammalian target of rapamycin.

but also seems to have higher sensitivity and specificity to certain markers, so that new markers can be found in the process of tumor development. Due to an expensive cost, we select 12 cases in our study, more research may be required in the future.

Our study may provide basic research for facilitating the identification of therapeutic target candidates for individualized treatment of ESCC, RNAscope detection method may be a more reliable exploration of new tumor markers and detection of tumor heterogeneity.

4. Materials and methods

4.1. Sample preparation

A retrospective analysis was made of 1632 patients who underwent surgery in the Fourth Hospital of Hebei Medical University from January 2008 to December 2010, and diagnosed esophageal squamous cell carcinoma by histopathology. A total of 1550 ESCC patients were followed up successfully, and 132 cases were lost. The follow-up rate was 91.9%. HE staining sections of all 1550 patients were examined and 106 ESCC patients with intratumoral heterogeneity were screened. All cases were reviewed by two pathologists. Two different, well-separated areas from each tumor were selected for assessment of intratumoral heterogeneity. There were significant histological or morphological differences in the two lesion regions in each case. All the 106 patients had no previous treatment before, no other tumor history, and all of them had complete clinical data.

4.2. Immunohistochemistry (IHC) and scoring

The expression of ZNF750 (Fuzhou Maxin Biological Technology Development Co., Ltd.), KMT2D (Fuzhou Maxin Biological Technology Development Co., Ltd.), EP300 (Fuzhou Maxin Biological Technology Development Co., Ltd.) and MTOR (Fuzhou Maxin Biological Technology Development Co., Ltd.) protein in the two lesion regions were measured by immunohistochemistry staining, and the difference was observed between different lesions. The regions were heated at 56°C for 1 night, deparaffinized in xylene, rehydrated in a graded series of alcohol. These regions were covered with 3% hydrogen peroxide solution for 10 min, heated in a pressure cooker with EDTA antigen repair solution repair for 5 min. The first antibodies of ZNF750, EP300, KMT2D and MTOR were added dropwise and incubated overnight at 4°C. PBS washed, dropping general-purpose secondary antibody, incubated at room temperature for 20–30 min, PBS washed. Diaminobenzidine (DAB) colored, observed under a microscope. Immunohistochemical grading was based on the percentage of positive stained tumor cells and the staining intensity. Staining extent was scored as 0 (< 5%) 0; 1(5–25%); 2 (25–50%); 3 (> 50%). Staining intensity was scored as 0, (nongranulated); 1 (light yellow); 2 (brownish yellow); or 3 (reddish brown). Total score was equal to staining extent multiplied by staining intensity. The high expression group was 4–9, the low expression group included negative expression or low expression with a total score of 0 to 3. Usually if a DNA amplification is detected from a single biopsy, then the tumor is considered positive for the mutation. To be consistent with this criterion, we decided that if protein expression was of high expression in one foci from a tumor, then that tumor was considered of high expression of that marker.

4.3. RNAscope and scoring

Using a novel method based on RNA in situ hybridization (RNAScope), 12 patients with formalin-fixed paraffin-embedded tissue were randomly selected from 106 patients with ESCC. The expression of ZNF750, EP300, KMT2D and MTOR mRNA was detected using the RNA scope FFPE 2.5 kit (ACD, USA). Paraffin sections were dried at 60°C oven for 1 h, conventional dewaxing and protease digestion, then RNA scope target repair reagents for 10 min. Plus ZNF750, KMT2D, MTOR, EP300 probe respectively. The housekeeping gene PPIB was for positive control, the bacterial gene DapB was for negative control probe. The samples were crossed in the hybridization oven (ACD, USA).Then the samples were eluted sequentially and signal amplified, diaminobenzidine (DAB) colored, observed under a microscope. The hybridization signals were counted under a 40-fold objective in 100 cancer cells and scored according to the following five grades: Staining was scored as 0 (less than 1 signal point in 10 cells); 1(1–3); 2 (4–10); 3 (> 10, with cluster Point of the positive cells less than 10%);4(> 10, with clusters of positive cells more than 10%).The number of signals in mRNA cells with high expression is more (3 or 4), and the number of signals in low mRNA expression is less (0–2).

4.4. Statistical analysis

Statistical analysis was performed by SPSS21.00. The correlation of the four markers expression with clinic pathologic characteristic of

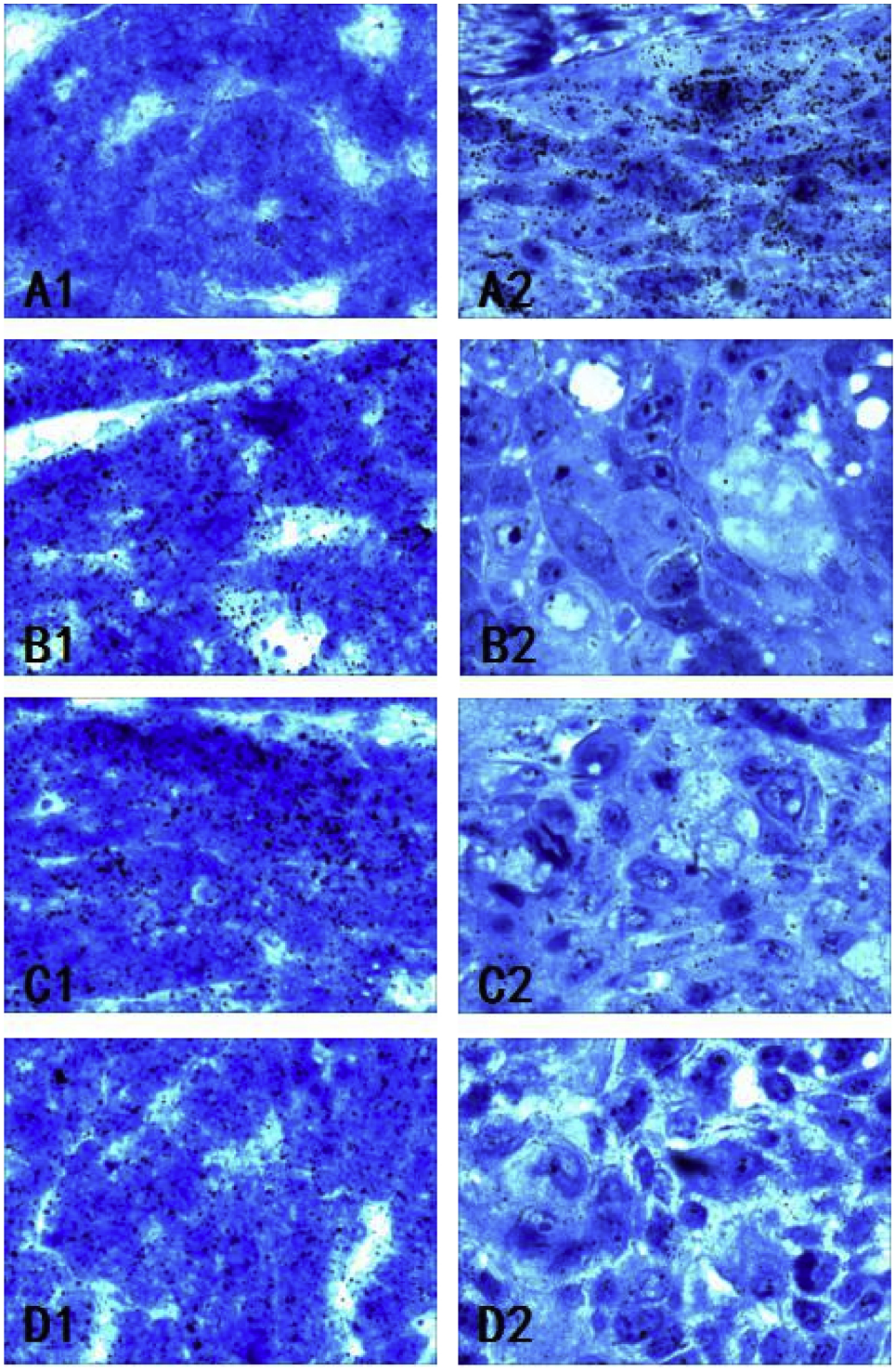

Fig. 4. RNAscope analysis of ERCC foci from the same tumor.
Representative foci stained for different markers showing heterogeneity, representative foci stained for different markers showing different scores (40X). A1&A2:ZNF750; B1&B2: EP300; C1&C2: MTOR D1&D2:KMT2D.

ESCC patients was assessed by the chi-square and Logistic regression analysis test. Kaplan-Meier single factor analysis was for survival analysis, COX regression for multivariate analysis and the log-rank test was used to compare differences in survival between groups. With $\alpha = 0.05$ as the test standard, $P < 0.05$ was statistically significant.

Conflicts of interest

The authors declared no conflict of interest.

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