CEP3 and CEP17 DNA probe potential in the genetic diagnosis and prognostic prediction of esophageal squamous cell cancer

MADINIYAT NIYAZ[1], ABLAJAN ABDURAHMAN[2], ABDUGHENI TURGHUN[2] and IDIRIS AWUT[2]

[1]Xinjiang Esophageal Cancer Research Institute, Medical Research Center; [2]Department of Thoracic Surgery, First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054, P.R. China

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Abstract. The aim of the present study was to investigate the clinical application of molecular pathological diagnosis for the prognosis of Kazakh patients with esophageal squamous cell carcinoma (ESCC) using centromere enumeration probes (CEPs) for chromosomes 3 and 17. A total of 40 patients with ESCC that had received radical surgical treatment and 10 healthy control participants were enrolled in the present study. Touch preparations of fresh cancerous and normal tissues were completed and fluorescence *in situ* hybridization (FISH) was performed to count the copy numbers of CEP 3 and 17, and abnormalities were analyzed, in comparison with routine pathological diagnoses. FISH analysis demonstrated that abnormal copy numbers of CEP 3 and 17 (aneuploidy) were detected in all 40 patients with ESCC. CEP 3 and 17 polyploidy rates differed significantly between poorly differentiated, moderately differentiated and well-differentiated ESCC groups (P<0.05): Well-differentiated, 35.38 and 30.92%; moderately differentiated, 55.81 and 44.43%; and poorly differentiated, 76.26 and 71.90%, respectively. Furthermore, polyploidy rates were significantly increased in the group with lymph node metastasis, as compared with the group without (CEP 3, P=0.0001; CEP 17, P=0.012). Variations in the copy numbers of CEP 3 and 17 were demonstrated to be correlated with the level of differentiation and lymph node metastasis in patients with ESCC. Therefore, the present results indicate that DNA probes may be used to predict prognostic factors in patients with ESCC. Furthermore, FISH technology is an objective and qualitative method that is capable of detecting variations in CEP 3 and 17; therefore, FISH may be used in the genetic diagnosis of ESCC in Kazakh patients.

Correspondence to: Professor Idiris Awut, Department of Thoracic Surgery, First Affiliated Hospital of Xinjiang Medical University, 137 Liyushan South Road, Urumqi, Xinjiang 830054, P.R. China
E-mail: edrs5@yahoo.com

Abbreviations: FISH, fluorescence *in situ* hybridization; EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; CEP, centromere of chromosome; SSC, standard saline citrate

Key words: DNA probe, fluorescence *in situ* hybridization, aneuploid, esophageal cancer, Kazakh patients

Introduction

Esophageal cancer (EC) is among the most common types of cancer associated with the digestive system. Due to a lack of efficient early diagnostic measurements, the majority of patients with EC are in an advanced stage when treatment is initiated, which is associated with a poor prognosis. Mortality rates of patients with EC are high (>300,000 patients per year), and the incidence of EC among Kazakh people in the Xinjiang Uyghur Autonomous Region of northwest China is 155.9/100,000, which is higher than any other ethnic group in China (1-3).

Conventional methods of pathological diagnosis provide crucial information regarding tumor differentiation and the morphological characteristics (4,5) in patients with EC. However, clinical diagnosis and decisions on treatment are difficult due to various limitations, including tissue disfigurement following extrusion, inadequate biopsy depth and discrepancies between pathological diagnosis and actual diagnosis. Therefore, a more objective and quantitative method is required to improve the accuracy of EC diagnosis (6).

Previous studies have suggested that the incidence and evolution of EC is associated with various chromosomal anomalies (7,8). During carcinogenesis, cells undergo molecular cytogenetic changes prior to any alterations in morphology. Nuclear chromosome abnormality, which is observed in cancer cells, is an early event during the process of tumorigenesis, and it has become the objective index for determining cancerous cells (9,10). Nuclear aneuploidy is a prevalent feature of various types of cancer, including EC (9,10); therefore, the detection of aneuploidy, which is usually found in aneusomic nuclei, may be used to detect cancerous cells. Fluorescence *in situ* hybridization (FISH) technology is a rapid and sensitive method used for the detection of aneusomy on a specific chromosome (11). FISH has been utilized for the diagnosis of various types of cancer with high sensitivity and specificity, including hematological malignancies and lung, breast and kidney cancer (12,13). The advantage of FISH is that it is an objective and quantitative method for identifying cancerous cells. Previous studies have

demonstrated that the priority of FISH in cancer detection is higher than conventional cytology and, therefore, may be advantageous to the early diagnosis of various types of cancer (14,15).

In the present study, chromosomal loci centromere of chromosome (CEP) 3 and 17 were selected to fabricate the DNA probe as they have an increased aberration rate in esophageal and lung cancer (16,17). In order to analyze the clinical application of FISH and any correlations between the prognosis of patients in the diagnosis of ESCC, 40 Kazakh patients with esophageal squamous cell carcinoma (ESCC) underwent FISH examination and conventional pathological diagnosis using surgical samples.

Patients and methods

Patients. Between June 2011 and September 2012, 40 Kazakh patients with ESCC were admitted to the Department of Thoracic Surgery at The First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) and underwent surgical resection. Among the 40 patients enrolled in the present study, 34 were male and 6 were female, with an average age of 57.4 years (Table I). None of the patients had previously received any preoperative radiotherapy, chemotherapy or other treatment. Pathology was graded according to the 7th American Joint Committee on Cancer staging manual (18). Final post-operative pathological diagnosis confirmed ESCC in all 40 cases, including well differentiated (n=13), moderately differentiated (n=16) and poorly differentiated tumors (n=11). Lymph node metastasis was detected in 55% (22/40) of cases; therefore, 45% (18/40) of the cases enrolled in the present study were non-metastatic. A total of 10 esophageal tissue samples (>5 cm distant from the tumor) were harvested as normal controls from healthy individuals. Informed consent was obtained from all patients.

FISH method. Touch preparations of cells were performed on glass slides from fresh specimens and air-dried for 24 h at room temperature and were subsequently stored at -80˚C in preparation for FISH. The same specimens were stained with hematoxylin and eosin for pathological evaluation (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China).

Cells were denatured with 70% formaldehyde at 74˚C in a water bath and subsequently washed twice with standard saline citrate (SSC; Abbott Molecular, Inc., Des Plaines, IL, USA) at room temperature. Slides were dehydrated through a graded ethanol series (70, 85 and 100%) prior to the application of 10 μl hybridization solution, containing 1 μl orange fluorescently-labeled CEP 3 probe, 1 μl green fluorescently-labeled CEP 17 probe, 7 μl hybridization buffer and 1 μl double-distilled water (all Abbott Molecular, Inc.). Slides were covered with a cover slip and sealed with rubber cement (Fixogum; Marabu GmbH & Co. KG, Tamm, Germany). Following incubation for 16 h at 42˚C in a humidity-controlled chamber, the slides were washed with SSC at 74˚C and at room temperature for 2 min. Subsequently, 5 μl diamidinophenylindole (DAPI II; Abbott Molecular, Inc.) was applied to each spot and covered with a cover slip.

Two fluorescently-labeled probes were used, Cy3 and FITC (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and each of the protocols included at least one normal esophageal tissue as a control. FISH signal analysis was performed according to the kit instructions (Vysis CEP 3 (D3Z1) SpectrumOrange Probe Kit and CEP 17 Probe Kit; Abbott Molecular, Inc.) A fluorescence microscope and an image acquisition and analysis system (DM6000; Leica

Table I. Clinical characteristics of the patients with esophageal squamous cell carcinoma.

Case	Age (years)	Gender	Pathology
1	71	M	T1bN0M0, IA
2	75	M	T2N0M0, IB
3	71	M	T3N0M0, IIA
4	55	M	T2N0M0, IB
5	58	M	T3N1M0, IIIA
6	54	F	T3N0M0, IIB
7	69	M	T2N0M0, IB
8	55	M	T3N2M0, IIIB
9	62	M	T2N0M0, IB
10	45	F	T3N1M0, IIIA
11	54	M	T2N0M0, IB
12	60	M	T2N0M0, IIA
13	57	M	T1N0M0, IA
14	70	M	T3N2M0, IIIB
15	58	F	T2N0M0, IIA
16	56	M	T3N1M0, IIIA
17	73	M	T2N1M0, IIB
18	68	M	T3N1M0, IIIA
19	40	M	T1bN0M0, IA
20	43	F	T2N1M0, IIB
21	54	M	T3N2M0, IIIB
22	47	M	T3N1M0, IIIA
23	44	M	T3N1M0, IIIA
24	56	M	T2N1M0, IIB
25	42	M	T3N1M0, IIIA
26	45	M	T3N0M0, IIB
27	50	M	T3N1M0, IIIA
28	56	F	T2N0M0, IB
29	72	M	T3N1M0, IIIA
30	65	M	T2N1M0, IIB
31	67	F	T3N1M0, IIIA
32	52	M	T1aN0M0, IA
33	50	M	T3N1M0, IIIA
34	48	M	T3N1M0, IIIA
35	62	M	T2N1M0, IIB
36	50	M	T2N0M0, IB
37	63	M	T3N0M0, IIB
38	55	M	T1bN0M0, IA
39	72	M	T3N1M0, IIIA
40	50	M	T2N1M0, IIB

Pathology graded according to the 7th American Joint Committee on Cancer staging manual (18). M, male; F, female.

Table II. Results of fluorescence *in situ* hybridization in normal esophageal tissue.

	Aneuploidy (CEP 3/CEP 17)			
Case	2 copies	3 copies	4 copies	Multiple copies
1	96/96	1/2	3/2	4/4
2	97/95	2/3	1/2	3/5
3	97/97	2/2	1/1	3/3
4	98/98	2/2	0	2/2
5	96/97	3/2	1/1	4/3
6	96/95	4/2	0/3	4/5
7	95/95	2/2	3/3	5/5
8	95/96	3/2	2/2	5/4
9	93/94	3/3	4/3	7/6
10	97/95	2/3	1/2	3/5

CEP, centromere of chromosome.

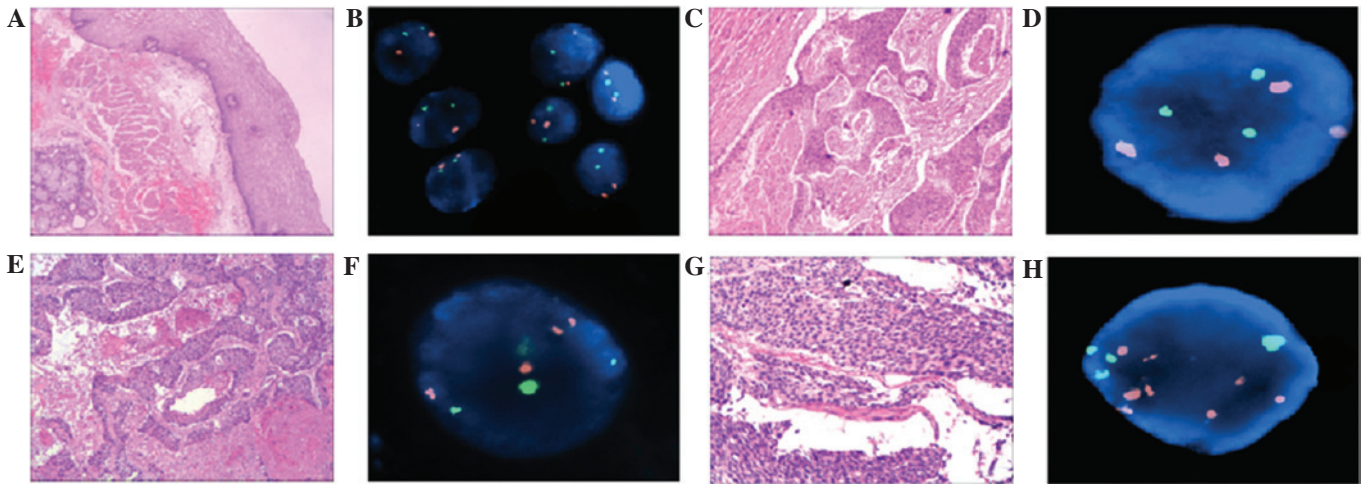

Figure 1. Comparison of hematoxylin and eosin (H&E) staining and fluorescence *in situ* hybridization (FISH) in normal and abnormal esophageal tissues. (A) Normal esophageal membranes following H&E staining (magnification, 40). (B) Normal representative nuclei carrying 2 copies of the centromeres of chromosomes (CEP) 3 (orange) and 17 (green). (C) Well-differentiated esophageal squamous cell carcinoma (ESCC) following H&E staining (magnification, 40). (D) FISH representative nuclei carrying 3 copies of CEP 3 and 3 copies of CEP 17. (E) Moderately differentiated ESCC following H&E staining (magnification, 40). (F) FISH representative nuclei carrying 4 copies of CEP 3 and 3 copies of CEP 17. (G) Poorly differentiated ESCC H&E staining (magnification, 100). (H) FISH representative nuclei carrying 7 copies of CEP 3 and 4 copies of CEP 17.

Microsystems GmbH, Wetzlar, Germany) was used to determine the signal count. Results were analyzed by two individual statisticians. All cells were evaluated, with the exception of damaged cells or those with overlapping nuclei. A total of 100 nuclei were counted from each patient, and the total number of centromeric signals was recorded. Cells were deemed positive for aneuploidy when the percentage of hyperdisomic nuclei with >3 copies of at least one nucleus was >10%.

Statistical analysis. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses in the present study. The Rank sum test and t-test were used to analyze the data from each group. Throughout the study, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Polyploidy rates of the centromeres of chromosomes 3 and 17 were detected using CEPs and FISH analysis. The percentage of diploid CEP3 and 17 was >93% in the control group (Table II). Abnormal polyploidy copy numbers of CEP3 and 17 were detected in all 40 ESCC specimens (Fig. 1).

CEP3 and CEP17 aberrations correlated with lymph node metastasis. Average mutation rates of CEP 3 and 17 were 61.2% and 50.95%, respectively (Table III). The sensitivity and specificity of the FISH results were 100% with no false positives or negatives, calculated according to the final pathological diagnosis. Lymph node metastasis was detected in 55% of cases, therefore, 45% of the cases enrolled in the present study were non-metastatic. Furthermore, polyploidy rates were

Table III. Results of fluorescence *in situ* hybridization in esophageal cancer tissue.

		Aneuploidy (CEP 3/CEP 17)				
Case	Pathology	2 copies	3 copies	4 copies	≥5 copies	Multiple copies
1	WDSCC	45/39	25/31	20/22	10/8	55/61
2	WDSCC	71/73	19/15	10/12	0/0	29/27
3	WDSCC	73/77	17/12	9/11	1/0	27/23
4	WDSCC	65/63	21/16	11/12	3/9	35/37
5	WDSCC	60/72	28/12	5/8	7/8	40/28
6	WDSCC	82/80	17/15	0/5	1/0	18/20
7	WDSCC	76/74	13/11	6/7	5/8	24/26
8	WDSCC	72/68	13/15	7/9	8/8	28/32
9	WDSCC	66/72	20/17	10/9	4/2	34/28
10	WDSCC	59/68	26/21	6/7	9/4	41/32
11	WDSCC	60/65	21/19	11/8	8/8	40/35
12	WDSCC	50/57	20/17	25/20	5/6	50/43
13	WDSCC	71/74	11/11	10/10	8/5	29/26
14	MDSCC	41/59	11/12	36/20	12/9	59/41
15	MDSCC	38/56	25/17	28/20	9/7	62/44
16	MDSCC	43/59	28/18	13/12	16/11	57/41
17	MDSCC	64/65	18/13	12/15	6/7	36/35
18	MDSCC	38/57	26/29	28/11	8/3	62/43
19	MDSCC	31/56	36/20	19/12	16/12	69/44
20	MDSCC	52/78	32/17	13/4	3/1	48/22
21	MDSCC	60/70	9/11	12/12	9/7	40/30
22	MDSCC	45/55	26/21	12/9	17/15	55/45
23	MDSCC	39/46	45/39	9/13	7/2	61/54
24	MDSCC	48/57	25/20	19/16	8/7	52/43
25	MDSCC	44/54	31/26	23/15	3/5	56/46
26	MDSCC	45/52	25/25	12/8	18/15	55/48
27	MDSCC	24/24	55/36	9/30	12/10	76/76
28	MDSCC	15/26	35/44	20/20	30/10	85/74
29	MDSCC	80/75	19/15	1/10	0/0	20/25
30	PDSCC	30/38	27/21	24/26	19/15	70/62
31	PDSCC	25/28	40/40	25/23	10/9	75/72
32	PDSCC	21/27	30/23	39/40	10/10	79/73
33	PDSCC	13/9	55/46	17/30	15/15	87/91
34	PDSCC	14/30	41/29	23/30	22/11	86/70
35	PDSCC	14/6	40/41	20/24	26/31	86/94
36	PDSCC	28/40	28/25	27/23	17/12	72/60
37	PDSCC	32/40	39/26	25/24	4/10	68/60
38	PDSCC	12/25	58/52	12/12	18/11	88/75
39	PDSCC	32/24	47/55	10/12	11/9	68/76
40	PDSCC	40/42	20/17	27/26	13/15	60/58

WDSCC, well-differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma; CEP, centromere of chromosome.

significantly increased in the metastatic lymph node group, as compared with the non-metastatic group (CEP 3, P=0.0001; CEP 17, P=0.012) (Table IV).

Results of differentiation degree of CEP3 and CEP17 in three groups. The polyploidy rates for CEP3 and 17 were 35.38 and 30.92% in well-differentiated ESCC, 55.81 and 44.43% in moderately differentiated ESCC, and 76.27 and 71.90% in poorly differentiated ESCC, respectively. Significant differences were detected between the poorly differentiated, moderately differentiated and well-differentiated ESCC specimens (CEP 3, P<0.05; CEP 17, P<0.05) (Table V).

Table IV. CEP3 and CEP17 aberrations correlated with lymph node metastasis.

Lymph node metastasis	CEP3	CEP17
Negative	44.38±3.820	40.57±3.486
Positive	66.32±3.946	56.21±4.898
T-value	-3.990	-2.639
P-value	0.000	0.012

CEP, centromere of chromosome.

Table V. Differentiation degree of CEP3 and CEP17 in the three groups.

Differentiation	CEP3	CEP17
WD	35.3846±12.2647	30.9231±10.9199
MD	55.8125±15.3849	44.4375±14.5692
PD	76.2727±9.5403	71.9091±12.0785
F-value	8.616	7.975
P-value	<0.05	<0.05

CEP, centromere of chromosome; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

Discussion

The incidence of EC is high in the Xinjiang Uyghur Autonomous Region of northwestern China, and the Kazakh ethnic group in particular suffers from an increased incidence of EC, as compared with other ethnic groups (2). Therefore, improved diagnosis and treatment of EC is required to enhance the survival rate and quality of life of patients with EC.

Previous studies have demonstrated that various genetic mutation occur during carcinogenesis (10,15). FISH is a sensitive and specific diagnostic technique that is capable of detecting various types of cytogenetic alterations, including aneusomy, amplification and deletion. This technique has become a widely used diagnostic method in cytogenetic studies (11,19); however, to the best of our knowledge there are no previous studies investigating the application of FISH in the diagnosis of Kazakh esophageal cancer.

Human chromosome 3 carries hyperdense genes, which are associated with numerous types of cancer, including raf-1 and erb-A; whereas chromosome 17 carries certain oncogenes and tumor suppressor genes, including p53, c-erbB2, BRCA1 and nm23 (9,14,20). Previous studies have demonstrated elevated aberration rates in CEP 3 and 17 in patients with EC, which indicates that EC may be correlated with constitutional cytogenetic alterations (12,20).

In the present study, CEP 3 and 17 DNA probes were selected, and the cut-off value for the percentage of hyperdisomic cells was set at 10%. Normal cells often have <6% hyperdisomic cells and this discrepancy is likely to be due to sister chromatids being counted as copies (6,8).

In a previous study conducted by Fiegl *et al* (21) FISH was applied in the diagnosis of lung, breast, liver and stomach cancer, and the confirmed diagnostic rate was increased. Furthermore, Fritcher *et al* (22) analyzed esophageal adenocarcinoma using the FISH method with c-Myc, P16, HER2 and 20q13 centromeric region probes, and demonstrated that the sensitivity of cytology was only 45% for the detection of esophageal adenocarcinoma; however, a detection rate of 100% was achieved using FISH.

In the present study, FISH was applied to Kazakh patients with ESCC to elucidate the diagnostic value of FISH in the detection of cancer cells and as a prognostic indicator. Polyploidy of CEP 3 and 17 was detected in all 40 ESCC specimens and significant differences in the rates of polyploidy were detected between the poorly differentiated, moderately differentiated and well-differentiated ESCC specimens for both CEPs (CEP 3, P<0.05; CEP 17, P<0.05). Furthermore, polyploidy was significantly increased in the metastatic lymph node group, as compared with the non-metastatic group (CEP 3, P=0.0001; CEP 17, P=0.012). The average mutation rates of CEP 3 and 17 were 61.2 and 50.95%, respectively.

The results of the present study demonstrated that the aberration rates of CEP 3 and 17 were correlated with the level of ESCC differentiation. This may due to the eating habits of the Kazakh population, in particular the over consumption of smoked meat, fermented food, heavy smoking or drinking, and the reduced consumption of fresh fruits and vegetables (2,23,24).

In conclusion, the present study successfully used CEP 3 and 17 probes to detect cancerous cells in Kazakh patients with ESCC. In particular, aneuploidy was significantly higher in poorly differentiated squamous cells and the metastatic lymph node group. Therefore, DNA probes may be used as predictive biological markers for the prognosis of patients with ESCC. Furthermore, as an objective and qualitative method, FISH technology is capable of detecting CEP 3 and 17 variations in the diagnosis of Kazakh patients with ESCC, which may be used to genetically diagnose EC in the future. Further studies are required.

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