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ORIGINAL INVESTIGATION

Analysis of Chromosome 3, 7 and 8 Centromeric Regions in Bronchial Lavage Specimens by FISH

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Abstract

OBJECTIVES: Multiple genetic changes are observed in malignant tumors but are rare or absent in benign conditions. Aneuploidy is the most common feature of solid tumors including lung cancer and diagnosis of malignant tumors is possible through detection of aneuploidy. The aim of this study was to investigate chromosomal abnormalities in cells from non-small cell lung cancer patients obtained bronchoscopically and to evaluate the suitability of fluorescence in situ hybridization (FISH).

MATERIAL AND METHODS: Bronchial lavage samples of 17 non-small cell lung cancer (NSCLC) patients were evaluated with four-color FISH using deoxyribonucleic acid (DNA) probes specific for the centromere regions of chromosomes 3, 7 and 8. tested specimens were first hybridized with probes, then visualized under fluorescence microscobeand captured with device's camera.

RESULTS: High number of aneuploidic cells were detected in all the samples. Increased or decreased abnormal copies or chromosomes 3, 7 and 8 were obserced in all the 17 patients. Aneuploidy of chromosome 3 (21.35%) was higher than those of chromosome 7 (9.06%) and chromosome 8 (15.47%). Moreover, our results were significant for monosomy and trisomy of chromosome 3, trisomy of chromosome 7, nullisomy, monosomy and trisomy of, chromosome 8 ($p< 0.05$).

CONCLUSION: It has been observed that FISH is a useful technique for detection of aneuploidy in bronchial lavage samples obtained by bronchoscopy. Interphase cells were evaluated without cell culturing with this method and high number of tumor cells were enumerated rapidly. Our study has demonstrated that, FISH technique may be used successfully in detection of chromosome number abnormalities in NSCLC patients and may facilitate evaluation of genetic abnormalities.

KEYWORDS: Lung cancer, bronchial lavage, cytogenetic, fluorescence in situ hybridization, aneuploidy

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INTRODUCTION

Lung cancer, the most frequent malignant tumor worldwide, is a complex disease occurring in one or both lungs as a result of a progressive accumulation of abnormal cells [1]. Lung cancers are divided into two main groups based on how the cells look under the microscope; small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) which accounts for 80% of all lung cancer cases.

Prognosis of lung cancer depends on the stage of the cancer, its size and position in the lung. The early stages associates with few specific symptoms and as a consequence most of the lung cancer cases are in the advanced stage when they are diagnosed and has cancer spread other parts of the body.With early stages, there is a chance of well providing control and relieving the symptoms of lung cancer, with reference to that, several researchers look for early and rapid diagnosis of the lung cancer in the recent years [2,3].

In the recent years, several chromosomal and molecular abnormalities have been found in NSCLC, even its early stages. Most of the studies show that lung cancer occurs gradually with the genetic and cellular changes accumulates in the bronchial epithelial, not with an immediate effect. While early stage tumors show relatively fewer genetic changes, numerous genetic changes are observed in the advanced stages.

Including lung cancer, aneuploidy is the most common feature of solid tumors and as a result, malignant tumors can be diagnosed by detecting aneuploidy. A number of molecular studies have been showed that lung carcinomas are characterized by both low level and high level widespread gains and losses of genetic material at various chromosomes [2-6]. Based on the hypothesis that chromosome aneuploidies contribute to tumorigenesis, numerical chromosomal

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abnormalities in cancer cells can be detected by the FISHmethod [3]. Fluorescence in situ hybridization analysis is a strong method for detecting chromosomal changes in tumor cells. In our study we targeted chromosome 3, 7 and 8 for possible three-probe combination for detecting NSCLC whether these abnormalities could provide an additional prognostic factor. We also aimed to visualize chromosomal abnormalities in bronchoscopically gained cells from NSCLC patients and tested suitability of an interphase FISH assay for detecting cancer cells in bronchial lavage specimens.

MATERIAL AND METHODS

Patients

This study was approved by the Research Ethics Committee of our hospital and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects.

Clinically suspected patients who underwent bronchoscopy with abnormal chest radiography or computed tomography (CT) findings at İstanbul University, Cerrahpaşa Medical Faculty, Chest Diseases Department between March 2013 - September 2014 were enrolled. Collected specimens were fixed in Carnoy's fixative and were kept at 4°C until the final histopathological diagnosis. After the definitive diagnosis was confirmed by cytologic examination, a total of 17 NSCLC patients were included in this study. All of the samples were residual specimens after diagnostic sampling.

The patients included 15 men and 2 women, with an average age of 65 years; range from 55 to 75 years. Clinical information (age, gender, job, family history and smoking status) was obtained from patient or family members. The final diagnosis of samples were made as follows; 13 squamous cell carcinomas and 4 adenocarcinomas (Table 1). To distinguish true aneuploidy levels for chromosome 3, 7 and 8 FISH was performed to peripheral blood cells from healthy individuals. Control group selected from healthy, non-smoking 11 donors. Specimens from patients and cultured peripheral blood cells from control donors were collected and FISH analyse was performed.

Table 1. Patients demographics

Characteristic	n
Sex	
Male	15
Female	2
Age, years	
Mean	64.71
Range	55-75
Smoking Status	
Non-smoker	1
Smoking	16
Family history of cancer	
Yes	9
No	8
Type of NSCLC	
Squamous	13
Adenocarcinoma	4

NSCLC: Non-small cell lung cancer.

Sample Preparation

Washing specimens were collected in 10 mL tubes and centrifuged 400 g for 10 mins. After removing supernatant, fresh Carnoy's fixative was added and this process was repeated 2 times. Following that step, cell pellet washed with 1 x PBS, centrifuged and permeabilized with hypotonic 0.075 mol/L KCl solution at 37°C for 20 mins. Lastly cell pellet was fixed in fresh Carnoy's fixative and stored at 4°C until they were used. Fixed cell samples were spotted on wet, cleaned microscobe slide and allowed to air dry overnight.

FISH Hybridization

Satellite probes used in this study were chromosome specific sequences generated from highly repeated human satellite DNA located in centromeric region of chromosomes. Satellite probes labelled with red fluorophore, green fluorophore and aqua fluorophore used respectively for centromeric regions of chromosomes 3, 7 and 8 were purchased (Cytocell Ltd UK) and four-color FISH was performed using 4', 6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), sulforhodamine 101 acid chloride (texas red) and aqua filters. The slides were immersed in 40 mL 10 mM HCl and 500 µL 0.5 mg/mL pepsin solution at 37°C for 8-10 mins, followed by soaking in 1 x PBS at room temperature (RT) for 5 mins. The slides were denaturated in %1 neutral buffered formalin solution at RT for 5 mins and soaked again in 1 x PBS for 5 mins. Slides were dehydrated in ethanol series (70%, 85% and 100%), each for 5 mins and allowed to air dry. Using fresh pipette tips, 3 µL of each probe and 1 µL of hybridisation solution were put in a microcentrifuge tube per test and gently vortex to mix. A total of 10 µL probe were placed on the slide, covered with coverslip, and sealed with rubber solution glue. After denatured at 74°C for 1.5-2 mins, slides were incubated 4 hours at 37°C in a humid, lightproof container. After removing coverslip and glue, slides were washed in 0.4 x SSC at 65°C for 2 mins and 2 x SSC, 0.05% Tween 20 for 30 seconds. 20 µL of the DAPI antifade was applied and allowed colour to develop in the dark for 1 hour at 4°C.

Slides were analyzed under the fluorescence microscope (Nikon Eclipse E600, Japan) with fluorescence filter sets and captured with device's camera (COHU Cooled CCD Camera Applied Imaging, Newcastle, UK). Captured images were monitorized with image analyser (MAC Probe 4.3 Applied Imaging, Ne castle, UK).

FISH Scoring

A total of 500 cells for control group and 100 interphase cell for patients were counted. FISH signals are seen as red (centromere region of chromosome 3), green (centromere region of chromosome 7), aqua (centromere region of chromosome 8) and nuclear counterstain are seen as blue.

To avoid bias, FISH slides were scored blindly by two independent observers. Cells did not count if they touch or overlap and counted ones had smooth, well-rounded borders. Cells with signals located on the extreme periphery of the nucleus did not evaluate. Two signals that were connected by a strand were counted as one signal.

Statistical Analysis

Data were analyzed by using the statistical package for thesocial sciences (SPSS v16.0) (Chicago, IL, USA) statistical software. Association between probe aneuploidy for chromosomes 3, 7 and 8 was defined with Mann-Whitney U test. All p values were two-sided and values less than 0.05 were considered statistically significant.

RESULTS

Disomic (normal) cells-the expected FISH result- for control group was detected in a large majority. FISH evidence for different levels of aneuploidy was observed in all 17 NSCLC specimens. For every case, each chromosome signals were counted independently from each other. Control groups were scored 500 consecutive nuclei from each sample by two analysts, each scorer was analyzed about 250 nuclei from a given sample. Typically, a normal cell FISH signals were appeared as 2 red, 2 green and 2 aqua signal each. For related chromosome probe, a cell was considered abnormal if it showed more than two signals which indicates chromosome gain and showed one or no signal which indicates chromosome lose (Figure 1).

Using satellite DNA probes for chromosomes 3, 7, and 8 simultaneous four-color FISH were performed to evaluate the frequency of nullisomic, monosomic, disomic, trisomic, tetrasomic, pentasomic, hexasomic, heptasomic, octasomic and nonasomic cells in specimens. Nullisomic cell lacks both representatives of a pair of homologous chromosomes. Monosomic cell has the homologous chromosomes are represented only once in a cell. Disomic or normal cell has the two homologous chromosomes. Trisomic cell has a single extra homologous chromosome. Tetrasomic cell has four homologues of the same chromosome. Pentasomic cell has five homologues of the same chromosome. Hexasomic cell has six homologues of the same chromosome. Heptasomic cell has seven homologues of the same chromosome. Octasomic cell has eight homologues of the same chromosome and nonasomic cell has nine homologues of the same chromosome.

Analyses of 17 cases by FISH showed normal, nullisomic, monosomic, trisomic, tetrasomic and polysomic (pentasomic, hexasomic, heptasomic and octasomic) cells for chromosome 3. heptasomic and octasomic cells found only in 1 case, hexasomic cells found only in 2 cases and pentasomic cells found only in 7 cases (Table 2). For the control group only normal, monosomic and trisomic cells were spotted for chromosome 3 (Table 3). For chromosome 7, 17 cases showed normal, nullisomic, monosomic, trisomic, tetrasomic and polysomic (pentasomic, hexasomic and nonasomic) cells. Pentasomic, hexasomic and nonasomic cells found only in 1 case (Table 4). For the control group only normal, monosomic and trisomic cells were spotted, 1 control showed tetrasomic cells for chromosome 7 (Table 5). Lastly for chromosome 8 we detected normal, nullisomic, monosomic, trisomic, tetrasomic and polysomic (pentasomic and hexasomic) cells. Pentasomic and hexasomic cells found only in 2 case (Table 6). For the control group only normal, nullisomic, monosomic and trisomic cells were spotted for chromosome 8 (Table 7).

Aneuploidy of chromosome 3 was especially high (21.35%) than the chromosome 7 (9.06%) and chromosome 8 (15.47%). Also our results are significantly different ($p< 0.05$) for monozomy 3, trizomy 3, trizomy 7, nullizomy 8, monozomy 8 and trizomy 8.

DISCUSSION

In the present study, the usefulness of FISH with centromeric DNA probes for bronchoscopically gained bronchial lavage samples and successful analysis of numerical chromosome changes were demonstrated. To visualize evaluation of

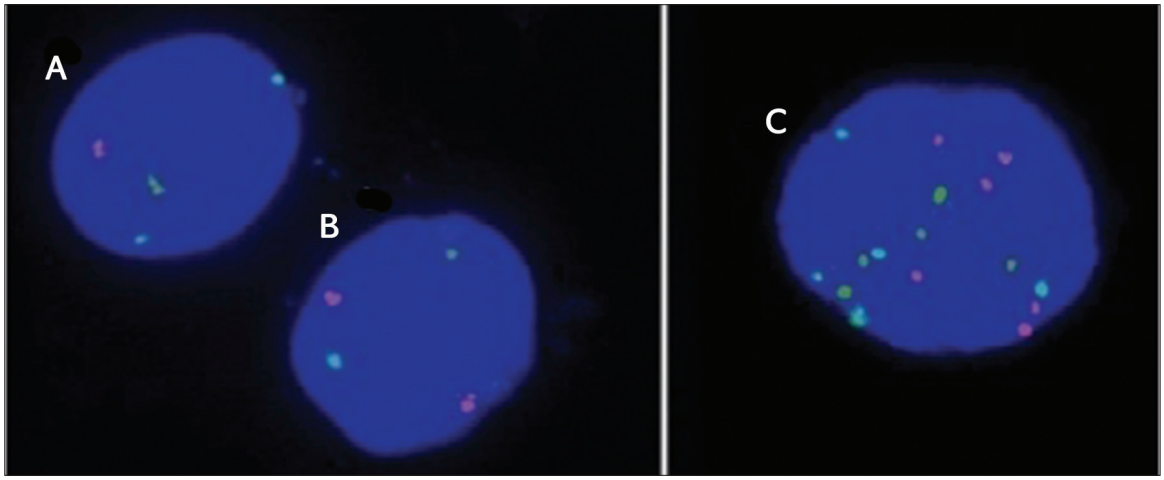

Figure 1. Examples of fluorescence in situ hybridization (FISH) abnormal cells found in patients. FISH clearly shows that cells are not disomic, demonstrating monozomy 3 and monozomy 7 **(A)**, monozomy 7 and monozomy 8, **(B)** and hexasomi 3, pentasomi 7 and pentasomi 8 **(C)**. FISH signals are seen as red (centromere region of chromosome 3), green (centromere region of chromosome 7), aqua (centromere region of chromosome 8) and nuclear counterstain are seen as blue.

Table 2. Result of CEP 3 probe in NSCLC patients

Case no	% of normal cells	% of nullisomic cells	% of monosomic cells	% of trisomic cells	% of tetrasomic cells	% of polisomic cells (> 4 signals)
1	64.62	0.77	8.46	17.69	7.69	0.77
2	72.30	-	3.08	21.54	3.08	-
3	69.12	-	4.41	14.70	7.35	4.41
4	91	-	2	3	3	1
5	91	-	3	4	2	-
6	90	2	5	2	1	-
7	88	-	2	7	3	-
8	69	-	-	5	10	16
9	77.97	-	6.78	13.56	1.69	-
10	71	1	7	16	4	1
11	85	-	7	5	3	-
12	83	1	4	8	4	-
13	89	-	6	3	2	-
14	71	-	3	19	5	2
15	81	-	4	11	4	-
16	81	-	4	11	4	-
17	79	-	5	13	1	2

CEP: Centromere enumeration probes, NSCLC: Non-small cell lung cancer.

Table 3. Result of CEP 3 probe in controls

Case no	% of normal cells	% of nullisomic cells	% of monosomic cells	% of trisomic cells	% of tetrasomic cells
1	98	-	0.60	1.40	-
2	97.20	-	1.12	1.68	-
3	96.80	-	1	2.20	-
4	95.40	-	1	3.60	-
5	98.20	-	1.80	-	-
6	96.60	-	1.40	2	-
7	96.80	-	3.20	-	-
8	99.60	-	-	0.40	-
9	97.20	-	2	0.80	-
10	96.20	-	2.40	1.40	-
11	98	-	1	1	-

CEP: Centromere enumeration probes.

aneuploidic abnormalities in NSCLC patients and provide an additional prognostic factor, we performed enumeration of three-probe FISH and demonstrated that four-color FISH is a feasible assay with an optimal probe combination for investigation of lung carcinomas. We examined interphase cells directly, without prior cell culture and rapidly counted a large number of tumor cells. Our molecular approach served beneficial technique fordetecting aneuploidy and has the potential for improving lung cancer detection. All of our cases showed chromosomal aneuploidy whichincreases mutation rate, gene amplification and/or genomic instability and accompanies tumor progression. Our findings are consistent with previous studies that also found FISH to be more useful and sensitive.

Studies showed that specimens not found to be positive for cancer with routine cytology (RC) were forwarded on for FISH analysis detected more peripheral lung cancers than RC alone [2,7-12]. Halling et al. showed that detecting peripheral tumors of lung cancer by FISH was able to increase the diagnostic sensitivity over routine cytology [8]. A subsequent study by Bubendorf and colleagues noted similar results in which the combination of routine cytology and FISH was increase the sensitivity compared with cytology alone [7]. In a study of 48 selected patients, Sokolova et al. found evidence

Table 4. Result of CEP 7 probe in NSCLC patients

Case no	% of normal cells	% of nullisomic cells	% of monosomic cells	% of trisomic cells	% of tetrasomic cells	% of polisomic cells (> 4 signals)
1	86.90	0.77	1.54	8.46	2.30	-
2	93.08	-	3.85	2.3	0.77	-
3	91.18	-	8.82	-	-	-
4	97	-	1	2	-	-
5	96	-	2	2	-	-
6	93	-	3	4	-	-
7	97	-	1	1	1	-
8	72	-	-	4	14	10
9	89.83	-	6.78	1.69	1.69	-
10	89	-	9	2	-	-
11	88	1	9	1	1	-
12	95	-	2	3	-	-
13	93	1	3	3	-	-
14	89	-	7	2	2	-
15	94	-	5	1	-	-
16	93	-	3	2	2	-
17	89	-	7	3	-	1

CEP: Centromere enumeration probes, NSCLC: Non-small cell lung cancer.

Table 5. Result of CEP 7 probe in controls

Case no	% of normal cells	% of nullisomic cells	% of monosomic cells	% of trisomic cells	% of tetrasomic cells
1	96.80	-	2.40	0.80	-
2	98.50	-	0.93	0.56	-
3	96.80	-	1.60	1.60	-
4	95.20	-	2.80	2	-
5	97.40	-	2.60	-	-
6	97.20	-	2.80	-	-
7	96	-	3.60	0.40	-
8	94.60	-	5.20	0.20	-
9	95.40	-	2.80	1.60	0.2
10	95.20	-	3	1.80	-
11	98.80	-	1	0.20	-

CEP: Centromere enumeration probes.

suggesting that the multitarget FISH test for simultaneous analysis of chromosome 1 and the 5p15, 7p12 and 8q24 loci might improve sensitivity for lung cancer detection [9]. In a study by Schenk et al. using FISH with DNA centromeric probes for chromosomes 3, 8, 11, 12, 17, and 18 aneusomy was present in all 10 primary tumors and 10 malignant effusions from 18 patients with lung cancer [13]. They also found FISH for chromosomes 7, 11, 17, and 18 to be positive in bronchial brushings from 5 patients with lung cancer.

Liu et al. developed a FISH assay to detect lung cancer by DNA centromeric probes for chromosomes 2, 3, 6, 7, 8, 9, 11, 12, 17 on 74 surgically resected NSCLC tissues, 32 operating margin tissue specimens without tumor and 174 bronchial brushing specimens [14]. Aneuploidy rates ranged from 62%-93% in tumor tissues and 29%- 70% in bronchial brushings of lung cancer patients based on individual probe data. The investigators determined that probes targeting chromosomes 3, 7 and 8 provided the optimal three-probe combination for detecting lung cancer. The three FISH probe combination had a similar sensitivity as RC. Based on this research, we tested chromosomes 3, 7 and 8 probe set for genetic abnormalities in NSCLC patients. In our study, the highest aneuploidy rate found was 21.35% for chromosome 3. chromosome 3 monosomy and trisomy were significantly higher ($p< 0.05$). Previous studies demonstrated copy number

Table 6. Result of CEP 8 probe in NSCLC patients

Case no	% of normal cells	% of nullisomic cells	% of monosomic cells	% of trisomic cells	% of tetrasomic cells	% of polisomic cells (> 4 signals)
1	91.54	3.08	2.30	2.30	0.77	-
2	91.54	3.85	3.85	-	0.77	-
3	91.18	-	5.88	2.94	-	-
4	95	-	4	1	-	-
5	93	1	6	-	-	-
6	86	-	12	2	-	-
7	78	1	16	4	1	-
8	71	1	5	16	5	2
9	79.66	1.69	11.86	6.78	-	-
10	77	2	22	-	1	-
11	73	2	22	2	-	1
12	71	4	22	3	-	-
13	91	-	9	2	-	-
14	87	1	9	3	-	-
15	87	-	10	3	-	-
16	92	-	3	5	-	-
17	84	-	12	2	-	2

CEP: Centromere enumeration probes, NSCLC: Non-small cell lung cancer.

Table 7. Result of CEP 8 probe in controls

Case no	% of normal cells	% of nullisomic cells	% of monosomic cells	% of trisomic cells	% of tetrasomic cells
1	98	0.20	0.8	1	-
2	98	-	1.86	0.19	-
3	98	-	2.8	0.2	-
4	97.40	0.20	2	0.4	-
5	97.60	-	2.4	-	-
6	95.80	-	2.4	1.8	-
7	95.80	-	4.2	-	-
8	99.80	-	0.2	-	-
9	96.40	0.20	2.6	0.8	-
10	96.20	-	3.2	0.6	-
11	96	-	4	-	-

CEP: Centromere enumeration probes.

changes on chromosome 3 are observed frequently and chromosomal gain of 3q associated with cancer formation from premalignant to invasive cancer. Also researchers suggested chromosome 3 probably contain many genes that could play oncogenic role [15-17]. Based on these findings, abnormalities of chromosome 3 are important for the development of lung cancer.

The role of epidermal growth factor receptor (EGFR) gene which locates at chromosome 7 in the pathogenesis and progression of various malignant tumors has long been known. The EGFR protein expression has an impact on prognosis, i.e. a higher expression correlates with a higher nuclear grade and larger tumors, and strong continuous membrane immunostaining is significantly associated with shorter survival [18-20]. Dordevic et al. showed EGFR overexpression is not associated with gene amplification but most likely with polysomy of chromosome 7 [18]. In our study the aneuploidy rate for chromosome 7 was %9.06. We hypothesized that increased expression of EGFR correlates with responsiveness to chemotoxic drugs in NSCLC, increased copy numbers of chromosome 7 could be the source of overall EGFR expression at the protein level. Waldman and colleagues demonstrated that the centromeric copy number of chromosome 7 is strongly correlated with tumor grade and labeling index in bladder cancer [21].

Clones with trisomy 7 as the sole abnormality have been reported in cultured malignant cells from NSCLC specimens.

Kubokura et al. investigated the c-myc gene amplification and the numerical abnormalities of chromosome 8 by FISH in NSCLC patients and obtained numerical chromosome 8 abnormalities correlated significantly with a poor prognosis [22]. The c-myc gene localized to 8q24 amplification seemed to be associated with tumor progression, and an overexpression of the c-myc gene protein may be related to metastasis of lung cancer [23,24]. Abnormalities of chromosome 8 was the second most numerical change in this investigation, being observed in 15.47% of the cells. Although there was no significant link between the amplification of the c-myc gene and clinical outcome, we hypothesized that the numerical chromosome 8 aberration might be a factor for survival.

This study is limited due to number of patients, therefore numerical chromosome aberation influences on clinical outcome could not be explained. Additional studies may be performed to explore utility of FISH in patients at an increased risk for developing lung cancer and a testing algorithm that includes other bronchoscopic techniques. For determining an optimal probe set of lung cancer diagnosis, studies should be conducted in representative patient populations.

As a result, our data indicate that FISH is a useful technique for successful detection of aneuploidy without cell culture in bronchial lavage samples obtained by bronchoscopy.

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