MiR-21, EGFR and PTEN in non-small cell lung cancer: an in situ hybridisation and immunohistochemistry study

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ABSTRACT

Aims To analyse microRNA (miR)-21 distribution and expression at the cellular level in non-small cell lung cancer (NSCLC). MiR-21 is an oncogenic microRNA overexpressed in NSCLC. In previous studies, overexpression of miR-21 was evaluated from the tumour bulk by quantitative reverse transcription PCR with results expressed on average across the entire cell population.

Methods We used in situ hybridisation and immunohistochemistry to assess the correlation between miR-21 levels and the expression of markers that may be possible targets (epidermal growth factor reaction) or may be involved in its upregulation (phosphatase and tensin homolog (PTEN), p53). The Pearson's χ^2 tests was used to assess correlation with clinicopathological data and with miR-21 expression both in tumour and tumour stroma.

Results Cytoplasmic staining and expression of Mir-21 were detected in the tumours and in associated stromal cells. Expression was highest in the stroma immediately surrounding the tumour cells and decreased as the distance from the tumour increased. No expression of miR-21 was found in normal lung parenchyma and a significant association was found between tumour localised miR-21 and PTEN.

Conclusions Presence of miR-21 in both cell tumour and stromal compartments of NSCLC and the relationship with PTEN confirms miR-21 as a microenvironment signalling molecule, possibly inducing epithelial mesenchymal transition and invasion by targeting PTEN in the stromal compartment possibly through exosomal transport. In situ immunohistochemical studies such as ours may help shed light on the complex interactions between miRNAs and its role in NSCLC biology.

INTRODUCTION

Lung cancer (LC) is the world leading cause of cancer-related mortality, accounting for approximately 1.76 million deaths per year worldwide. It amounts to 25%–30% of all US cancer deaths, more than colon and breast cancer combined. Non-small cell lung cancer (NSCLC) represents 85% of all LC and its current treatment has well-known limitations with a continuous need for research and identification of possible treatment targets.[1]

MicroRNAs (miRNAs or miR) are non-coding RNA molecules ranging in size from 20 to 25 nucleotides[2] that help to regulate more than 30% of protein-coding genes[3] and act as oncogenes on cell cycle control and differentiation.[4] Numerous reports have demonstrated miRNAs as diagnostic and/or prognostic markers in several human tumours,[5] including, leukaemia, lung, colon and thyroid cancer.[6–10] They also have been proposed as possible therapeutic targets[11 12] or as modulator of cytotoxic anticancer therapy.[13 14]

MiR-21 is one of the most studied miRNAs and is recognised as a ubiquitous oncogenic miRNA.[15–17] It is upregulated in most solid cancers[18] and several tumour suppressor proteins, such as phosphatase and tensin homolog (PTEN), have been shown to be among its main targets. Overexpression of miR-21 may result in a loss of PTEN expression[19] with increased cell proliferation, invasion and cell migration in vitro,[20] whereas in human NSCLC cells, triptolide was shown to reduce proliferation and enhance apoptosis through PTEN by targeting miR-21.[21]

P53 is one of the most commonly mutated genes in human cancer,[22] and by inducing apoptosis in response to DNA damage, it functions as a tumour suppressor gene.[23] Studies on several human cancers have shown that the p53 pathway may be a target of miR-21.[17 24–26] It has also been suggested that the epidermal growth factor reaction (EGFR) signalling pathway takes part in the positive regulation of miR-21 expression[27] since miR-21 is involved in the acquired resistance of EGFR tyrosine kinase inhibitors (EGFR-TKI) in NSCLC.[28]

Most studies on tumour-associated miRs have been based on RNA extracted from tumour bulk. While these studies were able to demonstrate a correlation between specific miRNAs and specific proteins, they were not specific regarding the cells expressing them. In an attempt to bridge this gap of knowledge, we constructed a study to evaluate the expression of miR21 using in situ nucleic acid hybridisation in formalin-fixed paraffin-embedded (FFPE) tissue sections of NSCLC. The aim of our study was to explore miR-21 expression differences in tumour and tumour-associated stromal cells (SCs) of NSCLC using immunohistochemistry (IHC), which is informative regarding cellular localisation of different proteins. We focused on the association between expression of miR-21 and the expression of markers, which may serve as its targets (such as PTEN and p53) or as its upregulators (such as EGFR) as well as its association with proliferation markers, such as Ki67.

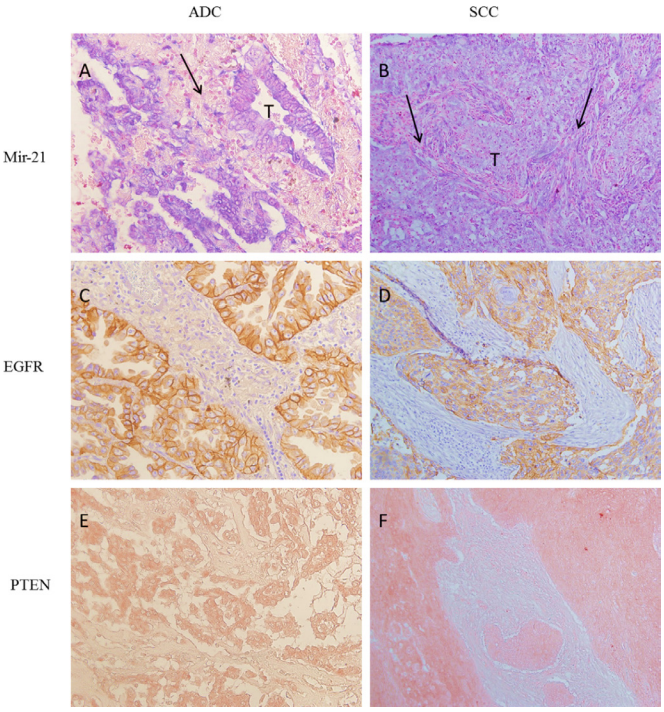

Figure 1 In situ hybridisation for miR-21 and immunohistochemistry for EGFR and PTEN. Adenocarcinoma (ADC) (A) and squamous cell carcinoma (SCC) (B) showing miR-21 expression as diffuse cytoplasmic blue staining in tumour (T) and adjacent stromal cells (black arrows). Tumour cells show strong membranous expression of EGFR (C, D). Weak cytoplasmic expression of PTEN (E, F) (A, C, E-magnification, x400) (B, D, F-magnification, x200). EGFR, epidermal growth factor receptor; miR, micro RNA; PTEN, phosphatase and tensin homolog.

MATERIAL AND METHODS

Patient samples

Tissue microarray cores (0.6 mm in diameter) samples were taken from 44 FFPE blocks of NSCLC patients diagnosed between 1999 and 2004 at the department of pathology, Sheba Medical Center, Israel and consisted of 22 adenocarcinomas and 22 squamous cell carcinoma. For each patient, two cores containing neoplastic tissue and tumour-associated stroma and two cores of normal lung tissue were sampled.

In situ hybridisation

The method used for miRNA hsa-miR-21 in situ hybridisation (ISH) was based on protocols detailed by Nielsen and Jørgensen *et al* with minor modifications.[29 30] The miRNA hsa-miR-21 used was a double-DIG labelled miRCURY LNA detection probe (Exiqon-Quiagen, Denmark). A U6 probe (Exiqon-Quiagen) was used as a positive control and a scrambled probe (Exiqon-Quiagen) as a negative control. Briefly, 6 µm thick lung adenocarcinoma and squamous cell carcinoma sections retrieved from TMA paraffin blocks were heated at 60°C overnight. After heating, dewaxing and rehydrating, the sections were treated with a proteinase-K (10 µg/mL, Roche Diagnostics, Germany) for 10 min.

Tissue sections were hybridised with a 60 nM double-DIG labelled LNA miR-21 probe at 53°C overnight. The immunological detection was performed with an alkaline phosphatase-conjugated antidigoxigenin (1:200, Roche Diagnostics, Germany). Nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) substrates (Roche Diagnostics, Germany) were added to the slides and counterstained with Nuclear Fast Red (Vector Laboratories, California, USA).

Table 1 Expression of stromal miR21 (miR21-S) with distance from tumour

miR21-S	Near tumour	Far from tumour	P value
Positive	37	0	<0.0001
Negative	7	44	

miR, micro RNA.

Immunohistochemistry

Four µm thick sections of tumour taken from TMA paraffin blocks were immunostained for surfactant A (ab49566, ABCAM, UK), PTEN (180256, Invitrogen, USA), EGFR (28–0005, Invitrogen), Ki67 (RM-9106-S0, Thermo Fisher Scientific, USA) and p53 (NCL-P53-1801, Leica, Germany). EGFR, Ki67 and p53 were fully calibrated by the Benchmark XT staining module (Ventana Medical Systems, Arizona, USA).

Surfactant A and PTEN immunostaining

Surfactant A (SP-A) and PTEN were manually immunostained. Slides were warmed up to 60°C for 1 hour, dewaxed in xylene and rehydrated. An endogenous peroxidase block was performed for 10 min in 3% hydrogen peroxid (H_2O_2)/ methanol (MeOH). After rinses in TBS spell out, sections were blocked with 10% goat serum for 30 min at room temperature and incubated overnight at 4°C with antisurfactant A (1:50) or anti-PTEN (1:50). Detection was performed by the Envision+System Horseradish peroxidase (HRP) Labelled Polymer (K4003, Dako-Agilent, California, USA) for PTEN and Envision+System HRP Labelled Polymer (K4001, Dako-Agilent) for surfactant A. The antibody binding was visualised with the substrate–chromogen 3-amino-9-ethylcarbazole (AEC) spell out. Sections were counterstained with haematoxylin and cover slipped with an aqueous mounting fluid (Glycergel, Dako-Agilent).

Table 2 MiR-21 expression in tumour cells and relationship with the other IHC marker

	MIR21-T expression level in tumour cells								
	MIR21-T positive				MIR21-T negative				
IHC markers expression level in stroma-S and tumour-T	Positive		Negative		Positive		Negative		
	N	%	N	%	N	%	N	%	P value
MIR21-S	30	91	3	9	7	64	4	36	**0.032***
P53-S	0	0	32	100	0	0	11	100	NA
EGFR-S	2	6	29	94	0	0	11	100	0.388
Ki67-S	1	3	29	97	0	0	11	100	0.540
PTEN-S	12	36	21	64	4	36	7	64	1.000
SPA-S	3	9	30	91	1	10	9	90	0.931
P53-T	14	44	18	56	6	55	5	45	0.536
EGFR-T	23	77	7	23	8	73	3	27	0.795
Ki67-T	10	33	20	67	5	45	6	55	0.475
PTEN-T	27	82	6	18	5	45	6	55	**0.019**
SPA-T	23	70	10	30	4	40	6	60	0.089

*statistically significant p<0.05
EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; miR, microRNA; PTEN, phosphatase and tensin homolog; SPA-S, Surfactant A in stromal cells.

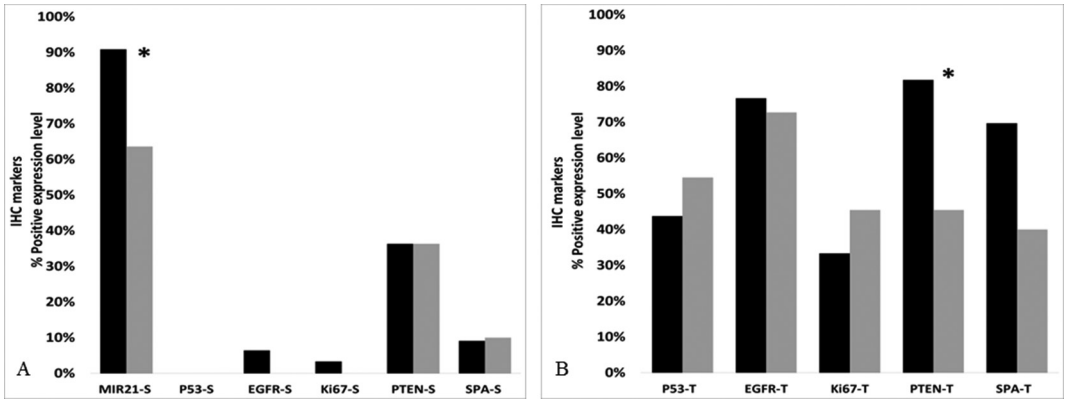

Figure 2 (A) Relationship between miR-21 expression in stromal cells and expression of IHC markers in stromal cells. Assessment of stromal expression relates to stroma close to the tumour cells, within the same section. (B) The relationship between miR-21 expression and other IHC markers in tumour cells. Black columns- MiR21-T positive expression; grey columns—MiR21-T negative expression. *P<0.05. EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; miR, microRNA; PTEN, phosphatase and tensin homolog; SPA-S, Surfactant A in stromal cells

EGFR, Ki67 and p53 immunostaining

The slides were warmed up to 60°C for 1 hour and subsequently processed to a fully automated protocol. Once sections were dewaxed and rehydrated, a CC1 Standard Benchmark XT pretreatment for antigen retrieval (Ventana Medical Systems) was selected for both Ki67 (1:300) and p53 (1:40). A protease1 Benchmark XT pretreatment (Ventana Medical Systems) was selected for EGFR (1:20). Detection was performed with an UltraView detection kit (Ventana Medical Systems) and counterstained with haematoxylin (Ventana Medical Systems). After a run on the automated stainer was completed, we dehydrated the slides. Before coverslipping, we cleared the sections in xylene and then mounted with Entellan.

Scoring of ISH and IHC

Representative viable tissue sections were anonymised and independently scored by two pathologists. In the event of discrepancies, the slides were re-examined and a consensus was reached by the observers.

Expression of miR-21 in tumour cells (TCs) and SCs was defined as 0—negative, 1—weakly positive, 2—moderately positive and 3—strongly positive.

EGFR intensity of staining was scored on a scale from 0 to 3 (0 for no staining, 1—for weak membrane staining, 2—for more intense membrane staining and 3—for intense brown staining of the membrane, stronger than score 2).

PTEN staining was scored as follows: 0—negative staining, 1—poorer staining than normal tissue, 2—staining similar to normal tissue and 3—staining higher than normal tissue.

SP-A staining, both cytoplasmic and intranuclear, was scored as follows: 0—negative, 1—sparsely and weakly positive, 2—diffusely and clearly positive and 3—strongly positive. As for P53 and Ki67, distinct, strong staining of nuclei was counted as a percentage of the total amount of nuclei. In addition, both p53 and Ki-67 were categorised as absent/low intensity (-) for <10% of nuclei positive TCs or significant intensity (+) for ≥10% of positive tumour nuclei.

Statistical analysis

We combined the expression level of all the IHC markers into 2 categories:

- 0—negative and weakly positive expression.
- 1—moderate or strong positive expression.

We used the χ^2 test to determine whether a significant association existed between cancer type and each of the IHC markers.

The test also determined if there was a significant association between the miR-21 in the TCs and each of the other markers, as well as correlating the staining of the SCs versus the TCs.

RESULTS

Mir-21

MiR-21 is upregulated in TCs as well as in tumour-associated SCs of NSCLC. Both tumour and SCs showed mainly diffuse blue staining of the cytoplasm (figure 1). Expression was highest in the stroma immediately surrounding the TCs and decreased as the distance from the tumour increased (p<0.0001; table 1).

Foci of carcinoma in situ showed minimal stromal expression. While normal lung parenchyma did not express miR-21, a weak positive expression was noted in alveolar macrophages, inflammatory cells and endothelial cells. A statistically significant positive association was found between miR-21 expression in tumour and SCs. MiR-21 showed a high rate of positivity in tumour-associated stroma (84%) regardless of miR-21 status in the TCs, but in tumours which were positive for mirR-21 the rate of stromal positivity was significantly higher than in tumours which were miR-21 negative (91% vs 64%, respectively; p=0.032; (table 2, figure 2).

Epidermal growth factor receptor

While EGFR tumour expression (table 2) was seen in 70% of the cases, stromal expression was very rarely observed (4.5%). Since miR-21 was almost always positive in the adjacent SCs, all NSCLC tumours with EGFR TC positivity demonstrated concurrent expression of miR-21 in the adjacent SCs.

Phosphatase and tensin homolog

A significant association was found between miR-21 and PTEN expression in TCs. PTEN TC positivity was seen in 72% of the cases and 82% of the miR-21 positive tumours showed a PTEN protein expression (p=0.019); (table 2, figure 2). This positive association was curbed in cases where extremely high miR-21 expressing TCs showed divergently low levels of PTEN. PTEN SC expression was independent of miR-21 tumour status as 36% of both miR-21 positive and miR-21 negative cases showed PTEN stromal expression.

Table 3 Expression rate of IHC markers in each of the cancer types (S-stromal cells, T-tumour cells)

	NSCLC subtype								
	Adenocarcinoma				Squamous cell carcinoma				
IHC/ISH markers expression	Positive		Negative		Positive		Negative		
	N	%	N	%	N	%	N	%	P value
MIR21-S	19	86	3	14	18	82	4	18	0.680
P53-S	0	0	21	100	0	0	22	100	0.945
EGFR-S	1	5	19	95	1	4	22	96	0.945
Ki67-S	0	0	20	100	1	5	20	95	0.323
PTEN-S	8	36	14	64	8	36	14	64	1.000
SP-A-S	1	5	20	95	3	14	19	86	0.317
MIR21-T	18	82	4	18	15	68	7	32	0.296
P53-T	7	33	14	67	13	59	9	41	0.091
EGFR-T	12	60	8	40	19	90	2	10	**0.023***
Ki67-T	7	35	13	65	8	38	13	62	0.837
PTEN-T	17	77	5	23	15	68	7	32	0.498
SP-A-T	15	71	6	29	12	55	10	45	0.252

*statistically significant p<0.05
EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; ISH, in situ hybridisation; NSCLC, non-small cell lung cancer.

Other IHC markers

The tumour tissue expressed p53, Ki67 and SP-A, but with no significant association to miR-21 expression (table 2). There was a trend for SP-A expression in TCs, to correlate with miR-21 expression in TCs but not in SCs (p=0.089).

In SCs, no expression of the IHC markers, p53, Ki67 was found.

Tumour subtypes, clinicopathological data correlation

Though not statistically significant, miR-21 expression seems to correlate with tumour subtype. We observed that 82% of adenocarcinomas were miR-21 positive, while only 68% of squamous cell carcinomas revealed miR-21 positivity (table 3). EGFR expression, on the other hand, did show significantly higher rates of positivity in the squamous cell carcinoma group over that seen in the adenocarcinoma group: a majority (90%) of squamous cell carcinoma cases were EGFR positive, whereas only 60% of the adenocarcinomas showed EGFR positivity (p=0.023); (table 3 and figure 3).

Table 4 EGFR and miR-21 expression in NSCLC

		EGFR-T				
		Adenocarcinoma		Squamous cell carcinoma		
		Positive	Negative	Positive	Negative	P value
Mir21-T	Positive	10	7	13	2	NS
	Negative	2	0	6	0	NS

EGFR, epidermal growth factor receptor; miR, microRNA; NS, not statistically significant; NSCLC, non-small cell lung cancer.

Correlation between EGFR and miR-21 expression in relation to tumour subtype showed indication of a positive correlation between miR-21 and EGFR in TCs, but without statistical significance (table 4).

DISCUSSION

MiR-21 has shown high diagnostic and prognostic value in association with several cancers, among them NSCLC.[31] It is one of the more profoundly upregulated miRNAs in NSCLC as compared with normal lung tissue.[18] However, most previous studies in NSCLC evaluated miR-21 expression by RT-PCR,[32–34] on bulk tumour tissue which could not assess its localisation, that is, expression in tumour and stroma separately, as we have done using ISH. One exception is Stenvold *et al*'s study in which miR-21 expression was also assessed using this method. They found differential expression of mir-21 in tumour and SCs in NSCLC and that high expression in SCs is a negative prognostic factor in NSCLC,[35] similarly to what has been demonstrated in other solid tumours such as oral squamous cell carcinoma[36] and colon.[37] However, at the cellular level, the precise role and molecular targets of miR-21 are not well known. In our study, we have also shown that miR-21 is upregulated in NSCLC TCs and in their associated stroma. Using ISH and IHC techniques, we were also able to analyse miR-21 expression and its relationships with some of its regulators and targets, in order to gain a better understanding of its role in LC.

In our study, most of the NSCLC cases showed concurrent expression of both EGFR and miR-21 in TCs, further supporting the idea that the EGFR pathway influences miR-21 expression. In addition, we found statistically significant higher expression of EGFR in TCs of adenocarcinoma than its expression in squamous cell carcinoma, contrary to previous findings by Veale *et al*.[38] The suppression of miR-21 expression by EGFR-TKI

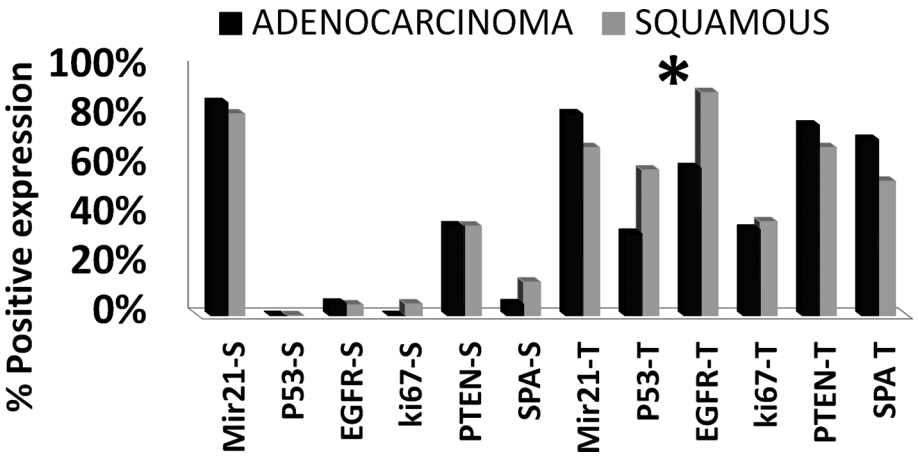

Figure 3 A positive expression of IHC markers in each of the NSCLC subtypes (S-stromal cells, T-tumour cells). *P<0.05. EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; miR, micro RNA; NSCLC, non-small cell lung cancer; PTEN, phosphatase and tensin homolog.

AG1478, an inhibitor of EGFR expression, suggests that the EGFR signalling pathway is involved in the positive regulation of miR-21 expression.[27 28]

Further studies are needed to ascertain these findings, since EGFR is now a clinically validated target in cancer therapy.[39] While both EGFR-TKIs and ligand-blocking antibodies are routine FDA-approved therapeutic options these days, there is still much to discover about EGFR in order to adjust tailored and effective EGFR-targeting therapies.[40 41]

Regarding the potential targets of miR-21, such as PTEN, several studies have shown downmodulation of PTEN expression by miR-21 in solid tumours.[42–45] In our study, somewhat unexpectedly, we found a positive relationship between miR-21 and PTEN expression in TCs. We saw a decrease in PTEN expression only when miR-21 levels were very high. High expression of miR-21 has also been correlated with poor prognosis in several cancers,[35–37 46] with miR-21 high expression associated specifically with tumour size and venous invasion.[47] It has been suggested that the downregulation of PTEN expression by miR-21 may in fact induce epithelial mesenchymal transition (EMT) activation,[43 48] thus rendering the TCs adjacent to the basement membrane to become invasive and migrate within the stroma. A study in colorectal cancer suggests that miR-21 plays a role in the regulation of EMT,[49] and another study shows that the medium of primary LC cells can induce EMT with miR-21 participation.[50] One study in oesophageal squamous cell carcinoma[51] has shown that cancer cells can release miR-21 into their surroundings. In this study, it was proposed that miR-21 may serve as a microenvironment signalling molecule, contributing to tumour growth and cancer progression. In our study, miR-21 expression decreased in the tumour-associated SCs with their distance from tumour TC cells, supporting the hypothesis that cancer cells may release miR-21 into adjacent SCs in a "gradient" associated manner.[52 53] Interactions between TCs and adjacent stromal tissue may be mediated via diffusion of exosomal miRNAs.[54–56] Indeed, high levels of miR-21 have been found in exosomes derived from NSCLC cell lines and exosome-mediated gefitinib resistance has been shown to be delivered via exosomal miR-21 in a LC cell line.[57–59] Furthermore, lack of syndecan-1 impacts the miRNA carried in exosomes in cell-based and animal models of LC[60] and plasma exosomal miR-21–5 p is among a panel of promising non-invasive prognostic biomarkers of NSCLC.[61 62]

In conclusion, our results may provide support for the concept of an exosomal miR-21 influx originating from the tumour towards the stroma where it may trigger certain down-stream pathways to promote tumour growth, angiogenesis and metastasis.[63–66] Investigation of tumour and SC communication via exosome networks may benefit from additional in situ immunohistochemical studies such as ours and provide a platform for a better understanding of the molecular mechanisms and targets of miR-21 in NSCLC.

Take home messages

- Presence of microRNA (miR)-21 in both tumour and stromal compartments of non-small cell lung cancer (NSCLC) and the relationship with phosphatase and tensin homolog (PTEN) confirms miR-21 as a microenvironment signalling molecule.
- Exosomal transport of miR-21 could allow for targeting PTEN in the stromal compartment and inducing epithelial mesenchymal transition and invasion.
- In situ studies are a valuable tool to shed light on complex miRNAs organisations in NSCLC.

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