SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 3: 549-553, 2012

Molecular pathological diagnosis for early esophageal cancer in Kazakh patients

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Received September 8, 2011; Accepted November 25, 2011

DOI: 10.3892/ol.2011.547

Abstract. Chromosome abnormalities in cancer cells occur early in carcinogenesis. We employed DNA probes for the detection of cancer cells in surgical specimens in Kazakh patients with suspected esophageal carcinoma, to analyze the application of this technique during the early diagnosis of esophageal cancer. Comparative analysis was used to compare the results of pathological diagnosis with the results of FISH. We performed esophagofiberscopic biopsy examinations in 50 Kazakh patients with suspected esophageal carcinoma, including 40 males and 10 females, with an average age of 56.8 years. The final diagnosis was esophageal squamous cell carcinoma in 47 patients, and adenocarcinoma, mucinous carcinoma and small cell carcinoma in one patient each. The pathological findings of the biopsy were positive in 45 cases, and false-negative in 5. The sensitivity and specificity of pathological diagnosis were 87.2 and 100%, respectively. Using FISH to examine the same tissues, we found that 48 cases showed aberrant copy numbers in either chromosome 3 or 17, and 2 cases were false-negative, with a sensitivity and specificity of 94.8 and 100%, respectively. The copy numbers of centromeres in chromosome 3 were significantly higher than the copy numbers of centromeres in chromosome 17 (P=0.0001). Compared with biopsy pathology, the FISH test was more sensitive. Being an objective and qualitative method, the technology of molecular pathological diagnosis may effectively increase the early diagnostic rate of esophageal cancer. In addition, the centromere probe in chromosome 3 may be the most sensitive probe for the diagnosis of esophageal cancer in Kazakh patients.

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Abbreviations: FISH, fluorescence *in situ* hybridization; EC, esophageal cancer; SSC, standard saline citrate

Key words: aneuploidy, fluorescence *in situ* hybridization, molecular pathology, esophageal cancer, Kazakh patients

Introduction

The incidence of esophageal cancer (EC) has been high (68.88/100,000) among the Kazakh people living in the Xinjiang Uygur Autonomous Region (Northwest of China) during the past 30 years. Nevertheless, early detection and treatment rates of EC remain low, and it consequently has a poor prognosis. If early diagnosis and treatment were possible, the 5-year survival rate could be increased to above 90% as in other countries/regions that have early detection programs (1-3).

Conventional pathological diagnosis plays a crucial role in the diagnosis of EC, and provides important information on tumor differentiation and the degree of morphological changes (4,5). However, due to the limitations of biopsy pathology, discrepancies between pathological diagnosis and actual diagnosis occasionally occur, making clinical diagnosis and treatment difficult. There is, therefore, a need to find a more objective and quantitative method to distinguish benign from malignant cells.

A number of studies suggest that the incidence and evolution of EC involves a variety of chromosomal anomalies. During carcinogenesis, a cell goes through molecular cytogenetic changes prior to showing morphological changes. Nuclear chromosome abnormality, which can be observed in cancer cells, is an early event during the process of tumorigenesis, and it has become the determining objective index of cancer cells.

Cell nuclear aneuploidy is one of the most common features of a number of types of cancer, including EC (6,7). Malignant cells are therefore capable of being diagnosed by detecting aneuploidy, usually found in aneusomic nuclei. Fluorescence *in situ* hybridization (FISH) technology is a rapid and sensitive method for detecting aneusomy of a specific chromosome and is widely used in the diagnosis of hematological malignancies, lung, breast and kidney cancer, with high sensitivity and specificity (8-11). The advantage of the FISH method has been considered to lie in its objective and quantitative evaluation of malignant cells.

Some studies have suggested that FISH has a certain value in detecting a variety of cancer cells in conventional cytology and early cancer diagnosis (12-14). However, no comparative study on conventional pathology with FISH using biopsy tissues for cancer cell detection has been reported previously.

In this study, 50 Kazakh patients with suspected EC underwent FISH examination and conventional pathological diagnosis using biopsied samples, to analyze the value of the clinical applications and prospective uses of the FISH method in the early diagnosis of EC.

Patients and methods

Patients. This study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University, China. Between March 2009 and December 2010, 50 Kazakh EC patients were admitted to the Department of Thoracic Surgery (First Affiliated Hospital of Xinjiang Medical University) and underwent resection. The patients included 40 males and 10 females, with an average age of 56.8 years (range 31-82).

Final pathological diagnosis confirmed esophageal squamous cell carcinoma in 47 cases post-operatively, including well differentiated tumors in 23 cases, moderately differentiated tumors in 18 cases, and poorly differentiated tumors in 6 cases; as well as poorly differentiated adenocarcinoma in 1 case, mucinous adenocarcinoma in 1 case, and small cell carcinoma in 1 case (Table I).

Table I. Patient charateristics.

Case	Gender	Age	Diagnosis
1	M	55	Sq
2	F	63	Sq
3	M	50	Sq
4	M	48	Sq
5	F	65	Sq
6	M	56	Sq
7	F	52	Sq
8	M	63	Sq
9	M	66	Scc
10	F	59	Sq
11	M	54	Sq
12	M	47	Sq
13	M	63	Sq
14	M	82	Sq
15	F	59	Sq
16	M	31	Sq
17	M	51	Sq
18	M	50	Sq
19	M	64	Sq
20	M	46	Sq
21	M	44	Sq
22	M	56	Sq
23	M	42	M-ad
24	M	61	Sq
25	F	48	Sq
26	M	42	Sq
27	M	39	Sq
28	M	71	Sq
29	F	60	Sq
30	M	56	Sq
31	M	45	Sq
32	M	64	Sq
33	M	72	Sq
34	M	65	Sq
35	M	67	Sq
36	M	65	Sq
37	M	58	Sq
38	F	54	Sq
39	M	50	Sq
40	F	56	Sq
41	M	65	Sq
42	M	56	Sq
43	M	63	Sq
44	M	69	Sq
45	M	71	Sq
46	M	65	Sq
47	M	62	Sq
48	M	56	Sq
49	F	37	Sq
50	M	56	Ad

M, male; F, female; Sq, squamous cell carcinoma; Ad, adenocarcinoma; Scc, small cell carcinoma; M-ad, mucinous adenocarcinoma.

Methods. To determine the diagnosis pre-operatively, all patients underwent esophagofiberscopic examination, and sites that appeared to be suspicious for malignancy were biopsied using standard biopsy forceps. After performing touch preparations of cells on glass slides with the specimen, the same specimens were used for conventional pathological diagnosis. Informed consent was obtained from all 50 patients.

Samples were stained with hematoxylin and eosin (H&E). Pathological evaluations were performed by three qualified pathologists from the Department of Pathology.

Pathomorphological classification of biopsy specimens were as follows: class I was mild grade squamous epithelial hyperplasia; class II was mild dysplasia; class III was moderate dysplasia, but without any malignant characteristics; class IV was severe dysplasia, i.e., carcinoma *in situ*; class V was typical cancer tissue. Classes IV and V were considered to be EC.

Touch preparations of cells were made on glass slides and air dried overnight at room temperature and then stored at -80˚C. Centromeric probes labeled with fluorochrome were used for the visualization and enumeration of copy numbers. Spectrum orange and green labeled probes were used to visualize centromeric regions of chromosomes 3 and 17. Reagents were purchased from Abbott Molecular, Inc. (Des Plaines, IL, USA).

Preparation of slides. Cells were denatured with 70% formamide and then washed twice in standard saline citrate (SSC) at 74˚C and at room temperature, respectively, for 2 min in a water bath. Then, slides were dehydrated through a graded ethanol series (70, 85 and 100%, each for 2 min). We then applied 10 μl of hybridization solution containing 1 μl of each of the DNA probes, 7 μl of hybridization buffer and 1 μl of double distilled water. This was covered with a cover slip and sealed with rubber cement. Following incubation for 16 h at 42˚C in a humidity-controlled chamber, the slides were washed with an SSC solution for 5 min at 74˚C, and at room temperature for 2 min. Diamidinophenylindole (DAPI, II) (5 μl) was applied to each spot and covered with a cover slip. The slides were observed under a fluorescence microscope that was connected to a cooled charge-coupled device camera

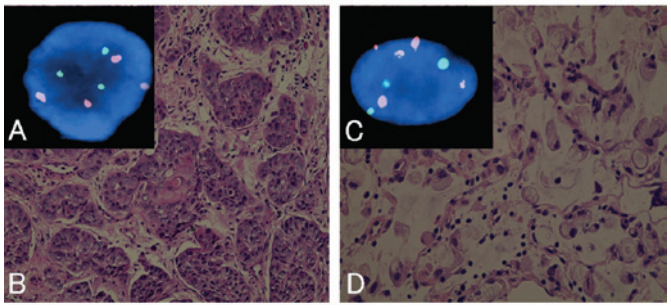

Figure 1. FISH analysis of the centromere of chromosome 3 (orange signals) and chromosome 17 (green signals). The normal representative is nuclei carrying 2 copies of the centromere of chromosomes 3 and 17. (A and B) The pathology is viewed by H&E staining; magnification x400. The results revealed squamous cell carcinoma (case 17) of grade V. FISH representative nuclei carry 4 copies of the centromere of chromosome 3, and 3 copies of the centromere of chromosome 17. (C and D) The pathology is viewed by H&E staining; magnification, x400. The results revealed adenocarcinoma (case 50) of grade V. FISH representative nuclei carry 5 copies of the centromere of chromosome 3 and 3 copies of the centromere of chromosome 17. H&E, hematoxylin and eosin; FISH, fluorescence *in situ* hybridization.

and an image analyzer system (Leica Microsystems, Ltd., Germany).

FISH analysis. FISH signal analysis was performed as follows: all cells, with the exception of damaged cells or those with overlapping nuclei, were evaluated. We counted 100 nuclei from each patient, and the total number of centromeric signals was recorded. When the percentage of hyperdisomic nuclei with more than three copies for at least one nucleus was >10%, we diagnosed malignancy.

FISH diagnosis was made without knowing the result of the conventional pathological diagnosis. Similarly, the results of FISH analysis were not shown to the pathologists. Thus, the two diagnoses were independently performed in a blind manner.

Statistical analysis. An IBM SPSS 16.0 statistical software package (IBM Corporation, NY, USA) was used for statistical analysis. The student's paired t-test was used to test the difference between the number of countable centromere signals of chromosome 3 and 17. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients and FISH analysis. Biopsy pathology yielded a diagnosis of primary EC in 45 patients, including squamous cell carcinoma in 42 cases, adenocarcinoma in 1 case, mucinous adenocarcinoma in 1 case, and small cell carcinoma in 1 case.

We classify the cases as follows: 3 cases were classified as class II; 2 cases as class III; 4 cases as class IV; 41 cases as class V. Five cases were false-negative but there were no false-positive cases. The sensitivity and specificity were 87.2 and 100%, respectively.

FISH analysis revealed that 48 cases had abnormal copy numbers in either chromosome 3 or 17. Representative findings of the pathology and FISH are shown in Fig. 1.

The FISH method yielded 2 false-negative and no false-positive cases, with a sensitivity and specificity of 94.8 and 100%, respectively (Table II). The copy numbers of centromeres in chromosome 3 were significantly higher ($P=0.0001$) than the centromeres of chromosome 17.

Discussion

The Xinjiang Uygur Autonomous Region is a multi-ethnic area located in the Northwest of China and the Kazakh ethnic group has a high incidence of EC. Improved early diagnosis of EC among this ethnic group is likely to lessen the burden of EC (1).

Aneuploidy is present in the nuclei of cancer cells. It is a common molecular pathological characteristic in human carcinoma (7,15,16). A number of studies have suggested that using DNA probes for the detection of aneuploidy in cancer cells may be a superior technique to conventional pathological diagnosis (8-12,14). Han *et al* examined 113 EC patients using specific centromere DNA probes 3, 8, 10, 12, 17 and 20, and found that chromosomal signal numbers and all chromosomes were found to have abnormal copy numbers (12). In their study, Fritcher *et al* analyzed esophageal adenocarcinoma using the FISH method with the centromeric region probes C-MYC, P16, HER2 and 20q13 (9). These authors found that the sensitivity of cytology is only 45% for the detection of esophageal adenocarcinoma, but FISH yielded a detection rate of 100%. The same study used FISH analysis with centromeric probes 7, 11, 12, 17 and 18. Aneusomy was not found in the normal controls of any chromosomes. By contrast, chromosomal abnormalities were found in all carcinoma specimens (13).

Using FISH technology, the genetic analysis of interphase nuclei closely reflected the real changes in chromosomes. Simultaneous use of two or more different fluorescent-labeled probes resulted in high sensitivity and specificity for the detection of esophageal, lung and breast cancer cells (9,11,14). Therefore, we performed FISH analysis and conventional pathological diagnosis for biopsied specimens in suspected EC patients to compare their sensitivity and specificity in order to analyze whether the early diagnosis of EC is possible or not and to obtain its clinical value. Our results showed that using the molecular pathological diagnostic method during the process of EC diagnosis is more accurate than conventional pathological diagnosis. This is consistent with the results of similar studies (8,12,14,17).

In our study, we selected the centromeres in chromosome 3 and 17 probes, and set the cut-off value of the percentage of hyperdisomic cells at 10%, whereas normal cells often have less than 6%. This discrepancy is probably due to counting sister chromatids as copies.

The sensitivity and specificity by biopsy pathology were 87.2 and 100%, respectively. Using FISH analysis, the sensitivity and specificity were 94.8 and 100%, respectively. These results indicated that FISH is more sensitive than biopsy pathology, the latter yielding 5 false-negative results: class II in 3 cases, class III in 2 cases. Post-operative final pathological diagnoses in all of these patients were esophageal squamous cell carcinomas. FISH yielded 2 false-negative results, both of which matched the 2 pathologically false-negative class II cases. This finding was probably due to the shallowness of the biopsies.

FISH successfully detected cancer cells in 3 cases in which biopsy pathology was false-negative. Post-operative

Table II. Results of FISH, biopsy pathology and final pathology.

Case	3 copies CEP3/CEP17	4 copies CEP3/CEP17	≥5 copies CEP3/CEP17	Hyperdisomy (%)	Biopsy pathology	FISH	Pathology
1	12/9	23/18	11/12	46/39	Sq, V	Positive	pT2N2M0
2	18/17	17/13	23/23	58/53	Sq, V	Positive	pT2N0M0
3	26/18	23/20	19/21	68/59	Sq,V	Positive	pT4N3M0
4	14/15	19/15	18/16	51/46	Sq,V	Positive	pT2N0M0
5	23/16	20/21	16/16	59/53	Sq, IV	Positive	pT3N1M0
6	27/23	20/17	27/26	74/66	Sq,V	Positive	pT2N1M0
7	15/17	25/19	25/20	65/56	Sq, V	Positive	pT2N1M0
8	11/8	11/16	19/20	41/44	Sq, V	Positive	pT3N2M0
9	31/28	25/20	33/32	89/80	Scc, V	Positive	pT2N1M0
10	25/26	30/25	18/18	73/69	Sq, V	Positive	pT3N1M0
11	22/20	27/28	20/23	69/71	Sq, V	Positive	pT2N0M0
12	33/29	30/31	19/12	85/72	Sq, V	Positive	pT4N1M0
13	3/1	1/2	1/0	5/3	[a]Negative, II	[a]Negative	pT1N0M0
14	35/27	29/26	31/29	95/82	Sq, V	Positive	pT3N2M0
15	19/13	22/25	22/19	63/57	Sq, V	Positive	pT2N0M0
16	19/15	22/22	17/18	58/55	Sq, V	Positive	pT3N1M0
17	24/20	30/26	14/17	68/63	Sq, V	Positive	pT2N1M0
18	12/9	11/14	21/15	44/38	Sq, V	Positive	pT3N1M0
19	23/17	23/20	21/21	67/58	Sq, V	Positive	pT3N0M0
20	9/11	12/12	9/7	40/30	Sq, IV	Positive	pT2N1M0
21	26/21	12/9	17/15	55/45	Sq,V	Positive	pT3N2M0
22	17/14	29/23	19/11	65/48	Sq, V	Positive	pT3N0M0
23	22/16	28/19	17/20	67/55	M-ad,V	Positive	pT4N2M0
24	29/16	25/25	17/18	71/59	Sq,V	Positive	pT2N0M0
25	2/2	0/2	1/0	3/4	[a]Negative, II	[a]Negative	pT2N0M0
26	13/15	7/9	8/8	28/32	Sq, V	Positive	pT3N1M0
27	17/15	10/15	5/5	32/35	Sq, V	Positive	pT3N0M0
28	58/52	12/12	15/13	85/77	Sq, V	Positive	pT3N1M0
29	30/24	22/16	20/21	72/61	Sq, V	Positive	pT3N1M0
30	55/47	21/19	9/9	85/75	Sq, V	Positive	pT4N2M0
31	45/39	9/13	7/2	61/54	Sq, V	Positive	pT3N1M0
32	13/11	12/13	5/2	30/26	[a]Negative, III	Positive	pT2N0M0
33	31/26	23/15	3/5	56/46	Sq, V	Positive	pT3N1M0
34	48/38	24/19	11/12	83/69	Sq, V	Positive	pT2N0M0
35	20/21	13/16	9/7	42/44	Sq, V	Positive	pT2N1M0
36	19/21	8/5	12/12	39/38	Sq, V	Positive	pT2N0M0
37	17/17	20/15	10/10	47/42	Sq, V	Positive	pT3N1M0
38	21/22	9/6	3/1	33/29	[a]Negative, III	Positive	pT1N0M0
39	25/20	19/16	8/7	52/43	Sq, V	Positive	pT2N1M0
40	13/11	6/7	5/8	24/26	[a]Negative, II	Positive	pT1N0M0
41	20/17	10/9	4/2	34/28	Sq, IV	Positive	pT2N0M0
42	15/12	9/11	6/7	30/30	Sq, IV	Positive	pT2N1M0
43	32/23	11/6	7/7	50/36	Sq, V	Positive	pT2N0M0
44	51/47	16/12	9/9	76/68	Sq, V	Positive	pT2N0M0
45	29/20	14/20	15/21	58/61	Sq, V	Positive	pT3N2M0
46	44/38	19/13	10/7	73/58	Sq, V	Positive	pT4N3M0
47	20/17	23/22	11/9	54/48	Sq, V	Positive	pT2N0M0
48	49/41	20/15	13/6	82/62	Sq, V	Positive	pT2N1M0
49	29/25	19/15	12/12	60/52	Sq, V	Positive	pT3N0M0
50	38/32	25/19	13/5	76/56	Ad, V	Positive	pT3N2M0

[a]False-negative. Sq, squamous cell carcinoma; Ad, adenocarcinoma; Scc, small cell carcinoma; M-ad, mucinous adenocarcinoma; CEP, centromeric enumeration probe ; II-V, grade; FISH, fluorescence *in situ* hybridization.

pathological staging confirmed stage IA in 2 cases, and stage IIA in 1 case, suggesting that FISH is capable of detecting the relevant chromosomal mutations in EC earlier. Therefore, FISH has its own clinical detection value for the early diagnosis of EC, and our findings have been supported by other studies (9,12,18). FISH may provide objective information on malignant cells, particularly in tissues with moderate or severe dysplasia. Therefore, we recommend FISH as an ancillary test in cases with moderate or severe dysplasia in order to avoid the misdiagnosis of EC (19,20).

The FISH results showed that the copy numbers of centromeres in chromosome 3 were significantly higher than those of chromosome 17 (P=0.0001). The results may be associated with the lifestyle of the Kazakh ethnic group, such as long-term excessive consumption of smoked meat, fermented foods, alcohol and tobacco, spicy foods, and lack of vegetables and foods rich in vitamins (1,20). In the future, the most sensitive probes should be selected to improve the early diagnosis of EC using molecular pathological techniques (9,10,21).

In this study, centromeres of chromosome 3 and 17 copy numbers and degree of aneuploidy were suggested to be correlated with the grade of tumor malignancy. These observations should be proven in future studies.

In conclusion, FISH technology is more sensitive than conventional pathology using biopsy specimens. Therefore, using an ancillary FISH test during the pathological diagnosis of cases with moderate or severe dysplasia may effectively improve the early diagnosis of EC. In addition, the centromeres of the chromosome 3 probe may be the most sensitive probe diagnostically in Kazakh patients with EC.

Acknowledgements

This study was supported by a grant from the Chinese postdoctoral fund of Xinjiang Medical University (20080-3014) and the Xinjiang Uygur Autonomous Region key disciplines Fund. Thanks are extended to the Department of Hematology, First Affiliated Hospital of Xinjiang Medical University for the technical support. We give thanks to Professor J. Patrick Barron, Professor and Chairman, Department of International Medical Communications, Tokyo Medical University for his review of this manuscript.

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