RESEARCH ARTICLE

DNA Replication Licensing Proteins for Early Detection of Lung Cancer

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Abstract

Background: To identify and characterize malignant and premalignant cells in sputum and matched tissue samples with reference to expression of minichromosome maintenance proteins (MCM2, MCM5) and cell division cycle protein 6 (CDC 6) and to assess their potential as biomarkers of premalignant and malignant lesions of the lung and associations with clinicopathological features. **Methods:** Expression of MCM2, MCM5 and 6 proteins in sputum samples and corresponding tissues was assessed by immunocytochemistry, and correlated with histological findings. **Results:** For characterization of malignant, metaplastic or dysplastic cells, CDC6 protein had the highest sensitivity of 87.7%. All the three markers together had a sensitivity of 94.4%. Furthermore these proteins could be employed to assess the proliferative potential of precancerous or atypical cells, as overexpression increasing with the stage of disease and degree of metastasis. **Conclusion:** The assessed markers can be utilized in routine cytopathology laboratories to supplement conventional morphological evaluation so that the sensitivity of sputum cytology can be enhanced. Potential applications in predicting the clinical behavior of lung lesions and predicting prognosis and survival deserve further attention.

Keywords: Minichromosome maintenance proteins- cell division cycle protein (CDC6)- sputum- immunocytochemistry

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Introduction

Despite recent advances in prevention, screening and treatment modalities, non-small cell lung cancer (NSCLC) remains as the leading cause of cancer related mortality worldwide, resulting in 1.6 million deaths each year and has a very poor survival rate, which has been attributed to the late diagnosis (Stewart and Christopher, 2014). Even in patients with stage I A tumors, there is a chance of recurrence in 33% of cases within 5 years after complete surgical resection (Martini et al., 1995). If early detection of lung cancer can be achieved by awareness programmes and more sensitive screening modalities, a longer average survival can definitely be offered (Ramnath et al., 2001)

Lung carcinogenesis is a multistep process characterized by the sequential accumulation of successive molecular, genetic and epigenetic abnormalities. Along with this, a series of morphological alterations of normal bronchial or bronchioloalveolar epithelium occurs, resulting in preneoplastic and neoplastic lesions. The major mucosal changes in the large airways that may precede or accompany invasive squamous cell carcinoma include hyperplasia (basal cell hyperplasia and goblet cell hyperplasia), squamous metaplasia, different grades of dysplasia (mild, moderate and severe) and carcinoma in situ (Brambilla et al., 2001). Atypical adenomatous hyperplasia is considered as a preneoplastic condition of bronchioloalveolar carcinoma, and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia is a proposed precursor of carcinoid tumors (Greenberg et al., 2002).

Sputum cytology has been recognized as the only noninvasive laboratory method of diagnosis for lung cancer, but it has very low sensitivity. The lack of adequate number of cells is one of the main reasons for its poor sensitivity (Palcic et al., 2002). Moreover, the reactive changes caused by different laboratory processing methods cause the cells to appear so atypical that distinguishing it as malignant / premalignant or benign is often difficult and can be rectified to an extent by robust techniques that can fish out the whole cell content of sputum samples. If a marker protein can be characterised to supplement the morphological evaluation, identification of malignant and premalignant cells becomes easier.

Abnormal cell proliferation, resulting from deregulation of the cell cycle is fundamental in tumorigenesis. The integrated mechanism that regulates the accurate replication of DNA and correct division of cells has a pivotal role in the neoplastic process (Evan and Vousden,

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2001; Lopez-Saez et al., 1998). Regulation of the cell cycle is a complex process and involves a wide variety of genetic mechanisms. Among them, the MCM proteins are the essential replication initiation factors. These proteins form a prereplicative complex by binding to DNA sites at which the origin recognition complex (ORC) and CDC 6 proteins have already sequentially bound. This complex acts as a license, permitting DNA replication and then dissociates irreversibly limiting replication to only once per cell cycle (Romanowski and Madine, 1997). During cell cycle, the MCM proteins form a hexameric complex, which is a key component of the prereplication complex that assembles at replication origins during early G1 phase (Kearsey et al., 1996). MCM proteins restrict DNA synthesis to occur only once per cell cycle (Todorov et al., 1995) and regulate DNA elongation. These functions of MCM proteins imply that they are correlated with cell proliferation, which has been consistently supported by experimental evidences (Stoeber et al., 2001)

The current study was aimed at characterising malignant cells and premalignant cells of sputum with Minichromosome Maintenance proteins (MCM2, MCM5) and Cell Division Cycle protein (CDC6) so that demonstration of these proteins can supplement the conventional sputum cytology. The association of these protein expressions with various clinicopathologic features were also analysed.

Materials and Methods

Subjects

The subjects for the study were selected from a cohort of 3185 patients referred from the Sanatorium for Chest Disease and Medical College Hospital from 2010-2015. All these patients were with chronic obstructive pulmonary disease and / or radiologic findings suspicious of malignancy. The subjects included both genders in the age group of 30-76 years. Among them, 90 cases had histologically proven malignant lesions which include 33 adenocarcinoma, 23 squamous cell carcinoma, 25 non small cell carcinoma and 9 small cell carcinoma. These cases were selected based on the adequacy of their sputum samples, availability of corresponding tissue samples and satisfactory clinical follow-up data. Among them, 5 cases were in stage 1, 16 cases were in stage 2, 29 cases in stage 3A and 15 cases were in stage 3B and 25 cases in stage 4.

In addition, 57 subjects were also selected based on sputum cytology. Among them 16 samples had atypical cells suspicious of malignancy, 20 samples with metaplastic cells and 21 cases with no evidence of malignancy. The clinical complaints and other clinico-pathological details were collected from patients' records and documented on a proforma. Subjects with any history of treatment for cancer or any such chronic ailments such as tuberculosis and subjects with inadequate sputum samples (number of pulmonary macrophages less than 5) were excluded from the study group. The study was approved by the Institutional review board and Human ethics committee (HEC No.31/2014) and informed consent was obtained from each participant.

Sample collection

Sputum samples were selected based on morphological evaluation and matched bronchoscopic biopsy samples were collected for comparison as a gold standard. Sputum samples were collected for 3-5 consecutive days, homogenized and processed using red solution (Cytorich® red Preservative Tripath Imaging Inc. Burlington NC, 27215, USA). The samples were vortexed with twice the volume of red solution and kept for 30 minutes. The mixed sample was then transferred to a 50 mL centrifuge tube and again vortexed, centrifuged at 600 g for 5 minutes. The pellet was re-suspended in buffer solution and again vortexed, centrifuged at 800 g for 10 minutes. The cell pellet was again vortexed and monolayer smear was prepared by using the settling chamber assembly provided by BD Surepath on pre-coated slides and the remaining samples were used for cellblock preparation (Veena et al., 2015; Sujathan et al., 2000).

Immunocytochemistry was performed in 5 micron sections from cell blocks/ monolayered smears and corresponding tissue samples according to standard ABC technique using DAB as chromogen. Sections were incubated with primary antibody for overnight and Novalink polymer was used as secondary system. Antigen retrieval was done by microwave technique in sodium citrate buffer (pH 6.0) at 700W for 15 mints. Primary antibodies were procured from Santha Cruz Laboratories (CDC 6 mouse monoclonal antibody, dilution 1:50, Positive control-tonsil tissue) and Novacastra (MCM2 and MCM5, mouse monoclonal antibody, dilution 1:25, Positive control- tonsil tissue). In monolayer smears, cell permeability was enhanced by treating with Sodium deoxycholate. Western blot analysis was performed for all the markers to assess the sensitivity of the antibodies. Immunoscoring was performed by two of the investigators independently. A repeat scoring was performed for samples having any dispute in diagnosis for sputum. Nuclear staining was considered as specific criteria for positive expression for all the proteins. The immunopositivity of tumor cells was assessed by counting a minimum of 200 cells from at least 3 representative high power fields. The H scores were then calculated as the product of intensity (0-3) and distribution (0-100 %) with H-score ranging from 0-300 and H-score 30 and above was taken as positive.

Analysis of MCM and CDC6 proteins and statistics

Statistical analysis was performed using SPSS-11 software. Sensitivity and specificity of each of the markers were assessed along with positive and negative predictive value taking histology report as gold standard. The comparison of expression pattern of all the 3 markers in cytology and histology samples was done by paired t-test.

Results

The correlation of the clinicopathological features with protein expression revealed that MCM2 proteins have significant association with tumor stage ($p = 0.04$) only (Table 1). Whereas, MCM 5 protein showed significant association with tumor stage ($p = 0.03$), histologic type

Table 1. Correlation of MCM2 and MCM5 Expression with Various Clinicopathological Features in Lung Cancer

Clinicopathological features	MCM2 protein expression status				P value	MCM5 protein expression status				P value
	Negative (37)		Positive (53)			Negative (30)		Positive (60)		
Mean Age(SD)	61.3 (7.0)		60 (10.9)		0.58	60.8 (6.3)		60.6 (10.8)		
	#	%	#	%		#	%	#	%	
Gender										
Male	35	94.6	46	86.8	0.298	29	96.7	52	86.7	0.262
Female	2	5.4	7	13.2		1	3.3	8	13.3	
Smoking status										
Smoker	22	59.5	39	73.6	0.284	22	73.3	39	65	0.57
Nonsmoker	5	13.5	3	5.7		3	10	5	8.3	
No information	10	27	11	20.8		5	16.7	16	26.7	
Histologic Type										
Adeno carcinoma	12	32.4	21	39.6	0.765	9	30	24	40	0.01
Squamous cell carcinoma	10	27	13	24.5		5	16.7	18	30.09	
Non small cell carcinoma	10	27	15	28.3		9	30	16	26.7	
Small cell carcinoma	5	13.5	4	7.5		7	23.3	2	3.3	
pStage										
I	4	10.8	1	1.9	0.04	1	3.3	4	6.7	0.03
II	6	16.2	10	18.9		5	16.7	11	18.3	
III	13	35.1	31	58.5		10	33.3	34	56.7	
IV	14	37.8	11	20.8		14	46.7	11	18.3	
pT classification										
T1	4	10.8	1	20.8	0.26	3	10	12	20	0.369
T2-T4	33	89.2	42	79.2		27	90	48	80	
pN classification										
N0	4	10.8	1	1.9	0.155	0	0	5	8.3	0.165
N1-N3	33	89.2	52	98.1		30	100	55	91.7	
pM classification										
M0	23	62.2	42	79.2	0.096	16	53.3	49	81.7	0.006
M1	14	37.8	11	20.8		14	46.7	11	18.3	
MCM5										
Negative	24	64.9	6	11.3	0.349	24	80	13	21.7	0.0001
Positive	13	35.1	47	88.7		6	20	47	78.3	

of tumor (p = 0.01) and metastasis (p = 0.006) (Table 1). Among the 3 markers, MCM2 and MCM5 had significant association (p = 0.0001) with each other and CDC6 had no significant association with clinicopathological features so it was not included.

Adenocarcinoma samples showed the highest H score of 66.7, 95 and 97.9 for MCM2, MCM5 and CDC6 respectively (Table 2). Mild, moderate or dense expression of MCM 2 (Figure 1 A-H), MCM 5 (Figure 2A-H) and CDC6 (Figure 3 A-H) were found in the nuclei of tumor cells and their expression patterns were similar in both sputum cell blocks, corresponding tissue samples as well as in the smears. The intensity of expression showed a slight variation between monolayered smears and cellblocks compared to tissue samples. The CDC6 proteins expressed weak positivity in the cytoplasm also in a few of the tumor cells. The samples designated as negative for malignancy had no expression for all the three markers, except for a few samples in which mild focal expression was noticed in a few cells. The majority of metaplastic cells also showed mild expression in a few samples. On the other hand, atypical cells showed dark or intense staining, but in a limited number of cells. The intensity of staining in different regions of same lesion also found to vary in tissue sections.

MCM2 proteins were found to have a sensitivity of 58.89 % (95% confidence Interval (CI): 48.02%-69.16%) and specificity of 73.68% (95% CI: 60.33% to 84.45%) for a diagnosis of malignancy. MCM5 proteins showed a sensitivity of 66.67% (95% CI: 55.94 to 76.2%) and a specificity of 70.18% (95 CI: 56.60% and 81.5%). CDC6 was found to have a sensitivity of 87.78% (95% CI: 79.18% to 93.7%) and specificity of 70.18% (95% C I: 56.6% to81.5%). (Table 3). The MCM2 and MCM5 proteins together had a sensitivity of 73.33% (95% CI: 62.97% to 82.10%) and a specificity of 59.56% (95% CI: 45.82% to 72.43%).The MCM2 and CDC6 proteins together had a sensitivity of 93.33% (95% CI: 86.04%

Table 2. Mean H -Score / Std.Deviation of Different Markers for Different Lung Lesions

Sample	Diagnosis	Number of Specimens	MCM 2 Mean H-core (Std.deviation)	MCM5 Mean H-score (Std.deviation)	CDC6 Mean H Score (Std.deviation)
Sputum	Negative for Malignancy	21	12 (14.5)	10.9 (14.2)	14.8 (27.6)
Sputum	Metaplastic cells	20	38.0 (51.3)	32.6 (39.2)	35.1 (40.6)
Sputum	Atypical cells	16	53.1 (47.9)	54.4 (43.0)	63.1 (45.6)
Sputum/ Cell block/ tissue	ADC	33	66.7 (54.5)	95.0 .(65.8)	97.9 (62.6)
Sputum/ Cell block/ tissue	SCC	23	52.6 (51.5)	64.1 (45.6)	79.6 (59.5)
Sputum/ Cell block/ tissue	NSCLC	25	45.2 (33.9)	68.4 (55.4)	64 (48)
Sputum/ Cell block/ tissue	SCLC	9	31.1 (32.6)	35.6 (41.6)	86.1(63.0)

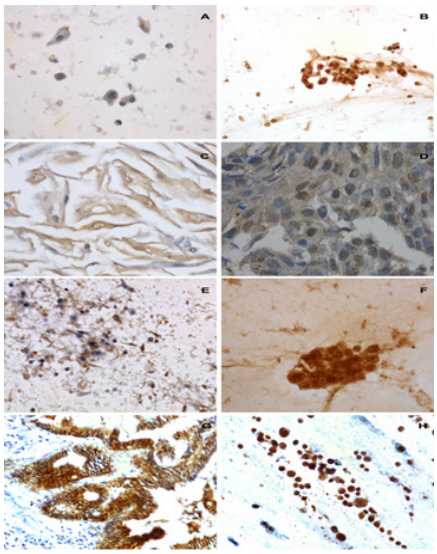

Figure 1. Figure 1A-H: A. Mild nuclear expression of MCM 2 in metaplastic cells (Sputum - 40x). B. Moderate nuclear expression of MCM 2 in atypical metaplastic cells (Sputum- 40x). C. Dense expression of MCM 2 in squamous cell carcinoma cells (Sputum cell block- 40x). D. Nuclear expression of MCM 2 in squamous cell carcinoma, occasional cells show dense staining and few cells show diffuse staining (Tissue- 40x). E. Intense nuclear expression of MCM 2 in squamous cell carcinoma cells (Sputum -40x). F. Dense nuclear expression of MCM 2 in adenocarcinoma cells (Sputum- 40x). G. Intense nuclear expression of MCM 2 in adenocarcinoma (Tissue- 40x). H. Nuclear expression of MCM 2 in small cell carcinoma cells (Sputum- 40x).

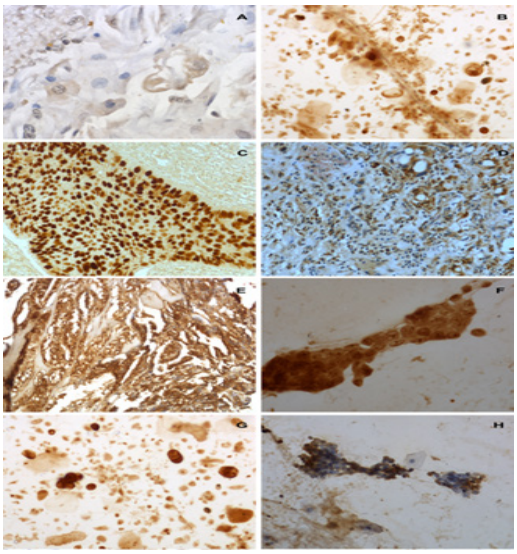

Figure 2. A-H: A. Mild nuclear expression of MCM 5 in metaplastic cells (Sputum - 40x). B. Moderate nuclear expression of MCM 5 in atypical metaplastic cells (Sputum- 40x). C. Dense nuclear expression of MCM 5 in squamous cell carcinoma cells (Tissue- 40x). D. Dense nuclear expression of MCM 5 in squamous cell carcinoma cells (Sputum- 40x). E. Intense nuclear expression of MCM 5 in adenocarcinoma cells (Tissue -40x). F. Dense nuclear expression of MCM 5 in adenocarcinoma cells (Sputum- 40x). G. Intense nuclear expression of MCM5 in non small cell carcinoma cells (Sputum- 40x). H. Diffuse nuclear expression of MCM 5 in small cell carcinoma cells (sputum- 40x).

Table 3. Sensitivity and Specificity of MCM2, MCM5 and CDC6 Based on H- Score

Markers	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predictive value (%) (95% CI)	Negative predictive value (%) (95% CI)
MCM2	58.89 (48.02-69.16)	73.68 (60.33-84.45)	77.94 (66.24-87.09)	53.16 (41.60-64.49)
MCM5	66.67 (55.94-76.25)	70.18 (56.60-81.56)	77.92 (67.02-86.58)	57.14 (44.75-68.91)
CDC6	87.78 (79.18-93.73)	70.18 (56.60-81.56)	82.29 (73.17-89.33)	78.43 (64.67-88.70)
MCM2 and MCM5	73.33 (62.97-82.10)	59.65 (45.82-72.43)	74.16 (63.79-82.86)	58.62 (44.93-71.40)
MCM2 and CDC6	93.33 (86.04-97.50)	59.65 (45.82-72.43)	78.5 (69.51-85.86)	85 (70.15-94.25)
MCM5 and CDC6	94.44 (87.5-98.15)	50.88 (37.29-64.37)	75.22 (66.22-82.86)	85.29 (68.93-94.99)
MCM5, MCM2 and CDC6	94.44 (87.50-98.15)	49.12 (35.63-62.71)	74.56 (65.55-82.25)	84.85 (68.09-94.83)

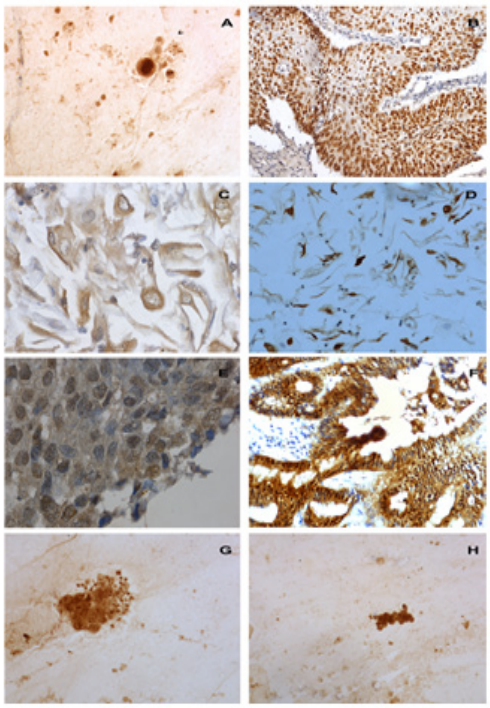

Figure 3. A-H: A. Dense nuclear expression of CDC 6 in metaplastic cells (Sputum - 40x). B. Dense nuclear expression of CDC6 in squamous cell carcinoma cells (Tissue- 40x). C. Moderate nuclear expression of CDC 6 in atypical metaplastic cells (Sputum cell block- 40x). D. Dense nuclear expression of CDC 6 in squamous cell carcinoma cells (Sputum- 40x). E. Intense and diffuse nuclear expression of CDC6 in Squamous cell carcinoma cells (Tissue -40x). F. Dense nuclear expression of CDC 6 in adenocarcinoma cells (Tissue- 40x). G. Intense nuclear expression of CDC 6 in adenocarcinoma cells (Sputum- 40x). H. Dense nuclear expression of CDC 6 in small cell carcinoma cells (sputum- 40x).

to 97.5%) and a specificity of 59.65% (95% CI: 45.82% to 72.43%). The combination of MCM5 and CDC6 together had a sensitivity of 94.44% (95% CI: 87.50% to 98.15%) and a specificity of 50.88 % (95% CI: 37.29% to 64.37%) (Table 3). All the three markers together showed a sensitivity of 94.44% (95% CI: 87.50% to 98.15%) and a specificity of 49.12% (95% CI: 35.63% to 62.71%) (Table 3).

Western blot analysis of tumor samples showed dense bands for MCM2 and mild bands for MCM5 and CDC6 in tumor samples, but normal samples had no expression for MCM5 and CDC6 and mild expression for MCM5 (Figure 4).

Discussion

MCM proteins are expressed in abundance in all phases of cell cycle, but are degraded in quiescent, senescent or differentiated cells and these proteins are present only in replicating cells (Stoeber et al., 2001; Gonzalez et al., 2005). Most of the cells in malignant and premalignant lesions are in dividing stage so there will be marked accumulation MCM proteins on it. Gonzalez and Tachbana (2005) has demonstrated a substantial increase in the number of cells expressing MCM proteins in malignant and premalignant lesions of differentiating

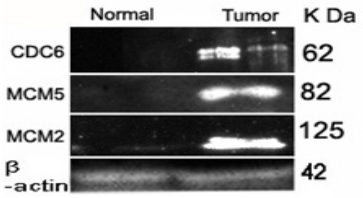

Figure 4.Western Blot Analysis of MCM 2, MCM 5 and CDC6 in Tumor Samples and Normal Samples

epithelia (Tachibana et al., 2005). bFreeman have reported that MCM proteins are more frequently detected in cells from malignant tissues than normal tissues suggesting the role of MCM proteins as a good indicator of proliferative potential of neoplastic tissues (Freeman et al., 1999). As the precancerous cells and malignant cells are proliferating continuously, expression of these proteins will be enhanced in these lesions and this will help to filter out these cells from their normal counterparts. In the present study, the MCM proteins showed weak positivity in normal and metaplastic cells, but intense expression was observed in atypical and malignant cells. All the three markers together showed a sensitivity of 94.4% for the identification of malignant cells. MCM5 and CDC6 together also had the same sensitivity suggesting that these markers can have a higher sensitivity when used in combination. It has been well demonstrated in many tumors that no particular MCM protein appears to be up-regulated in isolation, as it functions as a hexameric complex i.e. MCM 2-7 and that may be the reason for the higher sensitivity when used in combination. Studies employing these proteins in samples of uterine cervix have found to be advantageous for cervical cancer screening in low resource setting (Mukherjee et al., 2007). Moreover MCM 5 and other members of the MCM family of proteins, including MCM 2 and MCM 7 have been shown to be potentially useful markers for the detection of cervical lesions in tissue samples (Brake et al., 2003). The current study also observed immuno positivity in dysplastic cells and atypical cells compared to metaplastic cells in both sputum samples and tissue samples.

Even though analysis of the expression patterns of MCM2, MCM5 and CDC6 can be employed for assessing lung cancer risk and identifying precancerous lesions, some ambiguity exists while dealing with atypical cells or metaplastic cells. Most importantly, proliferation of cells, even though a hallmark of malignancy, also occurs as a component of inflammation and healing. Thus, metaplastic lesions usually originate in bronchial epithelium as a result of chronic irritation either by smoking or by chronic nonspecific inflammation and such lesions are present in 27% of current smokers and only 7% of former smokers (Morice et al., 1999). Furthermore, all high grade dysplasias need not necessarily lead to invasive cancer (Venmans et al., 2000) and it is often difficult to differentiate metaplastic cells with potential for progression from metaplastic cells originating due to inflammatory reactive changes.

The staining in the atypical / dysplastic areas vary greatly in intensity and pattern of staining from mild, diffuse staining to moderate, dense or intense staining.

This progressive variation can be explained by the proliferative behaviour of the lesions. As the MCM proteins are replication licensing proteins, they can be detected abundantly in continuously proliferating cells. The mean percentages of cells stained and intensity of staining were different across specimen categories and increased from normal mucosa to metaplasia and to dysplasia for all the marker proteins used in this study. In addition to their potential for detecting frank malignancy, the progressive increase in the expression pattern of these markers may also provide an estimate of the nature of potentially precancerous conditions (metaplasia and dysplasia) in the patient's airways. So it can be assumed that cells expressing intense and dense staining pattern may have the potential for proliferating into advanced lesions. This observation may be helpful in determining whether further investigations are needed for this patient to rule out any abnormal lesions in the lungs. This is very significant information for defining these proteins as markers for screening purpose. However, further studies with regular follow up and experimental demonstration in animal models are required to establish whether the progressive increase in the expression pattern correlates with potential of these lesions for progressing into malignancy.

Most of the previous studies in MCM proteins by immunohistochemistry (IHC) were performed in tissue samples and a very few reports were available analysing the role of MCM proteins as a predictive marker for lung cancer and precursor lesions in sputum samples. One of the studies detected these proteins in the peripheral blood of CML patients (Cai et al., 2015). To our knowledge this is the first study carried out in sputum samples and compared the expression pattern to that of corresponding tissue samples. The MCM proteins were abundantly present in cells at the surface of the metaplastic lung lesions which were more likely to be exfoliated into the sputum (Tan et al., 2001). So sputum samples can be an ideal platform for analysing premalignant lung lesions using MCM proteins. MCM-2 has been previously demonstrated as a sensitive marker for premalignant lung lesions in lung tissues and reported to be present in a greater percentage of cells than normal mucosa (Tan et al., 2001). It was also noticed that, majority of cells are MCM immunopositive in malignant and high grade premalignant lesions. As the dysplastic and malignant cells were continuously licensed for DNA replication and the presence of MCM proteins is manadatory for DNA replication. So these marker proteins are the ideal candidates for identifying premalignant and malignant lesions in the respiratory epithelium. As per the human protein atlas, lung cancer cells express this protein on a moderate intensity. (http://www.proteinatlas.org). Our study also supports this observation.

We have analysed the sensitivity of MCM proteins in sputum samples as a predictive marker for premalignant and malignant lesions. As MCM-positive cells usually appear at the surface of the abnormal epithelia, the cells exfoliating from this area i.e. cytology samples have higher sensitivity than histology samples (Stoeber et al., 2002; Davies et al., 2002). MCM proteins are therefore very promising biomarkers for early detection of malignancy and premalignancy in cytology samples. MCM positive cells in cytological preparations are easily identifyable due to crisp nuclear details; even at low magnification than histology samples (Williams et al., 1998; Chatrath et al., 2003) and these properties make MCMs highly reliable for detecting abnormal cells in cytological samples. This is particularly important when abnormal cells are rare, following sampling of a small lesion or only a small part of a larger lesion. MCM-based tests consequently show high sensitivity for detecting malignancy and premalignancy and can reduce the rate of false-negative results associated with conventional cytological screening (Andrew et al., 2014). The cytology samples obtained from sputum had the same expression pattern and sensitivity as that of tissue samples. So the current study suggests that these markers can be tried for population screening programme for lung cancer.

Another aspect revealed in our study was the significant association of MCM 2 with tumor stage and MCM 5 proteins with tumor stage, histological type of tumor and metastasis. This observation can be employed as a predictor of prognosis. These findings support the previous studies, which suggested that MCM7 markers can be used to predict tumor progression and prognosis of NSCLC patients (Toyonaka et al., 2011). Yang et al., (2006) also reported a significant association MCM2 expression with poor prognosis in patients with NSCLC, which suggests that identifying higher tumor proliferation may have an important role in predicting prognosis of NSCLC.

In conclusion, the present study has characterised the malignant cells, metaplastic cells and dysplastic cells of respiratory epithelium with MCM and CDC 6 proteins. This information can be utilised in routine cytopathology laboratories to supplement the conventional morphological evaluation so that the sensitivity of sputum cytology can be enhanced. The significant association of over expression of these proteins with the stage of disease and metastasis had potential application in predicting the clinical behaviour of lung lesions.

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