Pathol. Oncol. Res. (2012) 18:783–792
DOI 10.1007/s12253-011-9485-5

RESEARCH

# *RUNX3* Downregulation in Human Lung Adenocarcinoma is Independent of *p53*, *EGFR* or *KRAS* Status

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Received: 30 November 2010 / Accepted: 24 November 2011 / Published online: 24 June 2012


**Abstract** *RUNX3* aberrations play a pivotal role in the oncogenesis of breast, gastric, colon, skin and lung tissues. The aim of this study was to characterize further the expression of RUNX3 in lung cancers. To achieve this, a lung cancer tissue microarray (TMA), frozen lung cancer tissues and lung cell lines were examined for RUNX3 expression by immunohistochemistry, while the TMA was also examined for EGFR and p53 expression. RUNX3 promoter methylation status, and EGFR and KRAS mutation status were also investigated. Inactivation of RUNX3 was observed in 70% of the adenocarcinoma samples, and this was associated with promoter hypermethylation but not biased to *EGFR/KRAS* mutations. Our results suggest a central role of RUNX3 downregulation in pulmonary adenocarcinoma, which may not be dependent of other established cancer-causing pathways and may have important diagnostic and screening implications.

**Keywords** EGFR · KRAS · Lung adenocarcinoma · p53 · Promoter hypermethylation · RUNX3

## Introduction

Lung cancer is the most frequently occurring cancer worldwide, with the highest mortality rate. Approximately 90% of cases are due to exposure to cigarette smoke [1, 2] or environmental factors [3, 4]. Non-small cell lung cancers (NSCLC) represent 80% of lung cancers [5] comprising

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adenocarcinomas (ADC), squamous cell carcinomas (SCC) and large cell lung cancer. While genetic dysregulations in lung cancer have been observed [5–14], a clear understanding of its molecular pathogenesis is elusive [15], despite successes in other cancers [16, 17].

Molecular analyses of lung cancer have shown an association with the deregulation of certain genes such as p16[5, 6, 13], cytokinesis-Blocked Micronucleus Assay[8], Dkk-1 [14], cyclin E, VEGF-A, p27 and ß-catenin[5], p53, RASFF1, FHIT[13] and genes involved in the metabolism of tobacco smoke carcinogens such as CYP2D6[7, 12] and CYP1A1[9].; these associations have been postulated in both sporadic and familial lung cancer types[10, 11]. However, a reliable biomarker of lung cancer is elusive[15, 18], and indeed clear understanding of the molecular pathogenesis of this important malignancy is still lacking.

Runt-related transcription factor 3 (RUNX3, in chromosome 1p36.1), belongs to the RUNX family of transcription factor [19], which share a 128 amino acid highly conserved runt domain [20]. RUNX3 is an important target of the transforming growth factor (TGF-β) signaling pathway [21] and is an inhibitor of the Wnt pathway [22, 23]. It possesses two promoter regions separated by a large intron, with a 3478 bp CpG island in the proximal promoter [24]. Recent work revealed that the central role RUNX3 plays in tumour formation maybe due its developmental significance [25, 26]. Previously we established that RUNX3 dysregulation plays a leading role in the oncogenesis of breast[27, 28], gastric[29–31], colon[22] and skin[32] tissue types. Hypermethylation of the *RUNX3* proximal promoter CpG island is believed to be a major causation of silenced *RUNX3* expression and is linked to oncogensis[24], a direct correlation also reported in lung cancer [31, 33, 34].

p53 is a highly regulated transcription factor which plays a major role in protecting organisms against cancer formation [35]. p53 aberrations have been established to play a role in NSCLC and may be a poor prognostic indicator[36–38]. Another crucial gene in NSCLC formation is EGFR of which mutations characterize the lung cancer response of tyrosine kinase inhibitors[16, 39, 40]. *EGFR* and *KRAS* mutations are generally exclusive in lung ADC[41, 42] but rarely occur in the same case[43], and are associated with inverse prognosis and drug response[44].

Little work has been performed to compare RUNX3 to EGFR and KRAS status. KRAS mutations and RUNX3 expression was found to be mutually exclusive in a mouse RUNX3 knockout model[26]. Interestingly Tsunematsu et al. [45] found that EGF, a ligand of EGFR significantly enhanced RUNX3 expression in HOC621 cells, while Yanagawa et al. found an inverse correlation between EGFR mutation and RUNX3 methylation in Lung adenocarcinomas[46].

The aims of this study are to elucidate RUNX3 status in different lung cancer subtypes and its association with EGFR and p53 IHC expression and *KRAS* and *EGFR* mutations in ADC subtypes.

## Materials and Methods

This study was approved by our institutional ethics committee (NUHS DSRB Domain B/09/140).

### Tissue Microarray (TMA) Construction

Formalin-fixed, paraffin embedded (FFPE) lung cancer blocks were obtained from the Department of Pathology, National University Hospital (Singapore) and reviewed by a pathologist (MEN), for diagnostic confirmation. Two lung TMA blocks (TMA1 and TMA2) were constructed as described previously[32, 47], TMA1 consisted of thirty-one ADCs, sixteen squamous cell carcinomas (SCC), and twenty-eight corresponding normal lung parenchymal samples derived from tissue adjacent to several of the adenocarcinomas. TMA2 consisted of seventy-four other ADC and thirty-four other SCC samples. A basal cell carcinoma sample was also selected to act as a control for RUNX3 expression as established in our previous work[32].

### Frozen Lung Tissue

Four frozen ADC samples with matching non-tumour and a single non-tumour sample were obtained from the NUH-NUS tissue repository, while five SCC samples were obtained from the National Cancer Centre Singapore. Each sample was divided into portions DNA extraction and formalin-fixation and paraffin-embedding. Fixation was performed for 3 h in 10% neutral buffered formalin (SIGMA diagnostics), followed by dehydration with increasing concentrations of ethanol, xylene and paraffin and finally embedded into paraffin blocks.

### Cell Lines

Carcinoma cell lines NCI-H358 (CRL-5807), NCI-H1650 (CRL-5883) and NCI-H1975 (CRL-5908), SCC cell line NCI-H520 (HTB-182) and normal bronchus cell line NL20 (CRL-2503) were obtained from ATCC (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum and incubated in 5% $CO_2$ at 37°C. Cells were grown to confluence and pelleted for DNA extraction and paraffin embedding.

### Sectioning and RUNX3, EGFR and p53 Immunohistochemistry

FFPE blocks were sectioned at 4 μm and mounted on MAS-coated microslide glass (Superfrost; Matsunami, Tokyo,

**Table 1** Primer sequences used in Experiments

| Specific test | Primer Set | Forward Sequence | Reverse Sequence | Amplicon Size | PCR Annealing Temperature (°C) |
|---|---|---|---|---|---|
| *RUNX3* Promoter Hypermethylation Status | Methylated | ATA ATA GCG GTC GTT AGG GCG TCG | GCT TCT ACT TTC CCG CTT CTC GCG | 115 | 58 |
| | Unmethylated | ATA ATA GTG GTT GTT AGG GTG TTG | ACT TCT ACT TTC CCA CTT CTC ACA | 115 | 58 |
| *KRAS* mutation analysis | Exon2 | TCA TTA TTT TTA TTA TAA GGC CTG CTG AA | AAA GAC TGG TCC TGC ACC AGT A | 189 | 61 |
| | Exon3 | GAA GTA AAA GGT GCA CTG TAA TAA T | CAA TTT AAA CCC ACC TAT AAT GGT | 243 | 57 |
| *EGFR* mutation analysis | Exon 18 | GCT GAG GTG ACC CTT GTC TC | ACA GCT TGC AAG GAC TCT GG | 246 | 60 |
| | Exon 19 | AGC ATG TGG CAC CAT CTC | AGA CAT GAG AAA AGG TGG | 225 | 60 |
| | Exon 20 | CAT GTG CCC CTC CTT CTG | CTA TCC CAG GAG CGC AGA | 308 | 60 |
| | Exon 21 | AAT TCG GAT GCA GAG CTT | TAC AGC TAG TGG GAA GGC | 295 | 60 |

Table 1 shows all the PCR primer pairs that were utilized in the experiments. For the methylation status, bisulfite treatment was first performed; this was followed by two PCRs with different primers, one to detect the presence of a methylated sequence and the other to detect an unmethylated sequence. Two mutations were targeted for KRAS analysis while four mutations were targeted for EGFR analysis

Japan), deparaffinized with xylene and rehydrated with ethanol and finally water. RUNX3 antigen retrieval was performed using Target Retrieval Solution, Low pH (Dako, Denmark) and pressure cooking (T/T Mega, Milestone) at 120°C for 5 min. Sections were then incubated with Peroxidase Block (Dako) and serum-free blocking solution (Dako). RUNX3 Clone 6E9 was used for staining of the TMA [1, 2] slides and the BCC control slide, while clone 1E10 was used for staining the frozen lung samples and the cell pellets, both at 0.16 μg/mL [29, 48] and incubated overnight at room temperature. EGFR antigen retrieval was performed using 40 μg/ml proteinase K (Roche, Mannheim, Germany) for 15 min at 37°C on TMA1. Anti-EGFR clone H11 (Dako, Danmark) was incubated at room temperature for 1 h at 1/400 dilution. p53 antigen retrieval was performed in Target Retrieval Solution, Low pH (Dako, Denmark) and heating at 95°C for 20 min also performed on TMA1. The primary antibody, clone DO-7 was incubated at 1/200 dilution for 1 h. For all antibodies, the secondary antibody was allowed to bind for 30 min at room temperature and detected by a peroxidase-3,3'-diaminobenzidine–based detection system (EnVision+kit, DAKO).

## Scoring of IHC Protein Expressions

The IHC for TMA1, the frozen tissues and cell lines were scored by MFMO and MST, while TMA2 was scored by MFMO, BT and MST. RUNX3 was scored according to the 0 to 3 intensity classification, then divided into negative (0) and positive [1–3]. EGFR was scored positive with the presence of membranous staining in greater than 10% of the tumour cells. p53 were classified as positive for p53 protein expression if 5% or more of the tumour cells expressed nuclear p53 [49].

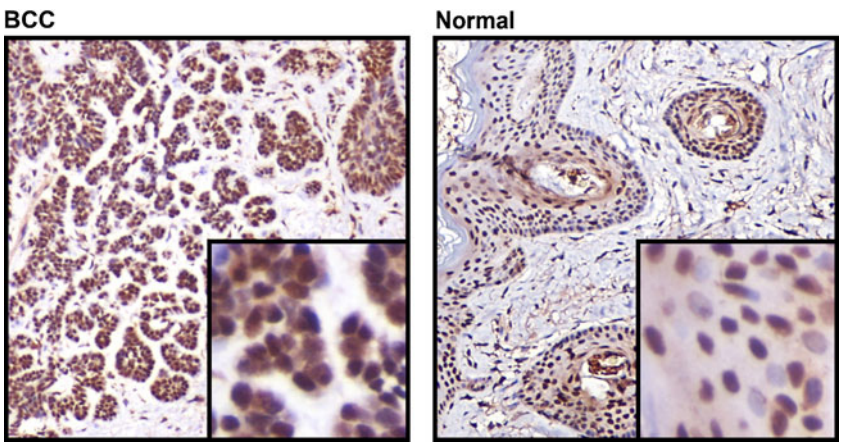


**Fig. 1** RUNX3 staining in BCC control slide. Nuclear RUNX3 expression in the BCC is strong and uniform while in the normal it is moderate and frequent

### RUNX3 Methylation Status in FFPE, Frozen Tissues and Cell Lines

DNA was extracted from the lung tissues and cells using the Puregene Cell and Tissue Kit (Gentra Systems, Minneapolis, MN). For the methylation studies, DNA was modified by sodium bisulfite (EZ DNA Methylation-Gold kit,Zymo Research Corporation, CA) followed by methylation-specific PCR[50]. A total of 4 μl of DNA was added to 2.5 μl 10× PCR buffer with 15 mM MgCl2, 10 μmol/l nucleotide triphosphates, 1 unit FastStart Taq Polymerase (Roche, Mannheim, Germany) and 10 μmol/L forward and reverse primers (1st Base Asia) and was run at 95°C for 6 min, followed by 40 cycles of 95°C for 30s, primer annealing (Table 1) for 30 s and 72°C for 30 s. A primer pair each was used for detection of the methylated and unmethylated sequences [24] as shown in Table 1. PCR products were visualized using the eGene HDA-GT12TM Genetic Analyzer (Qiagen, Irvine, CA).

### KRAS and EGFR Mutation Analysis

Mutation analysis was performed for *KRAS* exon 2 codons 12 and 13[51] and EGFR exon 18, 19, 20 and 21[52], using the five cell lines and a selection of nine ADC samples from the TMA. The primers and annealing temperatures are shown in Table 1. The PCR reaction was carried out in 25ul of 1X PCR buffer, 2.0 mM MgCl2, 0.2 mM dNTPs, 10pmol for each forward and reverse primers, 1U of Taq polymerase (AmpliTaq Gold, Applied Biosystems) and 100 ng of DNA template. Amplification was carried out for 4 mins at 95°C followed by 40 cycles of at 95°C for 1 min, primer annealing (Table 1) for 1 min, 72°C for 1 min and finally 72°C for 3 min. Sequencing was performed using the Big Dye V3.1 Terminator Kit (Applied Biosystems, Foster City, CA, USA), on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

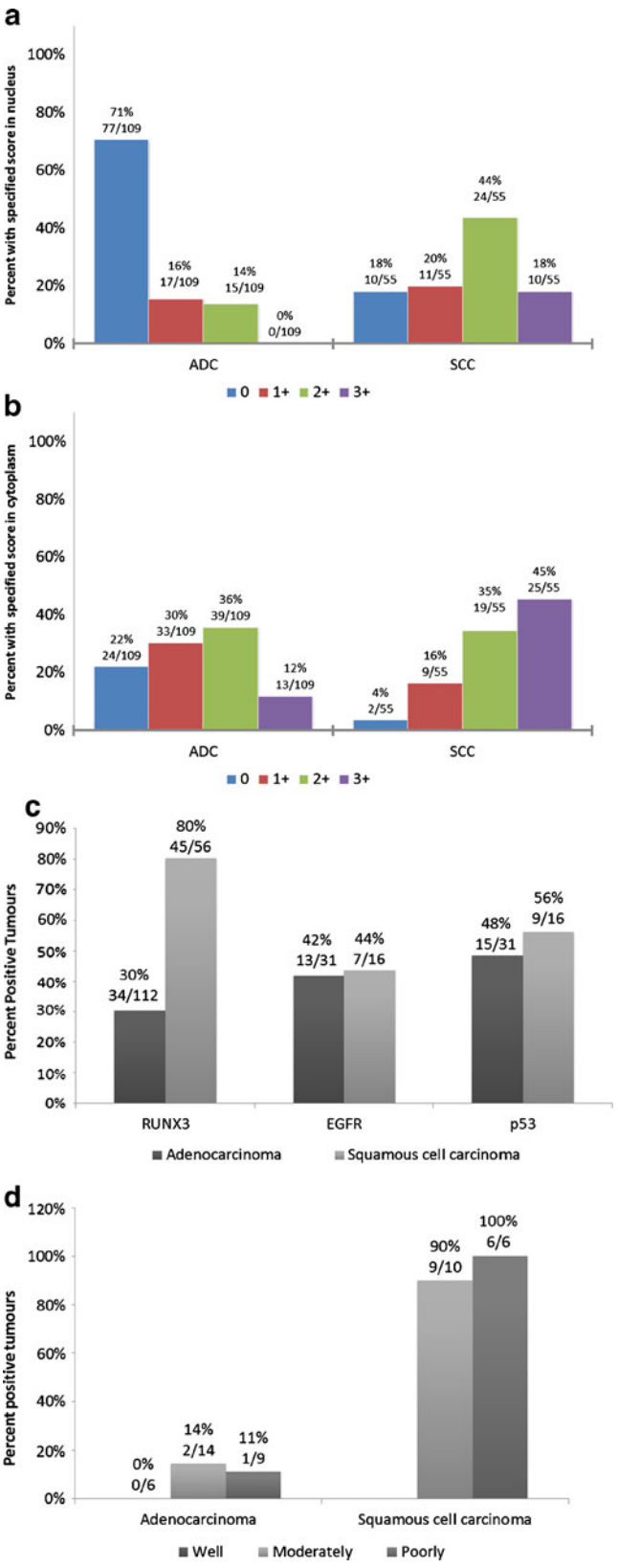


**Fig. 2** RUNX3 Staining localization in ADC and SCC. **a** Distribution of RUNX3 staining intensities observed in the nucleus of tumours. **b** Distribution of RUNX3 staining intensities observed in the cytoplasm of tumours. The full range of staining intensities based on cellular localization can be seen from panels a and b. The nuclear intensity in the ADCs are largely negative, with just a few cases in 1+ and 2+, with no 3+ staining, cytoplasmic staining is mainly positive. In the SCCs, nuclear staining in seen mainly as 1+,2+ or 3+while cytoplasmic staining is mainly 2+ and 3+. **c** RUNX3 (TMA1 and 2, frozen and cell lines), p53 (TMA1) and EGFR (TMA1) staining positivity in ADC and SCC. The localization patterns of staining for the two cancer types are clearly shown in the diagram above. Each bar represents the percentage of cases of the specific lung cancer subtype that is positive for expression of the three antibodies. SCC shows predominantly nuclear RUNX3 positivity and thus active RUNX3, while ADC presents a much larger percentage of nuclear negativity, thus inactive RUNX3. There is no distinguishing trend seen in the expressions of EGFR and p53 in the different cancer subtypes. **d** Percent positive tumours based on differentiation status. This panel shows the active RUNX3 from the TMA divided according to differentiation status. The ADCs are represented on the left, while the SCC are represented on the right. The ADC cases are made up of three differentiation levels and two levels for the SCC. As can be seen, the percent positive tumour does not vary according to differentiation status within a tumour subtype

### Statistical Analysis

The measure of association between ADC and SCC positivity (RUNX3, EGFR and p53); RUNX3 positivity against EGFR and p53 positivity; Methylation status to cancer subtype; EGFR IHC positivity to EGFR mutation; RUNX3 IHC expression to EGFR and KRAS mutation; and SCC positivity to differentiation level was performed using the Fisher's exact test. For comparison between RUNX3 nuclear score and cytoplasmic score between the cancer subtypes; and ADC positivity against differentiation level, Pearson's Chi-square test was carried out.

For all statistical tests, IBM SPSS Statistics (version 19.0, IBM, New York) was used.

## Results

### RUNX3 Expression in BCC Control

As with our previous study, RUNX3 protein is expressed in normal skin with distinct nuclear, moderate positivity in approximately 75% of epidermal cells present in all the epidermal layers (Fig. 1), while the BCC region showed strong and uniform (3+) nuclear expression of RUNX3.

### RUNX3, EGFR and p53 Immunohistochemistry

Having established that RUNX3 expression is in accordance with previous observations, we then went on to study the possible involvement of RUNX3 in the development of human lung cancers and its relation to EGFR and p53 protein expression. Two TMAs (TMA1 and TMA2, comprising one hundred and fifty-five lung tumour samples and twenty seven corresponding normal lung parenchymal samples), fourteen frozen lung samples and five cell lines were studied for RUNX3 expression by IHC. TMA1 was also tested for EGFR and p53 by IHC. The IHC results are summarized in Fig. 2, while Fig. 3 shows an archetypal staining pattern from the different cancer subtypes. IHC results from the cell lines are shown in Table 2. Figure 2 Panels A and B look specifically at RUNX3 staining, in the nucleus (A) and cytoplasm (B) in each of the tumour subtypes by showing the percentage of cases with at staining intensity level. The ADCs predominantly have a 0 score (underexpressed compared to the normal alveolar epithelium)

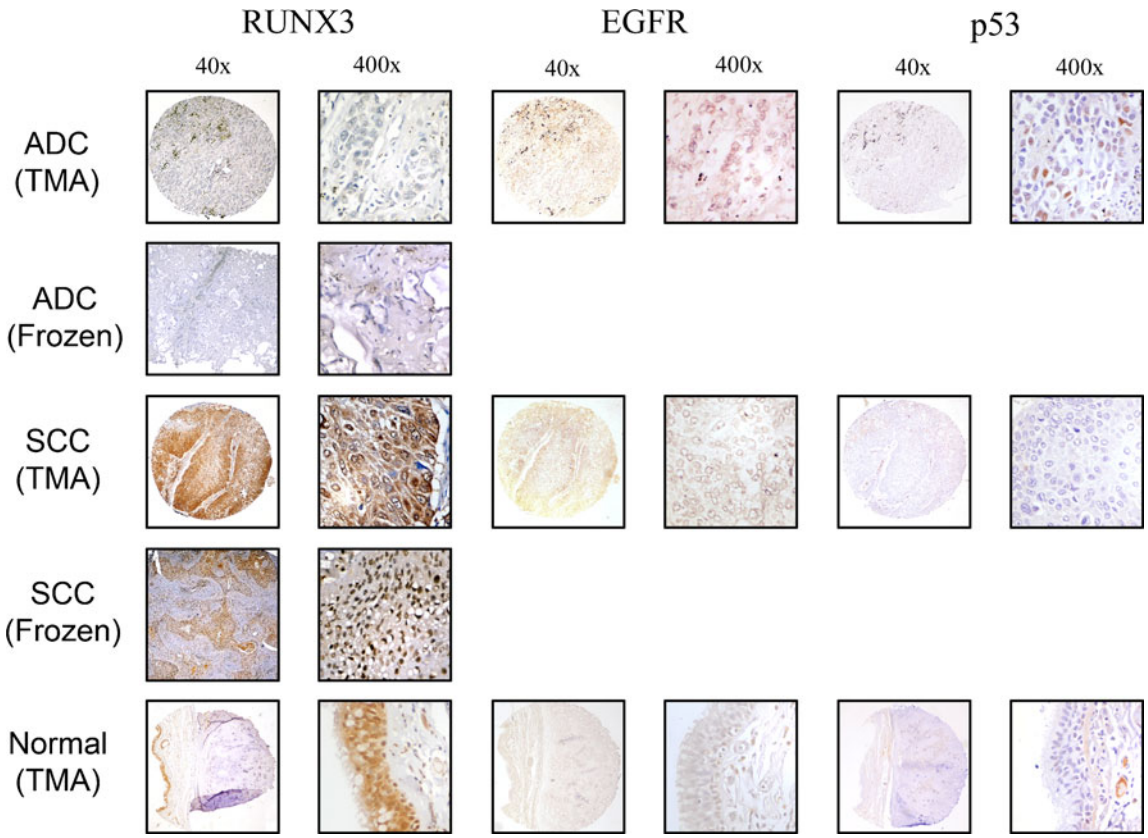


**Fig. 3** Examples of staining profiles seen in the TMA and frozen tissues. This figure represents the general staining profiles from the three different IHC markers, namely, RUNX3, EGFR and p53 in ADC from the TMA, frozen tissue ADC, SCC from the TMA, frozen tissue ADC and normal lung from the TMA. EGFR staining was only performed on the TMA samples and not the frozen samples. Images 40x and 400x magnification

**Table 2** Results observed in cell lines

| Cell Line | Cancer type | KRAS mutations | | EGFR mutations | | | | Methylation Status | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Codon 12 | Codon 13 | Exon18 | Exon19 | Exon20 | Exon21 | Methylated | unmethylated | RUNX3 +/− |
| CRL-5883 | Lung adenocarcinoma | WT | WT | WT | del 745-750 | WT | WT | - | + | - |
| CRL-5807 | Lung adenocarcinoma | 12>TGT | WT | WT | WT | WT | WT | + | - | + |
| CRL-5908 | Lung adenocarcinoma | WT | WT | WT | WT | WT | L858R | + | + | + |
| CRL-2503 | Normal Bronchus | WT | WT | WT | WT | WT | WT | + | - | - |
| HTB-182 | Squamous cell carcinoma | WT | WT | WT | WT | WT | WT | - | + | - |

Table 2 shows the results obtained from the lung cell lines. The cases are arranged according to cancer type. The KRAS and EGFR mutations are indicated. RUNX3 expression by IHC was seen in all three adenocarcinoma cases, while methylation was observed in two cases. Methylation was not observed in the squamous cell carcinoma. *WT* Wildtype (no mutation)

in the nucleus while the SCC are predominantly within the 1+−3+ scores (Pearson's Chi-square of 52.9, $p<0.0001$). The cytoplasmic staining is also significantly different, ADC expression is mainly 0-2+, while the SCC is skewed towards higher intensities (Pearson's Chi-square of 28.3, $p<0.0001$). Figure 2 panel C shows the percent of tumours positive for each of the studied markers according to the criteria described in the methods. Specifically for RUNX3, this indicates an active state, when it is localized in the nucleus, and an inactive state, when no nuclear expression is observed[27]. As can be seen, RUNX3 is downregulated and/or mislocalised (inactive) in ADCs (70%), while in the SCC RUNX3 seems less affected, i.e. normally expressed and active in 80% of cases. There was a statistically significant association between the RUNX3 positivity and lung cancer subtype ($p<0.0001$). No obvious distinction could be made with expression of EGFR and p53 according to the lung cancer subtypes (EGFR $p=1.000$, p53 $p=0.760$) (panel C). When analysed according to lung cancer subtype, no statistical association was observed between RUNX3 and EGFR positivity (ADC $p=0.58$, SCC $p=0.438$) or RUNX3 and p53 (ADC $p=1.000$, SCC $p=1.000$).

## RUNX3 Promoter Hypermethylation

Methylation-specific PCR was performed to investigate the involvement of gene silencing by methylation in RUNX3 underexpression in the lung ADC, a phenomenon we have previous shown in other cancer types [20, 27, 28, 32]. This involved carrying out two PCRs per samples, one to detect the methylated promoter and another for the unmethylated. The methylation status for the cell lines samples are summarized in Table 2 and frozen lung samples in Table 3. Figure 4 illustrates the results from the frozen lung samples. Unmethylated bands were observed for all the tissue samples but in only three of the five lung cell lines. As for the methylated bands, the results for the ADCs varied, three of four (75%) frozen lung samples showed methylated DNA, out of these, 2 corresponding normals were methylated, in addition, one normal (adjacent to tumour) without corresponding tumour sample showed methylation as well. In the cell lines, two if the three ADCs showed methylated bands, one of these also showed an unmethylated band. Methylation was also seen in the normal bronchus cell line. Methylated bands were seen in the SCCs for both the frozen samples and the single SCC cell line. Our results indicate that ADC exhibit RUNX promoter methylation while SCC lack methylation, and that this is statistical association ($p=0.005$).

**Table 3** RUNX3 protein expression and methylation status in the frozen lung samples

| | IHC Expression | Methylation Status | |
|---|---|---|---|
| Tumour | RUNX3 +/− | Methylated | unmethylated |
| AD1 | + | - | + |
| AD2 | - | + | + |
| AD3 | - | + | + |
| AD4 | + | + | + |
| N1 | - | - | + |
| N2 | - | - | + |
| N3 | - | + | + |
| N4 | + | + | + |
| N5 | - | + | + |
| SCC1 | + | - | + |
| SCC2 | + | - | + |
| SCC3 | - | - | + |
| SCC4 | + | - | + |
| SCC5 | + | - | + |

*AD* Adenocarcinoma, *N* Normal, *SCC* Squamous cell carcinoma

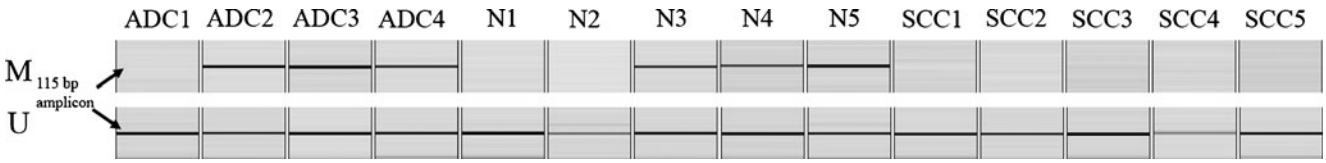


**Fig. 4** Diagram of the methylation results from the lung frozen samples. The above diagram shows the methylation status from all the frozen lung samples. A band along the M row indicates the presence of methylated promoter region, while a band along the U row represents an unmethylated promoter region. The adenocarcinoma samples (ADC1 to ADC4) have corresponding normal samples (N1 to N4). N5 is a normal lung sample that was adjacent to an adenocarcinoma tumour that was not included in this study. SCC1 to SCC4 represent the SCC samples. As can be seen, all samples exhibit an unmethylated band. While SCCs are negative for methylation, there is variability of methylation status in the adenocarcinoma and normal cases. M- Methylated primer set, U- Unmethylated primer set

### EGFR and KRAS Mutation Status

There is significant interest in the *EGFR* and *KRAS* mutation status of ADCs due to biological and diagnostic implications. We sought to investigate whether there may be a relationship between these two important biomarkers the phenomenon of RUNX3 downregulation in ADC by selecting a representative portion of RUNX3 negative ADCs of all differentiation levels in the TMAs and the cell lines. The mutation status of *EGFR* and *KRAS* are shown in Table 2 for the 5 cell lines and Table 4 for the TMA samples. The selected TMA cases (Table 4) provide a range of differentiation levels of the ADCs with all the cases exhibiting RUNX3 downregulation. *KRAS* mutations were observed in six out of fourteen cases (43%), while *EGFR* mutations were observed in five (36%), with one of the cases exhibiting both mutations, an overall mutation rate of 71%, all of which have been established in lung cancers[51, 52]. The absence of correlation between RUNX3 downregulation and *EGFR (p=0.505)* or *KRAS (p=1.000)* mutations indicates that RUNX3 observations are biologically unbiased to either one of these pathways. A comparison of EGFR mutation and EGFR IHC expression levels did not reveal any significant relationship ($p$=0.444).

## Discussion

Previous work has shown that the RUNX3 protein is only active when localized in the nucleus [27, 29], fulfilling its role as a transcription factor. Therefore, an analysis of subcellular localization gives an indication into the activity of RUNX3 in lung cancer subtypes. There is an obvious difference between the activity of RUNX3 in ADC and SCC (Fig. 2 Panel A). The absence of active RUNX3 in the ADCs strongly suggests that the aberrant downregulation of RUNX3 (70% of cases) is associated with the development of these tumours, an observation not made with the SCCs where only 20% of the cases lacked active RUNX3. Interestingly, when tested for the significance of the degree of differentiation against RUNX3 positivity for the ADC (Person's Chi-Square=0.932, $p$=0.627) or the SCC ($p$=1.000) (Fig. 2 panel D) a statistical effect was not found, suggesting pervasive inactivation of RUNX3 in adenocarcinoma development and a maintenance in SCC.

The paper by Araki et al.[53] is in contradiction with these findings in relation to a) the correlation to tumor severity in their case, and b) the differences in the normal RUNX3 subcellular localization. We believe that, in both

**Table 4** Results observed TMA samples selected for EGFR and KRAS mutation analysis

| Block | KRAS mutations | | EGFR mutations | | | | IHC results | | |
|---|---|---|---|---|---|---|---|---|---|
| | Exon 2 | Exon 3 | Exon 18 | Exon 19 | Exon 20 | Exon 21 | RUNX3 +/− | EGFR +/− | p53 +/− |
| AWD5 | WT | WT | WT | WT | NA | NA | - | + | - |
| AMD7 | 13>GAC | WT | WT | WT | NA | WT | - | - | - |
| AMD11 | WT | WT | WT | WT | WT | L858R | - | - | + |
| AMD15 | WT | WT | WT | del746-750 | CCC>TTC, pro>leu TCC>TTC, ser>leu | WT | - | - | - |
| APD1 | WT | WT | NA | WT | NA | WT | - | + | + |
| APD5 | 12>GTT | WT | WT | WT | NA | WT | - | - | + |
| APD6 | 12>GTT | WT | WT | WT | WT | WT | - | + | - |
| APD7 | 13>GAC | NA | NA | WT | NA | NA | - | + | - |
| APD10 | 13>GAC | WT | NA | WT | ACC>ATC, thr>ile | WT | - | - | - |

*NA* DNA not amplifiable, *WT* Wild type,

The table above shows the EGFR and KRAS mutation status of the selected adenocarcinoma cases from the TMA, with the RUNX3, EGFR and p53 IHC positive and negative expression. The specific mutations indicated are described in Krypuy et al. (2006) and Chin et al. (2007). AWD- adenocarcinoma (well differentiated), AMD- adenocarcinoma (moderately differentiated), APD- adenocarcinoma (poorly differentiated)

cases, these differences may be related to technical reasons (antibody type, scoring system, antigen retrieval technique etc.) which, in our experience, can affect both overall expression and subcellular expression. This was illustrated recently[54] in relation to another system.

*RUNX3* hypermethylation is well established as a cause for RUNX3 silencing [24, 33, 34, 53, 55–57]. Based on our findings, promoter methylation appears linked to ADC, however not to the SCC formation. None of the samples characterized as SCC showed any promoter methylation; instead, the maintenance of activity of RUNX3 correlates with the absence of hypermethylation. In the ADCs five of seven samples (71%) showed methylation of the RUNX3 promoter region. The presence of both methylated and unmethylated promoter regions as seen in several samples can be attributed to two possibilities. Firstly the presence of tumour (methylated) with admixed non-neoplastic cells (unmethylated), secondly, the occurrence of partial methylation, i.e. the methylation of only a single allele, as postulated previously [20]. The frequent methylation seen along with the frequent downregulation of the RUNX3 in the ADCs also indicates the RUNX3 has an early association with tumour development. This postulation is supported by observations in cases of atypical adenomatous hyperplasia, the equivalent of the adenoma in humans, where the early incidence of hypermethylation is seen in several genes including RUNX3, with a general increase in frequency with tumour severity[23], with similar observations seen in work on colorectal neoplasia[58] and in mouse models [26].

As with previous literature, we observe an inverse relationship between *EGFR* and *KRAS* mutation status in lung ADC [44, 59], with only one sample showing a mutation in both genes, a rarely observed occurrence[43, 44]. The single SCC showed no mutation while, interestingly, the single normal cell lines showed an *EGFR* mutation, perhaps highlighting the difficulty of producing "normal" cell lines with an intact genotype and a regeneration capacity. In general, the results indicate that the mechanism of *RUNX3* downregulation is not biased toward the *EGFR* or *KRAS* mutation pathways, specifically for KRAS this is consistent with work done in RUNX3 knockout mice[26]. This, together with the aberrant expression of RUNX3 in both the *EGFR* and *KRAS* mutation groups strengthens the argument that RUNX3 has an early association with ADC formation. The lack of any association between the EGFR and p53 protein expressions adds further to this notion.

At what level of the respective pathways does RUNX3 interact is an important question, not within the scope of this descriptive study.

Our results suggest that RUNX3 is downregulated in ADCs, however normally expressed in SCC. *EGFR* and *KRAS* mutations and EGFR and p53 protein expression were not correlated with the RUNX3 downregulated ADC samples. Our observations indicate that RUNX3 downregulation is an early indicator of ADC development which occurs independently of the degree of differentiation. It would be interesting to further this investigation into the expression levels of the TGF-ß and Wnt pathways, as these pathways appear to be regulated by RUNX3 expression levels, as we noted previously in intestinal tumours[22].

In essence, our study confirms that RUNX3 functions as an important biomarker in lung ADC development. In the right test validation environment, this may lead to a promising biomarker for early detection in biological samples, with applications as a screening tool.

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