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ORIGINAL ARTICLE

An approach of selecting appropriate markers from the primary tumor to enable detection of circulating tumor cells in patients with non-small cell lung cancer

Ujjwala M. Warawdekar[1], Mohamed M. Sirajuddin[1], Conjeevaram S. Pramesh[2], Rajesh C. Mistry[2] §

[1]*Mulherkar Laboratory, Advanced Centre for Treatment, Research & Education in Cancer, Tata Memorial Centre, Navi Mumbai;* [2]*Division of Thoracic Surgery, Department of Surgical Oncology, Tata Memorial Hospital, Parel, Mumbai, India* §*Present address: Kokilaben Dhirubhai Ambani Hospital and Medical Research Institute Andheri, Mumbai 400053, India*

Summary

Purpose: *Circulating tumor cells (CTCs) are rare and difficult to isolate, and require selecting minimal but appropriate markers. The aim of this study was to identify markers in the primary non small cell lung cancer (NSCLC) tissue to guide isolation of CTCs from the peripheral blood of patients with lung cancer.*

Methods: *The expression of CK-19, EGFR and MUC-1 was evaluated by RT-PCR in the NSCLC tumor and paired adjacent normal tissues from 27 patients. The normal cytology, and the neoplastic and fibrotic pathology of the tissue were analyzed by histochemistry. The expression of the markers was analyzed in relation to the stage and grade of disease.*

Results: *Expression analysis showed that 42% of the tumors were positive for CK-19, whereas 85% for both EGFR and MUC-1. Ninety two percent of the tumors expressed any one marker. All (100%) adjacent normal tissues were CK-19 negative, 52% EGFR negative and 44% MUC-1 negative. CK-19 expression was specific to the tumor tissue but it was expressed by only 42% of them, manifesting a need for at least three markers to guide the detection of CTCs isolated from the peripheral blood of NSCLC patients. Histopathology demonstrated that 58% were adenocarcinomas, 35% squamous cell carcinomas and 7% had mixed pathology.*

Conclusions: *This data serves as a prelude and emphasizes the importance of selecting markers expressed in the primary tumor tissue to facilitate and enable enumeration of CTCs.*

Key words: *cytokeratin -19, EGFR, MUC-1, NSCLC markers, squamous cell NSCLC*

Introduction

Lung cancer is the leading cause of cancer deaths among males and second amongst females, worldwide. Lung cancer accounts for 13% (1.6 million) of the total new cancer cases and 18.2% [1.38 million] of the total deaths in the world [1]. In India, lung cancer is the third most common fatal cancer among males accounting for 11.4%, whereas in females the incidence is low and accounts for 4% of the total cancer deaths in patients aged between 30 and 69 years [2]. NSCLC [3] constitutes 85% of the pathological subtypes of lung cancer, with adenocarcinomas and squamous cell carcinoma being the most frequent histologies observed [4]. Most patients present with advanced stage of disease, where potentially curative treatment cannot be offered [5]. Standard treatment for advanced disease is palliative chemotherapy and more recently, targeted therapy. Conventional methods of response evaluation rely mainly on patient symptoms, which are subjective; radiological imaging, which is expensive, frequently involves ionizing radiation and is labor-intensive

Correspondence to: Dr. Ujjwala M. Warawdekar, PhD. Mulherkar Lab, Advanced Centre for Treatment, Research & Education in Cancer (ACTREC) Kharghar, Navi Mumbai, 410 208, India. Tel: +91 22 27405000, Ext: 5418, Fax: +91 22 27405085, E-mail: uwarawdekar@actrec.gov.in
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to report. There have been several studies carried out worldwide to determine the prognostic value of CTCs [6] and disseminated tumor cells (DTCs) [7]. Most of these efforts have been towards ascertaining the value of monitoring CTCs, DTCs, or both, for prognostication and disease survival. Attempts have been made to correlate changes in CTCs with progression-free (PFS) and overall survival (OS) [8,9]. However, monitoring CTCs can be a challenge as the same tumor markers or epithelial proteins are not expressed in all primary tumors of a particular malignancy. Moreover, NSCLC is heterogeneous with respect to histology and biological characteristics. The heterogeneity of marker gene expression levels in NSCLC cells limits the reliability of an assay method with a single-marker detection scheme. Cytokeratin-19 (CK-19) is a frequently used gene marker for detecting CTCs in several epithelial types of cancer [7] and has been extensively used for monitoring CTCs in NSCLC [9-14].

Our initial efforts in standardizing cell-spiking experiments for detection of CTCs using quantitative RT-PCR, with NSCLC cell lines, particularly Hop-62, showed absence of CK-19, a marker commonly used for evaluating CTCs. The absence of CK-19 in Hop-62 could be a feature of the NSCLC tissue heterogeneity [15], necessitating selection of widely expressed as well as multiple markers for evaluating CTCs in the peripheral blood of patients with NSCLC. The observation of the total absence of CK-19 in Hop-62 initiated a study where 29 NSCLC tumor tissues and their paired adjacent normal tissue samples were screened to enable the selection of appropriate markers for tracking CTCs. This is the first study to have assessed marker expression in the primary tumor tissue and adjacent normal tissues using RT-PCR, while other studies have used immunohistochemistry (IHC) to evaluate CK-19, EGFR and MUC-1 levels.

Methods

Materials

TRIzol, First strand cDNA synthesis kit, PCR kit and agarose were obtained from Invitrogen (Carlsbad, CA, USA). DNase I kit and the100bp DNA ladder were procured from Fermentas (Ontario, Canada). The primers for CK-19, EGFR and Muc-1 were custom synthesized from Sigma (Bangalore, India). Plastic ware required for tissue culture was procured from Nunc A/S (Roskilde, Denmark). All other chemicals were purchased locally and were of analytical grade.

Cell lines

The cell line A-549 was purchased from the National Centre for Cell Science, Pune, India. MCF-7 [HTB-22] and Hop-62 were available in the lab, procured for ongoing studies.

Tissues samples

NSCLC tumor samples and adjacent normal tissue were obtained from chemo-naive patients at the time of surgery, snap-frozen in liquid nitrogen, and stored at -80°C. A total of 29 paired samples were collected. The specimens were processed for RNA isolation and tissue histology. Tissue samples collected were from an approved study and all the human subjects participating in the study had given the required informed consent. The study conformed to The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Cell culture

Cell lines A-549, Hop-62 and MCF-7 were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) and maintained with 5% CO_2 at 37°C under humidified conditions in an incubator. Cells grown in 100 mm culture dishes were harvested when 80% confluent and subsequently sub-cultured for maintenance. The breast cancer cell line MCF-7 was used as a positive control for the expression of CK-19 and Muc-1. The cell line A-549 was used as a positive control for EGFR.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from the cell lines Hop-62, A-549, MCF-7 and the clinical samples - NSCLC tissue and adjacent normal tissue using Trizol in accordance with the manufacturer's instructions. RNA was quantified and the ratio determined by a spectrophotometer reading at 260 nm and 280 nm [16]. The integrity of the isolated RNA was analyzed by denaturing formaldehyde gel electrophoresis [17]. Complementary DNA (cDNA) was synthesized from 5 µg of the total treated RNA using 5U of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase in 20 µl of the total reaction volume containing 100 ng random hexamers, and 1 µl of dNTP (10 mM). Annealing was carried out at 70°C for 10 min. After cooling on ice for 1 min, 1µl of RnaseOut (40 units/µl), 2 µl of reverse transcriptase buffer (x10), 2 µl DTT (0.1mM) and 4 µl MgCl2 (25mM) were added and the mixture was incubated at 25 °C for 2 min. Finally 1 µl of Super Script II Reverse Transcriptase (50 units/µl) was added and this 19 µl mixture was incubated initially at 25°C for 10 min and subsequently at 42°C for 50 min. The reaction was terminated at 70°C for 15 min and then chilled on ice. The mixture was centrifuged briefly and 1 µL of RNaseH was added to each

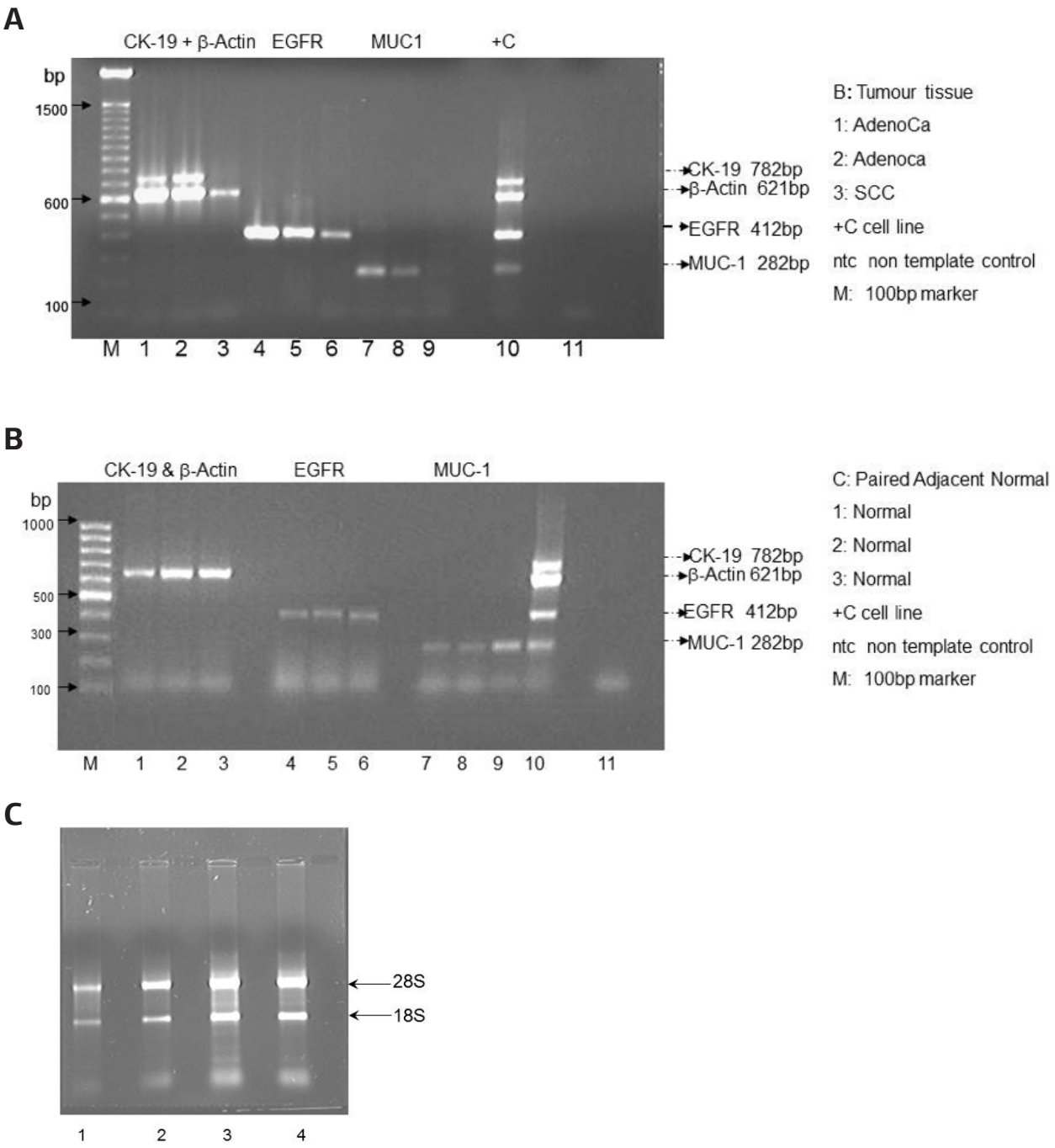

Figure 1. Marker expression in NSCLC tumor tissue and matched adjacent normal tissue. **A:** This panel shows a representative gel of tumor tissue samples with the expression of CK-19, β-actin, EGFR and MUC-1. Lanes 1, 4, 7 (adenocarcinoma), lanes 2, 5, 8 (adenocarcinoma); lanes 3, 6, 9 (squamous cell carcinoma) constitute three samples assayed for the three genes and β-actin as shown in the gel picture. Lane 10 is the control and lane 11 is the non-template control. For the positive control and the non-template control, PCRs were done as described in Methods, and the products obtained have been pooled and applied in the wells of lane 10 and lane 11 respectively, for ease of data presentation. **B:** This panel shows a gel of the paired adjacent normal tissue of the tumor samples in panel A. The normal tissue samples are PCR probed for CK-19, β-actin, EGFR and MUC-1. Lanes 1, 4, 7; lanes 2, 5, 8; lanes 3, 6, 9 constitute three paired adjacent normal tissue assayed for the three genes and β-actin as shown in the gel picture. Lane 10: control and lane 11: non-template control. For the positive control and the non-template control, PCRs were done as described in Methods, and the products obtained have been pooled and applied in the wells of lane 10 and lane 11 respectively, for ease of data presentation. **C:** Electrophoresis of RNA on a formaldehyde gel. The integrity of the RNA extracted from the tumor and paired adjacent normal tissue was determined using a denaturing formaldehyde agarose gel. Lanes 1 and 2 are two representative samples of tumor tissue RNA and lanes 3 and 4 the paired adjacent normal tissue. The picture shows the 28 S and 18 S RNA species.

tube and incubated for 20 min at 37°C. Prior to cDNA conversion, RNA was treated with DNaseI to eliminate any contaminating genomic DNA.

Amplification of cDNA by PCR

Expression of the genes encoding CK-19, EGFR, and MUC-1 was analyzed with RT-PCR using multiplexing for CK-19 and β-actin, the house-keeping gene. The primers for CK-19 [*Forward primer 5'ACAGCCACTACTACACGAC 3'; Reverse primer 5'ACTTGATGTCCATGAGCCG 3'*], EGFR [*Forward primer 5'GAAGCAACATCTCCGAAAGC 3'; Reverse primer 5' TGATAGGCACTTTGCCTCCT 3'*] and MUC1 [*forward primer 5' TGAGCGAGTACCCCACCTAC 3'; Reverse primer 5' ACCTGAGTGGAGTGGAATGG 3'*] were designed from the human genes and cDNA and for β-actin [*Forward primer 5'ACACTGTGCCCATCTACGAGG 3'; Reverse primer 5'AGGGGCCGGACTCGTCATACT 3'*]were selected as used by Wei et al. [18]. The PCR conditions were set such that CK-19 and β-actin were multiplexed and reactions for MUC-1 and EGFR were done individually. In short, the PCR programs were designed with the annealing temperature ranging from 55° C to 59° C, selected according to the Tm of the primers. The initial denaturation was for 5 min at 94°C, followed by a cycle of denaturation, annealing and extension, repeated sequentially for 35 cycles, and completion of the PCR by termination with final extension at 72°C for 10 min. The PCR products were separated with electrophoresis on agarose gels containing ethidium bromide, and visualized using an UV Gel Imager. Tissue samples that were negative for β-actin were excluded from analysis. A total of 27 samples were available for analysis.

Tissue pathology

Haematoxylin & Eosin [H&E] staining of the sections from the tumor tissue and the adjacent normal tissue was carried out as routinely done to assess the total percentage of tumor in the NSCLC samples and the absence of tumor cells in the adjacent normal tissue. These slides were examined and analyzed by a pathologist to score the percentage of tumor and normal tissue in the given specimen. All the tumor specimens had more than 70% of the tumor component and no tumor cells were present in the adjacent normal tissue in any of the samples.

Candidate markers for detection of circulating tumor cells

The criterion for a candidate marker for the detection of CTCs in the peripheral blood of a patient with NSCLC would be primarily the expression, or an overexpression of this gene in the tumor tissue. Selection of the marker would be directed by the prevalence of expression of this gene in the specific tumor type. An ideal marker would be such that it is completely absent in the adjacent normal tissue. For determining the expression of candidate markers, RNA was isolated, quantified and analyzed for integrity, as previously described.

Results

Importance of multiple markers

Representative gel pictures of RT-PCR for the selected markers and the house-keeping gene β-actin in NSCLC tumor tissue and adjacent normal tissue are depicted. Figure 1A shows the expression of CK-19 in adenocarcinomas and its absence in squamous cell carcinomas. The squamous cell carcinomas sample was a high grade, T2N0M0 tumor from a 40-year-old male. This specimen was positive for EGFR but negative for MUC-1, whereas its paired adjacent normal tissue was also negative for CK-19 but positive for MUC-1 and EGFR (Figure 1B). All the paired adjacent normal tissues (N=25) were negative for CK-19 as shown in Figure 1C. One of the samples screened was a dysplasia (data not shown) and lacked expression of CK-19, EGFR and MUC-1, whereas its paired adjacent normal tissue showed expression only of MUC-1 among the three markers. Dysplasia, being a precancerous lesion, was not included in the analysis. The distribution of marker expression in the tumor tissue and its paired adjacent normal tissue emphasizes the absence of CK-19 expression in 58% of the tumor specimens (excluding the precancerous lesion) and 100% of the paired adjacent normal tissue.

For a reliable expression analysis, it is important to assess the integrity of the isolated total RNA in both the tumor tissue and the paired adjacent normal tissue. A denaturing formaldehyde gel done to confirm the presence of the major 28S and 18S species of the ribosomal RNA (rRNA ; Figure 1C) in a 2:1 ratio also helped determine the integrity of the mRNA, which constitutes 4% of the total RNA. The characteristics of the study subjects are described in Table 1.

Marker distribution and disease characteristics

The distribution of the three markers was analyzed and correlated to the tumor tissue characteristics. Figure 2A and 2B are representative images which show the histology of the H&E stained sections of the tumor tissue specimens and adjacent normal tissue, respectively. There was a higher representation of adenocarcinomas as compared to squamous cell carcinomas in the sample size. CK-19 showed positive expression in 56% (9/16) of adenocarcinomas as compared to 20% (2/10) of squamous cell carcinomas. Adenosquamous carcinoma has been grouped with adenocarcinomas, and similarly the subtype keratinizing squamous

Table 1. Patient and disease characteristics

Characteristics	*N (%)*
Sample size	N = 29*
Gender	25
Males	25 (86.2)
Females	4 (13.8)
Age, years, median	59
Range	37-80
SE	± 2.2729
Disease stage	
I & II	18 (69.2)
III & IV	8 (30.8)
Differentiation	
Good	0
Moderate	8 (30.8)
Poor	18 (69.2)
Histology	
Adenocarcinoma	15 (55.55)
Squamous cell carcinoma	9 (33.33)
Adenosquamous carcinoma	1 (3.7)
Keratinizing squamous cell carcinoma	1 (3.7)
Dysplasia**	1 (3.7)

*Two samples, both tumor and paired adjacent normal tissue, showed degraded RNA. Therefore 27 samples were included in the study.
** Precancerous lesion
SE: standard error

cell carcinomas has been grouped with squamous cell carcinomas for analysis pertaining to expression of all three genes. Both these subtypes expressed CK-19, were high grade tumors, and were in stage IV and II, respectively. The stage I CK-19 negative tumors constituted 30% (5/16) of adenocarcinomas and 40% (4/10) of squamous cell carcinomas. Of the two stage III/high grade adenocarcinomas negative for CK-19, one was also negative for EGFR and both showed poor survival (< 18 months after surgery). Figure 2 C shows the distribution of the three markers according to the stage of disease. Seventy five percent of stage III-IV samples were positive for CK-19 expression as compared to 28% of stage I–II. The survival benefit would logically be towards the early stage of disease. However, from the 72% (N=13) of stage I-II CK-19 negative samples 38% (N=5) were high grade squamous cell carcinomas and the follow-up showed that the patients did not survive beyond one year post-surgery, whereas 23% (N=3) were low grade squamous cell carcinomas and showed a survival of 2-5 years. It needs to be mentioned that one case of low grade squamous cell carcinoma positive for CK-19 survives beyond 5 years and is due for follow-up in 6 months. The representation of stage I CK-19 negative adenocarcinomas was 38% (N=5), all these being positive for the other two markers (EGFR and MUC-1). Sixty percent of them (N=3) were high grade tumors and 2/3 did not survive beyond 10 months.

Discussion

Studies in the field of CTCs have shown that the best markers are the epithelial intermediate filament proteins from the cytokeratin family [9,19] and, also membrane proteins like EGFR family proteins [20] and the mucin family proteins [21,22]. The clinical relevance of the detection of CTCs would be determined by the presence of a viable circulating cell which can be assessed by isolating the total RNA and assaying quantitatively for the expression of the selected candidate markers in the enriched CTCs component. Also its visual characterization based on the presence of these markers and the nucleus, with immunocytochemistry and imaging techniques will enable confirmation of the CTC identity.

We were able to isolate good quality non-degraded RNA from 27 tumor tissue samples (N=29); two samples showed fully degraded RNA in both the tumor and adjacent normal tissue and were not included in the study. Degradation of the isolated RNA is usually observed on the gel as a reduction in the 2:1 ratio of the rRNA species, or as smear which would reflect total degradation. Some studies report that, despite a reduced ratio of the 28S:18S, the mRNA is not drastically affected and expression studies can be done successfully with such a total RNA isolate [23-25]. Our, as well as other studies, have shown the necessity to assay for multiple markers, mainly because the expression level of the same marker may vary in different tissue samples due to histological differences and the extent of disease. Reports of heterogeneous gene expression in isolated CTCs further complicate, increase the difficulty of detecting CTCs [27] and confirm the need to assay multiple markers. As described, we have selected CK-19, EGFR and MUC-1 and assayed their expression in the tumor tissue as well as the paired adjacent normal tissue.

In the context of marker selection to track CTCs, a study by Liu et al. [27] which reported a 58% positive detection rate, and an older study by Peck et al. [28] which obtained a 40% positive detection rate in the CTCs from the peripheral

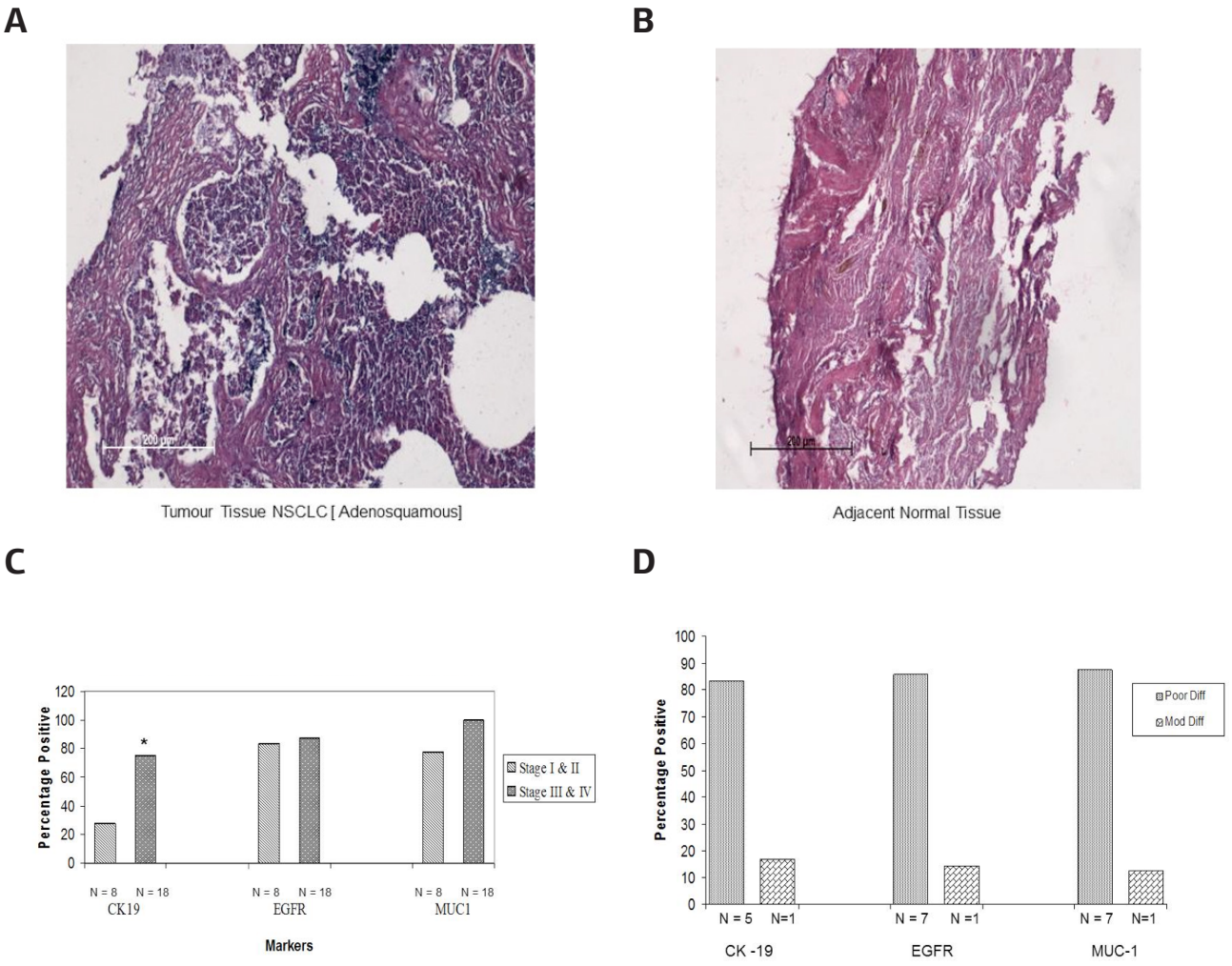

Figure 2. A: Tissue section of high grade adenosquamous carcinoma (stage IV disease). **B:** Tissue section of adjacent normal tissue. Both sections were stained with hematoxylin and eosin at x40 magnification. Scale bar=200μm. **C:** Positive expression of markers according to disease stage. The lower disease stages (stage I & II) are grouped together (N=18) and also similarly stage III & IV (N=8) are grouped together. The histogram shows a comparison of percentage of tissue samples positive for expression of each gene, according to the groups as described above. The three genes shown here are CK-19, EGFR and MUC-1. *p=0.0384. **D:** Expression of markers according to the disease grade in stage III and IV. The two major grades present in the tumor samples are the moderately differentiated and the poorly differentiated. The histogram shows a comparison of percentage of tissue samples positive for expression of each gene, according to the grades as described above. Statistical analysis was not possible (moderately differentiate group, N=1). The three genes shown here are CK-19, EGFR and MUC-1

blood of patients with lung cancer, when only CK-19 was used as a marker, coincides with our expression data obtained from the NSCLC tissue samples and is possibly due to the absence of expression of CK-19 in the tissue of origin, i.e. the NSCLC tumor tissue. Besides, there are reports in the literature on the presence or absence of CK-19 expression and the malignant nature of tumors. Kanaji et al. [29] have shown that in NSCLC cell lines, when sublines were experimentally selected for invasive properties, there was an associated lowering of CK-19 expression. Also, when these same subline cells were transfected with CK-19, the presence of exogenous CK-19 decreased their invasiveness. The loss of expression of cytokeratin in the tumor cells probably is due to the acquisition of mesenchymal molecular characteristics and the associated invasiveness [30]. Nevertheless, these observations reiterate the validity of assaying appropriate and multiple markers.

Our study is the first to report assessing the RNA for CK-19 in tumor tissue and confirms earli-

er observations of lowered protein staining. It has been reported that CK-19 weak staining by IHC in squamous cell carcinoma correlates with poor survival [31,32].

A correlation of marker detection with disease shows that, in terms of disease stages, the number of tumor tissue samples with CK-19 expression was higher in patients in stage III and IV than in stage II and I (p=0.0384). This difference was not observed with EGFR and MUC-1 expression. Though the numbers are small, in stage III and IV the distribution of positive expression of CK-19, EGFR and MUC-1 was higher in the higher disease grades as seen in Figure 2D. For advanced stages, histologically all were adenocarcinomas (8/8) and constituted 50% (8/16) of the sample size with the expression of all 3 markers obtained in a higher percentage of samples; 75% (6/8) for CK-19, 87.5% (7/8) for EGFR and 100% (8/8) for MUC-1. This data shows that at an advanced stage, 75% of the samples showed more than two markers, whereas in stage II around 56% showed more than two markers and in stage I only two markers were expressed in 77% (7/9) of the samples, with complete absence of one marker (CK-19).

Our study has some limitations, most notably the small sample size. So, even though the numbers are low, the presence of CK-19 expression and the associated state of differentiation possibly determines the survival benefit. Kosacka and Jankowska [32] have also shown that a strong CK-19 protein staining in the tumor tissue correlates with reduced CYFRA 21-1 levels in the serum.

Summing up, these observations unravel questions pertaining to the intermediate filament metabolism in the NSCLC tissue and, whether the loss of CK-19 is a cause or a consequence of the aggressiveness of the disease.

Also, the loss of CK-19 with an associated expression of EGFR in stage I disease probably contributes to the invasiveness of the tumor and poor survival of the patient [33,34].

EGFR has been widely reported to assess CTCs [6,35-37] and was explored as a candidate marker suitable for tracking CTCs. Though the EGFR gene has been documented to be overexpressed in NSCLC [38] and other cancers [39,40], it was important to confirm and assess its expression in the NSCLC tumor tissue prior to its selection as a marker to track CTCs. The expression of EGFR is also associated with epithelial mesenchymal transition (EMT), is linked with invasive tumor characteristics and documented as a marker for EMT [33,34]. Our summarized results show that EGFR expression was present in 85% of the tumor tissue (N=22/26) and 48% (N=12/25) of the adjacent normal tissue. According to histological type 94% (15/16) of adenocarcinomas as compared to 70% (7/10) of squamous cell carcinomas express the EGFR gene. The samples negative for EGFR constitute 15% (4/26) of the total, and one of them was a high grade stage III adenocarcinoma negative for both EGFR and CK-19.

MUC-1 is a widely expressed antigen in lung cancer and has been extensively explored as a marker for disease prognosis [41] as well as for therapeutic strategies [42-44]. This study, initiated in early 2006, probed the merit of using MUC-1 as a marker to track CTCs and subsequently the AdnaTest Breast Cancer for CTCs has shown that MUC-1 can be used as one of the markers to assess the presence of CTCs [45]. Our data is similar to a recent report of MUC-1 expression according to histological type: 100% (16/16) of adenocarcinomas expressed the MUC-1 gene as compared to 60% (6/10) of squamous cell carcinomas [46]. It has been shown that the presence of MUC-1 on the cell surface contributes to the invasiveness of the disease by mechanisms that facilitate adhesion of CTCs to the microvasculature leading to intravasation and probable promotion of metastases [47,48].

This study has emphasized the importance of selecting multiple markers for the enumeration of CTCs from peripheral blood by highlighting differences observed in the expression of commonly used epithelial markers in the primary NSCLC tissue and, particularly, the necessity of multiple markers when the disease has not progressed and is at an early stage.

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