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Sputum screening using high resolution image cytometry

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Abstract

Screening using chest X-ray combined with sputum cytology failed to decrease lung cancer mortality in an NCI-sponsored study. However, several attempts have been performed recently to increase the sensitivity of lung cancer screening. Our approach is the application of high resolution cytometry to sputum cytology. High resolution cytometry system can measure 120 nuclear features per cell, therefore microscopically invisible nuclear morphological changes could be evaluated. The phenomenon called Malignancy Associated Changes (MACs) was extensively analyzed in this study and authors evaluated the expression of MAC in each sputum specimen. A total of 106 sputum specimens (52 from lung cancer patients and 54 from apparently healthy individuals) were collected and slide pairs were made. One slide was stained by Papanicolaou for conventional sputum cytology and the other was stained by Feulgen staining for cytometry. The sensitivity of conventional sputum cytology was 19% by the experienced cytopathologists in this study. Cytometry revealed 70% sensitivity at 77% specificity by measuring the MAC expression. In this study, the possibility of increasing the sensitivity of sputum cytology was suggested by employing the high resolution cytometry and the MAC concept.

Introduction

Lung cancer is the leading cause of cancer death throughout the world. To reduce the mortality of lung cancer, prevention, early detection, and improvement of treatment are indispensable. Among these, early detection is considered to be most important. The combination of chest X ray and sputum cytology has been the initial screening method of lung cancer[1)]. Chest X ray may not be sufficiently sensitive to screen peripheral lesions with ground glass appearance (GGA) features which are considered to be precancerous/early cancerous lesions[2-4)]. Helical CT has been extensively used to detect such lesions and the sensitivity of the detection of tiny nodules improved greatly compared to that of chest X-ray[2-4)]. Altough sputum cytology is the sole method to detect central-type early stage lung cancer, the sensitivity of this method in such lesions has been reported to be less than 70% and only 10-20% for overall lung cancers[1)]. Randomized control studies using mass surveys in the US (Mayo Clinic, Memorial Sloan-Kettering, Johns Hopkins) failed to show a decrease in lung cancer mortality by screening programs including chest X ray and sputum cytology[5-9)]. As that study was performed almost 30 years ago, the results may not be equally interpreted at present considering the improved state of the art of diagnostic and therapeutic modalities[10)11)]. Recently, the interest in early detection has become intense and several attempts have been made using molecular techniques[12-19)], specific antigens for lung cancer[20-23)] and cytochemical analyze[24-26)]. In this manuscript, we introduce our approach using high resolution cytometry, which can objectively analyze 120 nuclear features per cell. We extensively studied the

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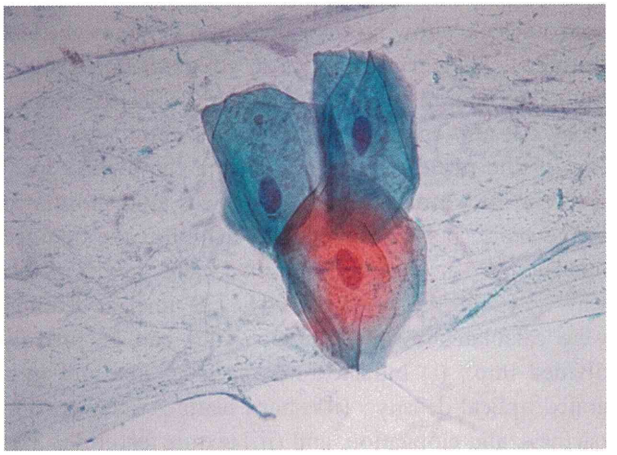
phenomenon called Malignant Associated Changes (MACs) by analyzing nuclear features. MACs are subvisual morphological changes in the nuclei of normal cells found in the vicinity of the malignant growth and have also been reported in sites distant from the primary sites of malignancy (Fig. 1). The concept of MAC was firstly reported by Nieburg in 1959 in buccal cells[27]. The existence of MACs has been shown in several organs ; uterine cervix, colon, buccal smear, blood, lung and breast[24–41]. The authors postulate the existence of cancer can be highly suspected if MAC cells are present, even in cases in which cancer cells are absent, therefore increased sensitivity for cancer would be obtained by clinical application of MACs. This paper reports the expression of MACs in normal exfoliated cells in sputum in cancer patients and normal individuals and discussed the possibility of applications for lung cancer screening.

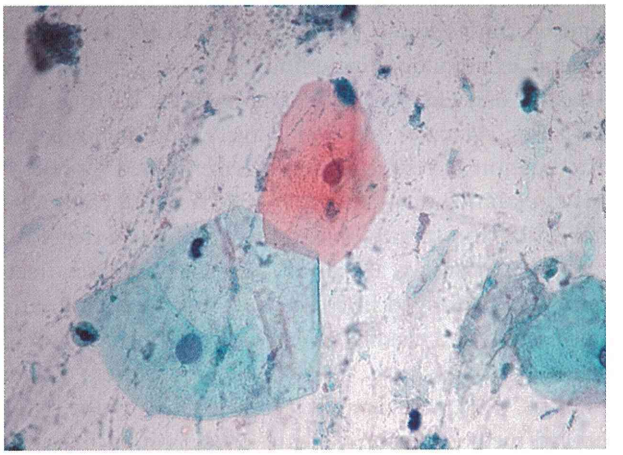
Material and Methods

Subjects

Sputum samples were collected by from 130 heavy smokers or ex-smokers (>30 pack-years), aged 45–75 years. As 24 samples turned out to be unsatisfactory specimens after staining and were eliminated from the study. Therefore a total of 106 sputum samples were studied, the characteristics of which are shown in Table 1. These were patients with known or suspected lung cancer included for diagnostic work-up. A total of 52 samples were from lung cancer patients and 54 from non-cancer patients or healthy individuals. The non-cancer group consisted of 36 healthy individuals with or without inactive chronic inflammation, 2 cases confirmed to have metaplasia and 16 postoperative



Case 1



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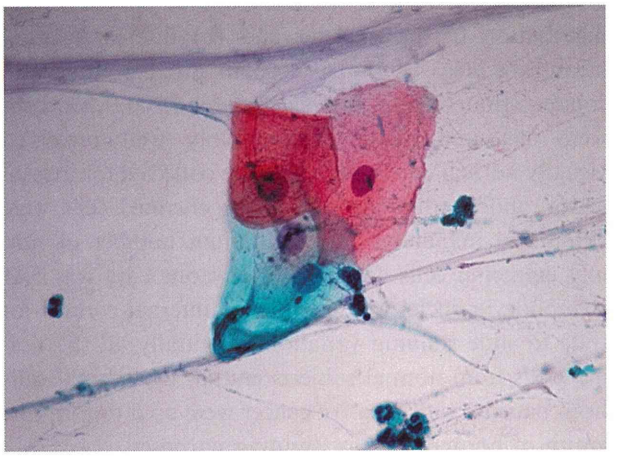

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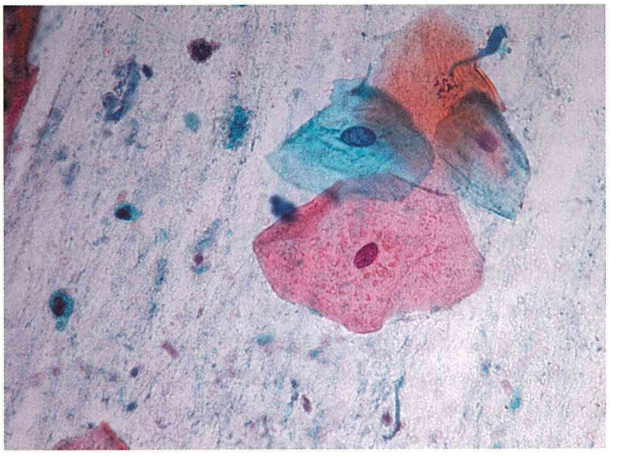

Case 4

Fig. 1 Figure 1 shows representative true normal cells and apparently similar MAC cells. All cases were classified as microscopically normal by experienced pathologists, but there are morphological differences which can be recognized only by mathematically programmed high resolution cytometrical evaluation of cell clusters (i.e. not merely individual cells).
Case 1 : MAC cells, 68-year-old man, squamous cell carcinoma
Case 2 : MAC cells, 72-year-old man, adenocarcinoma
Case 3 : true normal cells, 72-year-old man, apparently healthy individuals
Case 4 : true normal cells, 63-year-old man, apparently healthy individuals

Table 1 A total of 106 sputum specimens (52 from lung cancer patients and 54 from apparently healthy individuals) were studied.

		Central type	Peripheral type
Malignant : 52 cases			
Squamous cell carcinoma	21	6	15
Adenocarcinoma	25	0	25
Large cell carcinoma	1	0	1
Small cell carcinoma	5	2	3
Stage I		22	
Stage II		5	
Stage III		25	
Benign : 54 cases			
Normal		36	
Post curative operation		16	
Dysplasia		2	

disease-free patients with stage I lung cancer for at least 3 years. For definitive diagnosis, each patient received chest X ray, chest CT and bronchoscopy as well as general examination including blood test, pulmonary function and electrocardiogram. Among 52 lung cancer cases, 44 cases received surgery and pathological examination of resected lungs was performed by experienced pathologist in all cases. The rest of 8 cases were treated by non-surgical method after definitive diagnosis of lung cancer was obtained. Endoscopic laser treatment was performed in 3 cases due to central type early stage cancer and chemotherapy was performed in 5 small cell lung cancer cases. All cases were current or ex-smokers. Sputum samples were collected by the 3-day pooled method by spontaneous expectoration or saline induction if necessary. A total of 4–6 slides (2–3 slide pairs) were made for the study. For each case, one slide pair was selected and one slide was stained by Papanicolaou staining for conventional sputum cytology and the other was stained by Feulgen staining for cytometry. Feulgen stain is used in histology to identify chromosomal material or DNA in cell specimens.

Conventional cytology

The Papanicolaou stained slides were screened in the Department of Pathology, Tokyo Medical University according to their standard sputum cytology interpretation. Cytotechnologists screened slides initially and experienced cytopathologists made the final diagnosis. Both of them were blinded to all clinical information.

Cytometry

The Feulgen stained slides were scanned by the AcCell-Savant system (Accumed Inc., Chicago, USA), which was originally developed for automated prescreening of cervical specimens[43–45]. The key element of this system is its light transducer, a charge-coupled device, with 1,320×1,035 individual sensor elements. This results in the aquisition of chromatically and geometrically correct images with square pixels. On average over 500 measurements (pixels) are made over each nucleus, allowing the calculation of many nuclear features. The device employs an automatic focus algorithm such that all nuclear images are collected in exact focus. It also employs algorithms for automated detection of the nuclear boundaries along the highest local gradient between the stained nucleus and unstained cytoplasmic background ; thus defining the stained nucleus in a precise, objective and reproducible way[43–45]. In the present study, 120 nuclear features were calculated by the system. They can be broadly divided into ; (i) bulk features, such as area and integrated optical density ; (ii) shape features, such as compactness, and elongation, and (iii) texture features. The texture features can be further divided into two subcategories : (a) continuous analyzing the optical density distribution of the nucleus, and (b) discrete features measuring areas of the nucleus in discrete chromatin condensation states. For each slide, 1,200–1,500 cells were measured automatically. Only well preserved visually normal columnar cells were collected for investigation and overlapping cells or degenerated cells were eliminated manually. The minimum number of normal epithelial cells collected in any one case was 530. Lymphocyte nuclei were used as an internal control for slide to slide staining variations. Initially all the normal cells from normal subjects and all the normal cells from patients with invasive cancer were used to form the group of normal cells for training purposes.

Statistical Analysis

The authors postulate there may be subtle morphological difference between normal cells obtained from cancer patients and those from healthy individuals according to the MAC concept. Nuclear images were captured and mathematically analyzed by the system. To create the test set, 200 normal cells were randomly

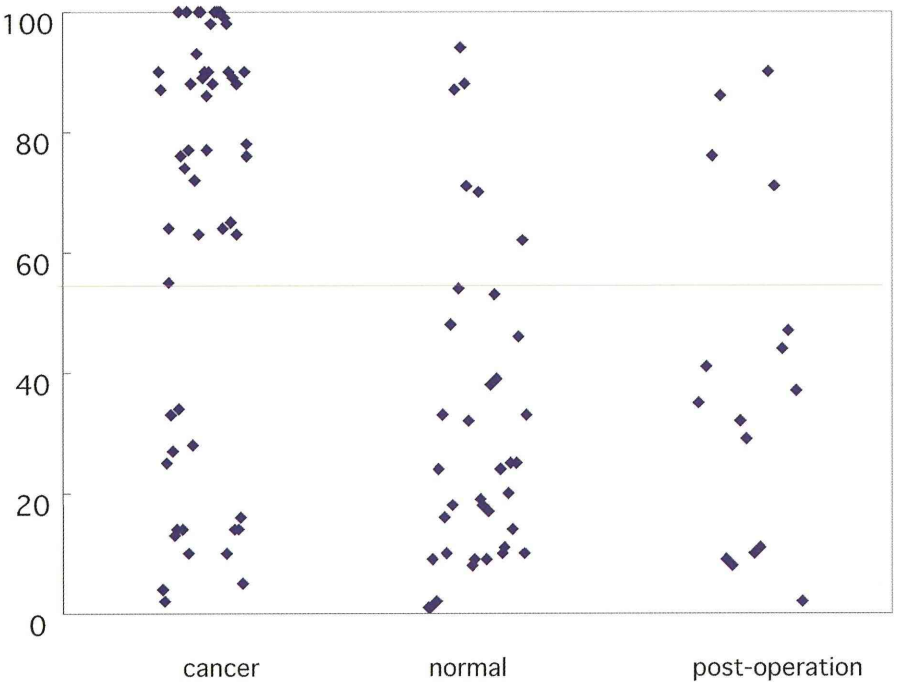

Fig. 2 The MAC expression of each case was plotted. There was a statistical difference of MAC expression between the cancer group, after curative operation group and the non-cancer group. The sensitivity for cancer was 70% with 77% specificity using the threshold at 50% MAC expression.

collected from 36 normal subjects (control group : total 7,200 cells) and from 25 stage III cancer cases (MAC group : total 5,000 cells). Multivariate analysis (cell by cell stepwise discriminant function analysis) was performed to determine the powerful nuclear features discriminating normal cells of MAC group from those of control group using the S-PLUS (Mathematical Systems Inc., Tokyo, Japan). Based on these results, the optimal combinations of the discriminating nuclear features were decided and the definitions of MAC cells were obtained. The criteria of MAC cells created by the test set were then applied in every case and the expression rate of MAC cells was evaluated in each case for validation.

Results

Conventional sputum cytology

A total of 10 slides were diagnosed to be positive for cancer among slides obtained from 52 cancer patients, therefore the sensitivity of conventional sputum cytology was calculated to be 19% (10/52) in this series.

Cytometry

Multivariate analysis revealed differences OD kurtosis, DNA Low vs Med & High, High density object, and high F values of Run per 45 between normal cells. Obtained from cancer patients (MAC cells) and those from healthy individuals, and moreover that, the combination of these four features was very powerful indiscriminating between these two groups. These four features mathematically expressed the chromatin distribution in the nucleus. Thus MAC cells were defined by the combination of these four features and the expression of MAC cells per cells measured was calculated in each case. The MAC expression was plotted in each case in Fig. 2. The sensitivity of cytometry was 70% with 77% specificity when the cut-off line of the expression rate was 50%. In this experimental group, sensitivity of sputum cytology was enormously improved (19% to 70%) by the application of the MAC concept compared to that of conventional cytology. Also the MAC expression decreased in the disease-free status after curative operation of lung cancer. Among lung cancer cases, there was no statistical difference of MAC expression in relation to stage or histologic type (Table 2).

Table 2 There was no statistical difference of MAC expression in relation to stage or histologic type.

Squamous cell carcinoma	64.8±22.9	N.S.
Adenocarcinoma	60.1±19.8	
Stage I	65.1±24.6	N.S.
Stage II	59.4±18.7	N.S.
Stage III	63.7±21.5	

Discussion

Lung cancer is the most common cause of cancer death, but an over 90% 5-year survival rate has been obtained if it is detected as carcinoma in situ or in the microinvasive stage[46–57]. Also less-invasive endoscopic laser treatment have shown favorable therapeutic out-

come[56)57)]. Much effort has therefore been made to detect early stage lung cancer and even precancerous lesions. Sputum cytology has been commonly used since the 1970's, especially to detect central-type lung cancer, and has been believed to be the only method to detect occult lung cancer[1)]. However approximately 40% of subjects cannot produce appropriate sputum specimens and 30% of occult lung cancer cases are fail to be diagnosed by sputum cytology[1)]. Thus there seem to be rooms for improvement in sputum cytology. For peripheral early cancer, helical CT has been extensively used and conventional sputum cytology is not expected to play an important role. However, in this study, the sensitivity for adenocarcinoma (peripheral location) was equal to that of squamous cell carcinoma (central location). This preliminary result suggests that MACs may be applicable to future lung cancer screening. Recently, several attempts have been tried using molecular biology techniques to identify early stage lung cancer cases as well as latent cancer cases or individuals at high risk for cancer[12–19)]. Tockman et al proposed the use of specific monoclonal antibodies to detect lung cancer[20–22)], and referred sensitivity and specificity of 64% and 88%, respectively[20)]. That study evaluated only dysplastic cells, but it is unusual for a large number of dysplastic cells appear in the same slide. We employed a different approach analyzing only normal cells using high resolution cytometry. Quantitative cytometry has been extensively studied by the Vancouver group of Palcic et al and they proved the existence of the MAC phenomenon by analyzing nuclear morphology with high resolution cytometry[24–26)43–35)]. We also subsequently postulated that detection of early stage or precancerous lung lesions could be enhanced by using the MAC expression rate as a biomarker index. MACs have been discussed for more than 70 years in the literature, but these early works were qualitative and difficult to reproduce. In 1959, Nieburgs defined the characteristic microscopic findings of MAC cells[28)] : curved chromatin, rows and quadrant formations of circular areas, etc but these were also only qualitative descriptors[29–35)]. If MACs could be measured in an objective and reproducible way, they may be useful clinically. Previous work has shown that MAC cell features are strongly related to nuclear chromatin distribution[24–35)]. Thus, high resolution image cytometry may enable more accurate diagnosis of MAC cells. MacAuley et al. analyzed the nuclear features of bronchial biopsy specimens in the opposite side of the lung cancer location by quantitative microscopy. They reported that MAC expression was detected in over 80% of bronchial biopsy specimens from apparently normal sites of patients with lung cancer[24)]. They also stated that there is no difference in MAC expression between smokers and non-smokers. Therefore MAC expression is not influenced by smoking status and is only related to the existence of cancer. One of the present authors (N.I.) obtained normal cells via the bronchoscope from normal sites in the contralateral lung from that with primary lung cancer and high MAC expression was observed[26)]. Payne analyzed MAC expression in cancer-negative cytology slides of subjects evaluated in the Mayo Lung Project who developed lung cancer 2–3 years later. MAC expression was analyzed in the same manner as the present study and increased expression was observed in cases with future development of cancer. Future cancer development was predicted with 40% sensitivity and 90% specificity from negative slides[25)]. Palcic et al. conducted a multicenter field study and collected a total of 833 sputum samples (177 cancer, 98 dysplasia and 558 control). Specimens were evaluated by image cytometry and the result was compared to the conventional cytology. The sensitivity of 60% can be achieved with 90% specificity by cytometry, compared to 14% sensitivity of conventional sputum cytology. Similarly, 45% sensitivity with 90% specificity was obtained by MAC technology for stage 0 and I lung cancer compared to 14% sensitivity obtained by conventional cytology[27)]. We obtained similar results that there was no difference in MAC expression according to stage of lung cancer. At present, the mechanism of diagnosis based on MACs is not yet established. One hypothesis is that growth factors or cytokines excreted from cancer cells may affect the surrounding normal cells. Another theory is that some genetic abnormality also exists in the ostensibly normal cells. MacAulay and co-workers reported that MAC expression decreased to within the normal range after curative resection of lung cancer[24)]. We also observed decreased MAC expression in disease-free cases after currtive operation. Further investigations are necessary to reveal the mechanisms of MAC, but if our postulate is correct, MAC can be used as a biomarker to evaluate therapeutic effects as well as a screening tool.

The reasons for the false positives obtained were not clear, but these cases might develop cancerous or precancerous lesions which could not be detected by our series of examinations. Further study of a large number of cases and long-term follow up of MAC positive subjects confirmed not to have cancer is required. False positives and false negatives are also related to the choice of the cutoff decision boundaries between MAC positive and negative samples.

This study demonstrates the feasibility of the application of MAC quantification to sputum specimens. The presence of MAC cells in sputum specimens would suggest the existence of latent cancer or high risk for cancer development even if cancer cells cannot be observed. MACs may also be useful in predicting the out-

come after surgical resection or detecting recurrence. Multicenter control trials for a long follow up period and an adequate threshold setting are necessary before clinical application[58].

Comprehensive analysis of lung cancer using biomarker produced by moleclular biology, proteomics and image cytometry might evaluate the risk of cancer and improve the diagnostic rate of early cancer.

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High resolution image cytometry を用いた喀痰スクリーニング

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【要旨】 米国国立がん研究所が行った調査で、胸部単純レントゲン撮影に喀痰細胞診を組み合わせたスクリーニングでは、肺癌による死亡率は減少しなかった。しかしながら、肺癌スクリーニングの感度を高めるために、最近いくつかの試みが行われている。我々の試みは high resolution cytometry を喀痰細胞診に応用することである。High resolution cytometry は細胞 1 つあたり 120 の核特徴を測定可能で、その結果、極めて微細で目に見えない核形態変化が評価出来るようになった。Malignancy Associated Changes (MACs) と呼ばれる現象を、この研究で詳しく分析し、個々の喀痰試料における MAC の発現を評価した。Malignancy Associated Changes (MACs) とは癌病変の周辺で発見された、正常細胞の視覚上の核形態変化のことで、癌原発巣から離れた部位でも、同様な報告がなされている。この存在は子宮頸部、結腸、頬部口腔粘膜擦過、血液、肺そして乳腺などの器官でも示されている。今回我々は合計 106 の喀痰試料(肺癌患者 52 検体、健常者 54 検体)を集め、スライドをペアで作製した。一方ののスライドは従来の喀痰細胞診のためにパパニコロ染色し、そしてもう一方には high resolution cytometry のために Feulgen 染色を行った。従来の喀痰細胞診の感度はこの研究において、経験豊富な細胞病理医によって 19% であったが、High resolution cytometry では、MAC 発現を測定することによって、70% の感度と 77% の特異性を示した。この研究で high resolution cytometry と MAC の概念を用いることによって、喀痰細胞診の感度を高める可能性が示唆された。

〈キーワード〉 肺癌、喀痰細胞診、早期発見、肺癌検診、Malignancy Associated Changes