Akciğer kanseri hastalarında dolaşımdaki tümör hücrelerini nasıl tespit ederiz?

akım sitometrisi ile yapılan kısa bir çalışma

Tuğba Kevser UYSAL[1], Ender ŞİMŞEK[1], Ahmet ÇARHAN[1], Özen ÖZENSOY GÜLER[1]

Öz

Dolaşımdaki tümör hücreleri (CTC'ler) karsinomların metastatik yayılımında önemli role sahiptir. Bu nedenle, son yıllarda birçok kanser türünde hastalığın seyri ve tedavi etkinliğinin anlaşılmasında yardımcı olabileceği düşünülen CTC'ler üzerine yoğunlaşılmıştır. Teknik yaklaşımlardaki gelişmeler ile özellikle akciğer kanseri gibi dokularına ulaşılması zor olan tümörlerde sıvı biyopsi olarak tüm kandan CTC tespitinin değeri her geçen gün artmaktadır. Tanı anında sıklıkla metastaz yaptıkları teşhis edilen akciğer kanseri, hem kadınlarda hem de erkeklerde kanser ölüm nedenlerinin birincil sebebidir. Çalışmamızın amacı akım sitometrisi ile akciğer kanserli hastaların periferik kan örneklerinde (7.5 mL) CTC tespitidir. Zenginleştirme ve saptama adımlarından oluşan modifiye ettiğimiz yöntemimiz ile CTC sayısının tespit edilmesi için 9 akciğer kanserli birey ve 9 sağlıklı birey çalışmaya dahil edilmiştir. Uyguladığımız metotta zenginleştirme basamağı için fikol yoğunluk gradiyent ayrımı ve immünomanyetik ayırma tekniği (CD45 negatif seçilim) gerçekleştirilmiştir. Sonrasında, zenginleştirilmiş hücreler arasında CTC'leri tespit etmek için, anti-epitelyal hücre adhezyon molekülünün ve sitokeratinlerin ekspresyonuna dayanan çok parametreli akım sitometrisi ile analiz yapılmıştır. Çalışmamız sonucunda akciğer kanserli hastaların tümünde CTC gözlenirken, sağlıklı bireylerde gözlenmemiştir ($Z=3.823$; $p<0.001$). Böylece, modifiye ettiğimiz metodun akciğer kanserinde CTC tespitinde kullanılabilirliği gösterilmiştir.

Anahtar Kelimeler: Akciğer kanseri, akım sitometresi, dolaşımdaki tümör hücreleri, kanser, sıvı biyopsi

Yayın Bilgisi

Gönderi Tarihi:28.03.2018

Kabul Tarihi:26.04.2018

Online Yayın Tarihi:30.09.2018

DOI: 10.26453/otjhs.410582

Sorumlu Yazar

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How could we detect circulating tumor cells in lung cancer patients?

A brief study by flow cytometry

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Abstract

Circulating tumor cells (CTCs) play a crucial role in the metastatic spread of carcinoma. Therefore, CTC has been interest of a subject in the past few decades in terms of prognosis and response to the therapy in several cancer diseases. Recent improvements in technical approaches maintain to identify CTCs from whole blood have demonstrated the potential value of CTC detection as a liquid biopsy especially in those tumors where tissue accessibility is often challenging as in lung cancer. Lung cancer is the most common cause of death from cancer worldwide in both men and women which is commonly metastasize before it is diagnosed. The aim of this study is to enumerate of CTCs in peripheral blood sample (7.5 mL) of lung cancer patients by flow cytometry. Our modified method which consists of enrichment and detection steps get involved in 9 patients with lung cancer and 9 healthy volunteers. We performed a density-based ficoll gradient centrifugation and a immunomagnetic separation technique (CD45 negative selection) for the enrichment step. Next, multi-parameter flow cytometry based on the expression of anti-epithelial cell adhesion molecule and cytokeratins was used to detect circulating tumor cells among enriched cells. According to our results, circulating tumor cells were not detected on healthy volunteers but circulating tumor cells were found in all of patients with lung cancer ($Z=3.823$; $p<0.001$). We demonstrate that circulating tumor cells were detectable in peripheral blood sample of lung cancer patients by our modified method.

Keywords: Cancer, circulating tumor cells, flow cytometry, liquid biopsy, lung cancer

Article Info

Received:28.03.2018

Accepted:26.04.2018

Online Published:30.09.2018

DOI: 10.26453/otjhs.410582

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INTRODUCTION

Lung cancer is the most common cause of cancer deaths worldwide.[1] According to the current cancer statistics, lung cancer is the first and fourth most common type of cancer among males and females respectively in both Turkey and the World.[1,2] Furthermore, both subtypes of lung cancer (small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)) have a poor survival rate[3] and it is estimated to be responsible for approximately one in five of total deaths worldwide (1.59 million deaths, 19.4% of the total).[4] Because of its high fatality (the overall ratio of mortality to incidence is 0.87),[4] it has become an important health problem throughout the world.

Since lung cancer is usually asymptomatic in the early stages, the patients are detected in either advanced stage (stage IV) or in locally advanced stage (stage IIIA and stage IIIB).[5] Due to the difficulties in early diagnosis and lack of effective therapeutic methods, the mortality of lung cancer is high.[6] The 5-year mortality from the time of diagnostic is at approximately 85% to 90%.[7]

Metastasis, an important characteristic in cancer progression and is associated with mortality.[8] The metastatic process has a series of steps that takes place to spread primer tumor cells from their original residing of residence to the distant organs. Circulating tumor cells (CTCs) which shed into the peripheral blood from solid tumor, play a critical role in cancer progression.[3,9] Last decades, CTCs called "liquid biopsy" can be analyzed non-invasive and simple blood tests. Thus, they may be useful for early diagnosis, understanding metastasis development, identification of therapeutic targets and resistance mechanisms (predictive information) and real-time monitoring of therapies.[10] CTC analyzes have been useful in tumors where especially tissue accessibility is challenging such as lung cancer and some advanced applications can detect CTCs in whole blood to assess metastasis and prognosis.[3,11]

In literature, the researches have mostly focused on CTCs enumeration and the process involves in two important steps: enrichment and detection.[12,13] CTC enrichment step might be size-based, density-based, immunomagnetic separation or microfluidic-based. And, the CTC detection approaches include nucleic acid-based techniques and/or protein-based techniques.[13] In our study, we used our modified method described previously by our group.[14] The CTC enrichment step of our modified method was performed by using a combination of two separate methods: density-based ficoll gradient centrifugation and immunomagnetic separation (CD45 negative selection). In the final step, the CTCs were analyzed by flow cytometry. The aim of our study is to examine the applicability of our modified method for the detection of CTCs in patients with lung cancer.

MATERIALS AND METHODS

Sample Preparation

Human blood samples were obtained from Ankara Atatürk Training and Research Hospital affiliated with Ankara Yildirim Beyazit University, Turkey. The patient group was composed of lung cancer patients who were newly diagnosed, untreated and identified stage while the control group consisted of healthy volunteers carrying no suspicion of cancer in this study. All experiments were approved by the social and human sciences ethics committee (AYBU, Ankara/Turkey, 03.02.2015-12) and the informed consent forms obtained from all patients and healthy volunteers. The peripheral blood samples (7,5 mL) from 9 lung cancer patients and 9 healthy volunteers were collected into EDTA-coated tubes. Blood samples were stored at room temperature and then the protocol of CTC detection defined in our previous study was applied.[14]

Detection of CTCs

The detection of CTCs were performed as our published protocol.[14] Enrichment steps were implemented in the CTC enumeration process to increase the detection success rate. Improved enrichment methods using specific markers aim to identify the cells by distinguishing them from leukocytes. In this study, we used a density gradient separation (ficoll) and an immunomagnetic separation [Cluster of Differentiation 45 (CD45) for the negative selection] in the enrichment step. Enriched cells were incubated with the anti-epithelial cell adhesion molecule (Anti-EpCAM), the anti-cytokeratins (anti-CKs) and the leukocyte specific marker (anti-CD45). After, flow cytometry (The BD FACSAria™ III Cell Sorter) was used for the identification of CTCs in a highly specificty with multiple parameters. Consequently, Anti-CK14,15,16,19(+), Anti-CK7,8(+), Anti-EpCAM(+) and Anti-CD45(-) phenotypes were defined CTCs and counts of them were obtained.

Statistical Analysis

The IBM SPSS Statistics 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) software was used to perform statistical analysis. The distribution of age and CTC counts was examined by Shapiro-wilk test. The CTC counts were expressed in terms of median (min-max: minimum-maximum), categorical variables such as gender were expressed in number (%). The Mann-Whitney U test was used for comparison between groups. All data were subjected to the above statistical methods, and P-values less than 0,05 were considered significant.

RESULTS

The characteristics of patients and healthy volunteers

In the present study, the average age of the lung cancer patients and healthy volunteers

regardless of gender was 63,78±8.01 and 61,67±11,38 years, respectively. The average age was 62,72±9,61. There were 8 male and 1 female in both the patient and control group. The clinical characteristics of patients are presented in Table 1.

Comparisons of CTC levels

We compared CTC levels in patients with lung cancer and healthy volunteers. As shown in Table 2, the CTC levels in lung cancer patients (median 8 CTC) was significantly higher than healthy volunteers (median 0 CTC, p <0,001). However, about CTC levels there was no statistically significant difference in patients with stage III and stage IV (p=0,111; Table 2). Additionally, we did not find a significant difference in CTC levels between SCLC and NSCLC (p=0,333; Table 2). The results suggested that patients with lung cancer have high level of CTCs compared with healty volunteers. The results of flow cytometry are presented in Fig. 1 and Fig. 2 respectively for P1 patient, C1 volunteer.

DISCUSSION AND CONCLUSION

Although CTCs were observed more than 100 years ago, for the first time, their clinical significance has recently recognized.[15] Detection of CTCs might be difficult because of their rare numbers comparing with the number of leukocytes (~1 CTC per 10^5-10^8 leukocytes) in blood.[13] Although there are many different techniques applied for the detection of CTC, only the CellSearch system which has been approved by the U.S. Food and Drug Administration is often preferred in CTC researches.[16] In this system, CTCs were immunomagnetically enriched with EpCAM and immunofluorescent staining was performed using CD45 for excluding the leukocytes and CK 8/18/19 and DAPI are used for identification of CTC.[3,17] The cut off value was identified in breast, colorectal and prostate cancers for determining the efficacy of treatment and prognosis.[13] On the other hand, the CellSearch system has not yet been approved for lung cancer and there is still no accepted cut off value. However, many studies have been conducted using the CellSearch system on the role of CTCs in lung cancer lately and significant data have been reported.[3] One of the first study using the Cellsearch system in lung cancer patients and reported that CTC was detected in three out of four additional SCLC patients and CTCs may be useful for SCLC diagnosis.[18] In another study, CTCs in NSCLC and SCLC patients by CellSearch system were detected in 30,6% of lung cancer patients and in 12,0% of non-malignant patients and there were differences between stage I and stage IV tumors.[19]

The prognostic significance of CTC detection by the CellSearch system in 101 chemo-naive stage III–IV NSCLC patients was demonstrated by Krebs et al. The results showed a

significantly higher CTC count in stage IV than that in stage III (60 and 27, respectively) and the CTC numbers were associated with prognostic factor for progression-free survival (PFS) and predictor of overall survival (OS) after one cycle of chemotherapy.[20]

There are other studies about besides CellSearch in CTC detection. Krebs and colleagues analyzed CTCs in patients with NSLC using epithelial marker-dependent CellSearch system and epithelial marker-independent the size-based discrimination method (ISET). Although both technology platforms detected CTCs, ISET counted higher numbers of CTCs compared with using CellSearch.[21] However, as a result of other studies with the filtration system, it has been determined that this method is not specific and sensitive.[22] Because, even in the same patient, the size of tumor cells that have been captured has been changed.[23]

Unlike filtration and CellCearch, in our study, we prefered the Ficoll gradient centrifugation as the first step. After centrifugation, the PMBC layer containing CTCs were separated erythrocytes and granulocytes in the blood samples.[24] We performed magnetic-activated cell sorting (MACS) for an immunomagnetic depletion of leukocytes in collected PBMCs and thus CTCs are enriched with negative selection. For the detection of CTCs, the negative selection has higher efficiency compared to the positive selection or the combination of negative and positive enrichments.[25]

Although a wide variety of methods were preferred in the detection step applied after the enrichment step, CTCs were detected by flow cytometry (FC) using with fluorescent monoclonal antibodies (anti-CD45, anti-EpCAM, anti-CK 7, 8 and anti-CK 14, 15, 16, 19) in our study. EpCAM is an ideal tumor antigen that is overexpressed in various cancers including lung cancer and widely used for the diagnosis of CTCs.[26] Another protein family commonly used in CTC detection is CKs. They are expressed in very specific patterns according to epithelial types and CK markers are generally identificated for cancer classification. The analysis of CK-5/6, CK-7, CK-10/13, CK-14, CK-17 and CK-18 expressions in lung cancer provides important information about cancer progress.[27] We aimed to eliminate false positives in the blood samplesof healthy volunteers using our selected markers together.

Due to the higly heterogeneous nature of CTCs, quantitative analysis of these rare cells appears advantageous. Quantifiable fluorescence-based FC analysis is therefore an attractive alternative to CTC detection. FC which is used clinically in many disciplines including hematology and oncology has been proven to be an extremely powerful technology.[13] FC also provides advantages such as the ability to easily perform multiple marker analysis on the same sample, the ability to measuring and quantifying the level of expression, and the ability to collecting

sorted cells.[13] Therefore, FC was used in our CTC detection step.

More recently, Huang et al. identified CTCs from patients with NSCLC using FC to observe the effects of surgical approaches on the levels of CTCs. The cells were enriched immunomagnetically with EpCAM (positive selection) were analyzed by FC with anti-CK-PE and anti-CD45-FITC fluorescent-labeled monoclonal antibodies for CTC enumeration.[28] In another study, immunomagnetic nanobeads were effectively used for the detection of CTCs, CTCs were enriched with CD45 and analyzed by FC in 84 blood samples of lung cancer patients. Three of them were NSCLC patients and detection rate was 100%.[25]

In our study, 9 patients with lung cancer and 9 healthy volunteers were studied and the CTC count was determined as 0 in all healthy individuals and different number of CTCs was detected in all of patients in Table 3.

In addition to the significance of the CTCs in lung cancer, some studies suggested CTCs related with disease stage.[19,20] The potential of differentiate among disease stages of CTC counts was demonstrated in 150 NSCLC patients by Tanaka and colleagues reporting that CTC was in different counts between stage I and stage IV.[19] Krebs et al. reported the prognostic role of CTCs in 101 NSCLC patients with stage III-IV and CTC counts were found to be significantly higher in stage IV patients than in stage III patients.[20]

Several studies have indicated that CTCs are detected in different types of cancer patients as an important clinical marker for the diagnosis of cancer, monitoring of disease progression, treatment efficacy.[29] Similarly, we suggest that CTC can be a multifunctional biomarker due to the successful detection of CTC using our modified method in lung cancer patients. In our study, there was no significant difference between CTC counts and lung cancer subtypes, although the CTCs in peripheral blood of patients with lung cancer was successfully detected. In present study, the number of individuals was low; however, more extensive study will be performed in future study. In addition, CTCs will be sorted with the fluorescence active cell sorting (FACS) device and molecular characterization will be performed too. Therefore, more information about the biology and pathology of cancer disease can be obtained and more effective and successful treatment can be provided by applying for individualized treatment therapies.

Acknowledgments: The authors thank to Ankara Ataturk Training and Research Hospital (Ankara, Turkey) Oncology Depertmant staff for providing their wide experiences on patients.

Conflict of interest: The authors report no conflict of interest.

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a novel metastatic tumor marker. Exp Ther Med. 2011; 2(3): 385-391.

Table 1. Clinical characteristics of lung cancer patients.

Features	Individual numbers and percentage distributions n (%)
Stage	
IIIA	2 (22.2%)
IIIB	2 (22.2%)
IV	5 (55.6%)
Histology	
SCLC	2 (22.2%)
NSCLC	7 (77.8%)
Subgroups of NSCLC	
Adenocarcinoma	5 (71.4%)
Squamous cell carcinoma	2 (28.6%)
Sites of 5 metastatic patients	
Brain	3 (60.0%)
Bone	2 (40.0%)

SCLC: small cell lung cancer; NSCLC: non-small cell lung cancer

Table 2. Distribution of CTC counts in control and patient groups.

Group variable	CTC Counts[1]	Test statistic	p value
Group		3,823	<0,001
Patients	8,0 (4,0-15,0)		
Control	0,0 (0,0-0,0)		
Histological types		1,176	0,333
SCLC	11,0 (9,0-13,0)		
NSCLC	6,0 (4,0-15,0)		
Stage		1,722	0,111
Stage III	5,0 (4,0-10,0)		
Stage IV	9,0 (6,0-15,0)		

[1] Median (min-max); CTC: circulating tumor cell; SCLC: small cell lung cancer; NSCLC: non-small cell lung cancer

Table 3. CTC counts of control and patient groups.

Control group	CTC counts	Patients	CTC counts
C1	0	P1	13
C2	0	P2	6
C3	0	P3	4
C4	0	P4	15
C5	0	P5	10
C6	0	P6	9
C7	0	P7	8
C8	0	P8	5
C9	0	P9	5

CTC: circulating tumor cell

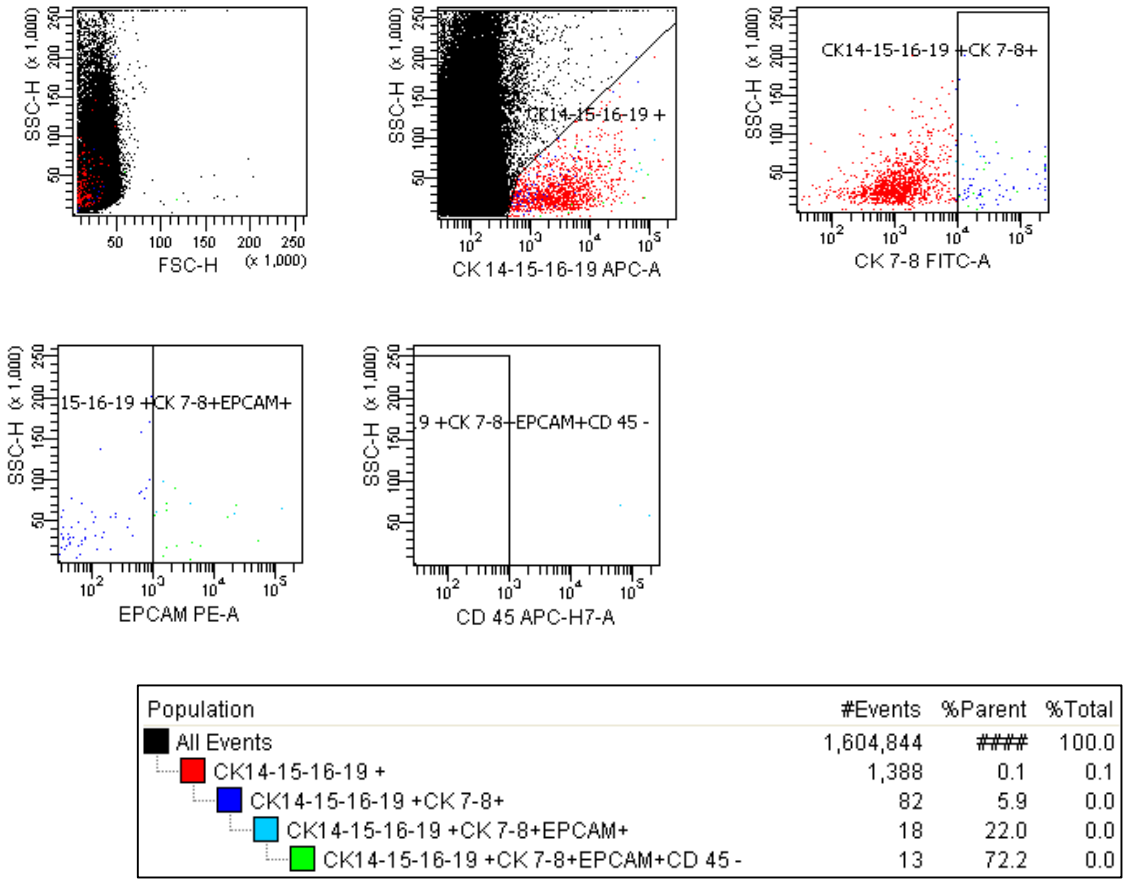
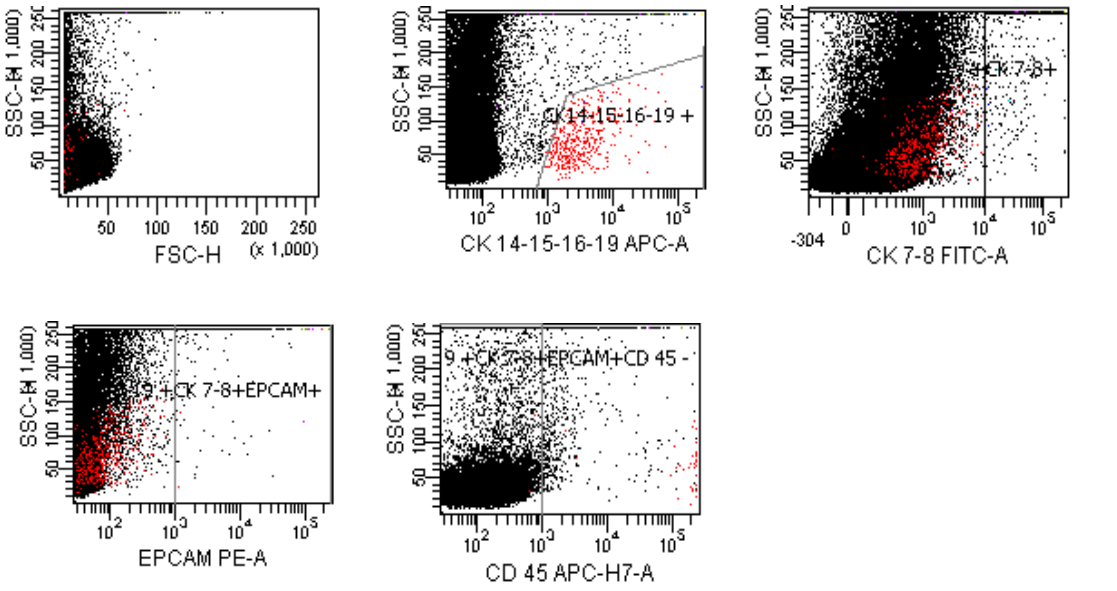


Figure 1. The result of flow cytometry for the P1 patient.



Population	#Events	%Parent	%Total
All Events	800,805	####	100.0
CK14-15-16-19 +	425	0.1	0.1
CK14-15-16-19 +CK 7-8+	6	1.4	0.0
CK14-15-16-19 +CK 7-8+EPCAM+	2	33.3	0.0
CK14-15-16-19 +CK 7-8+EPCAM+CD 45	0	0.0	0.0

Figure 2. The result of flow cytometry for the C1 volunteer.