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Glypican-3 is overexpressed in lung squamous cell carcinoma, but not in adenocarcinoma

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Glypican-3 is a membrane-bound proteoglycan whose expression has been linked to malignancies through the existence of both mutations and aberrant protein expression. Reports on glypican-3 expression in lung cancer were limited, with some evidence for loss of expression, which suggested a tumor-suppressor role. We sought to evaluate glypican-3 expression in lung cancer at the protein and mRNA levels and correlate it with clinical, histological and genomic characteristics such as *RAS* mutation status. We used immunohistochemistry on tissue microarray to study glypican-3 expression in 97 patients, evaluated glypican-3 mRNA levels by quantitative polymerase chain reaction in 143 patients and identified *RAS* mutations by allele-specific oligonucleotide hybridization. We correlated the results with clinical and histological data. Glypican-3 immunostaining was negative in all normal lung tissues, but positive in 23% of lung carcinoma samples. High protein and mRNA expression was associated with squamous histology (positive stain in 55% of squamous cell carcinoma *vs* 8% of adenocarcinoma, *P*<0.0001 for both immunostaining and mRNA). *RAS* mutations were highly associated with adenocarcinoma and low glypican-3 mRNA expression (*P*<0.0001 for both). Among smokers, glypican-3 mRNA expression was reduced in adenocarcinoma patients (*P*=0.013), and was elevated in those with squamous cell carcinoma (*P*=0.03, interaction *P*=0.0009). These opposing associations also correlated with the smoking burden. Patients with tumors staining positively for glypican-3 smoked significantly more than patients with tumors staining negatively (*P*=0.013). No association was found between glypican-3 expression and patient outcome. In conclusion, glypican-3 was overexpressed in cancerous compared with normal lung tissue. Adenocarcinoma and squamous cell carcinoma had differential expression of glypican-3, with predilection to squamous cell carcinoma patients who smoked. Glypican-3 expression in squamous cell carcinoma as an oncofetal protein renders it a potential candidate marker for early detection of lung squamous cell carcinoma.
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Glypican-3 belongs to a six-member (glypicans 1–6) family of extracellular, membrane-bound heparin sulfate proteoglycans, with peak expression during fetal life that is downregulated after birth.[1–4] All glypicans have a common structure that includes a glycosylphosphatidylinositol anchor to the cell membrane, an N-terminal globular cysteine-rich domain and a C-terminal heparin sulfate glycosaminoglycan attachment site.[5–7] The glypican-3 gene is localized to chromosome Xq26 and was first identified for its association with the X-linked Simpson–Golabi–Behmel syndrome.[8] In this pre- and postnatal overgrowth syndrome, the glypican-3 gene is functionally inactivated by either point mutations or micro-deletions.[9] In addition to

visceral and skeletal abnormalities, Simpson–Golabi–Behmel patients have increased incidence of embryonal tumors (Wilms' tumor, neuroblastoma and hepatoblastoma).[8,10] This combination of findings suggested that glypican-3 has an inhibitory effect on cell proliferation and could function as a tumor-suppressor gene.[11] Indeed, few tumors, including ovarian carcinoma,[12] mesothelioma[13] and breast carcinoma,[2,14] show lower glypican-3 expression relative to normal tissue.

In contrast, other tumors overexpress glypican-3, whereas its expression in normal tissues is silenced. The most prominent example of this is hepatocellular carcinoma where glypican-3 expression can serve as a diagnostic serum and immunohistochemical marker.[3,15] Other tumors with high glypican-3 expression include Wilms' tumor,[16,17] neuroblastoma,[16] hepatoblastoma,[17] melanoma[18] and testicular germ-cell tumors.[19] There is evidence that depending on the cellular context, glypican-3 may activate either the canonical[20] or non-canonical Wnt-signaling pathways.[21] Altogether, it appears that glypican-3 expression is silenced in tumors derived from tissues that might normally express it, whereas it is increased in tumors arising from tissues where glypican-3 is normally silenced.[19]

To date, only one study has investigated the expression and role of glypican-3 in lung cancer.[22] This study involved cDNA microarray analysis, which was followed by validation studies. The study found that glypican-3 was suppressed in tumor compared with normal tissue and in smokers relative to non-smokers, suggesting that glypican-3 is a tumor-suppressor gene for lung carcinogenesis. A meta-analysis of publicly available gene expression databases found that glypican-3 expression predicted longer survival in lung adenocarcinoma.[23] On the basis of uncertainties arising from these studies and the small number of patient samples analyzed, we have studied further glypican-3 expression in a large cohort of non-small-cell type lung carcinoma patients at both protein and mRNA levels.

Materials and methods

Immunohistochemistry Study Materials

Construction of lung cancer tissue microarray has been approved by the University Health Network (UHN) Research Ethics Board. The tissue microarray contains samples from 107 non-small-cell type lung carcinoma from patients who underwent surgical resection at the UHN from 1995 to 2000. Each patient was represented by one core of normal lung tissue and three cores of tumor from formalin-fixed, paraffin-embedded tissue. All cores were of 0.6 mm in diameter. Clinical data and follow-up were available for all patients whose samples were included in the tissue microarray (Table 1).

Glypican-3 Immunohistochemical Staining

Glypican-3 immunohistochemical staining was performed using the 1G12 monoclonal antibody according to the manufacturer's protocol (BioMosaics, Burlington, VT, USA). Briefly, endogenous peroxidase was blocked using 3% hydrogen peroxide and antigen was retrieved in 0.01 M sodium citrate buffer (pH 6) using a pressure cooker. Sections were blocked in non-serum blocker (DakoCytomation, Carpinteria, CA, USA) followed by incubation with mouse 1G12 monoclonal anti-human glypican-3

Table 1 Patient characteristics

	Immunostaining	mRNA	Both
No. of patients	97[a]	143	58
Age (year)			
Range	42–82	39–86	43–82
Median	68	68	68
Gender			
F	40	63	28
	−41%	−44%	−48%
M	57	80	30
	−59%	−56%	−52%
Histology			
Adenocarcinoma	59	89	37
	−61%	−62%	−64%
Squamous cell carcinoma	31	54	21
	−32%	−38%	−36%
Large cell carcinoma	7	0	0
	−7%	0%	0%
Stage			
1	73	89	45
	−75%	−62%	−78%
2	17	37	10
	−18%	−26%	−17%
3	7	17	3
	−7%	−12%	−5%
Smoking history			
Yes	79	114	45
	−81%	−80%	−78%
No	17	29	13
	−18%	−20%	−22%
Unknown	1	0	0
	−1%	0%	0%
Pack-year of past smokers			
Known	39	75	26
	−49%	−66%	−58%
Unknown	40	39	19
	−51%	−34%	−42%
Range	3–150	1.6–150	7.5–150
Median	45	45	42.5
RAS			
Mutant	27	38	16
	−28%	−27%	−28%
Wild type	70	88	42
	−72%	−62%	−72%
Unknown	0	17	0
	0%	−12%	0%

[a]Ten patients were excluded from the initial 107 patients included in the tissue microarray, since they had less than two scoreable tumor cores in the stained tissue array.

(20 μg/ml; BioMosaics) for 2 h at room temperature. Incubation with a rabbit anti-mouse secondary antibody (Biocare Medical, Concord, CA, USA) for 20 min was followed by incubation with a horseradish peroxidase complex, Mach-3-M-Polymer HRP (Biocare Medical), for 30 min at room temperature. The sections were developed with the 3,3′-diaminobenzidine tetrahydrochloride chromogen and counterstained with Gill's hematoxylin.

Scoring of Glypican-3 Immunohistochemical Staining

Membrane and cytoplasmic staining were scored for both intensity of staining (0–3) (Figure 1c–f) and percent of positively stained tumors cells, evaluated per core. Two pathologists (S Aviel-Ronen and S Jothy) independently evaluated and scored the immunostains. Only cases with at least two evaluable tumor cores were included in the analysis, and the final score of each criterion was the average of the evaluable cores.

Glypican-3 mRNA Expression Study by Quantitative Polymerase Chain Reaction

The mRNA expression study was carried out on 143 lung cancer samples, of which 58 patients were also in the immunohistochemistry cohort (Table 1). Total RNA was isolated from fresh frozen tissues using the guanidium thiocyanate–phenol–chloroform method, using DNAse I treated with DNA-free DNAse (Ambion, Austin, TX, USA), and column purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). Five nanograms of total RNA were reverse transcribed using Superscript II reverse transcription reagents and oligo-dT (Invitrogen, Carlsbad, CA, USA) to produce cDNA. Primers were designed using the Primer Express software version 2.0 (Applied Biosystems, Foster City, CA, USA). To exclude amplification of contaminating pseudogene sequences, primers (sequences provided in Table 2) were first aligned using the BLASTN program, followed by dissociation curve and primer efficiency tests. Quantitative polymerase chain reaction (qPCR) assays were conducted in duplicate in a 384-well plate using the SYBR Green assay in ABI PRISM 7900-HT (Applied Biosystems), using 2 ng equivalent of cDNA for a 10 μl qPCR reaction. The reactions were activated at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 15 s and extension at 72°C for 20 s. Transcript amount per nanogram of cDNA was obtained from standard curves generated with a pool of 10 non-tumor lung genomic DNAs.[24] Technical replicates were collapsed by averaging. Normal-
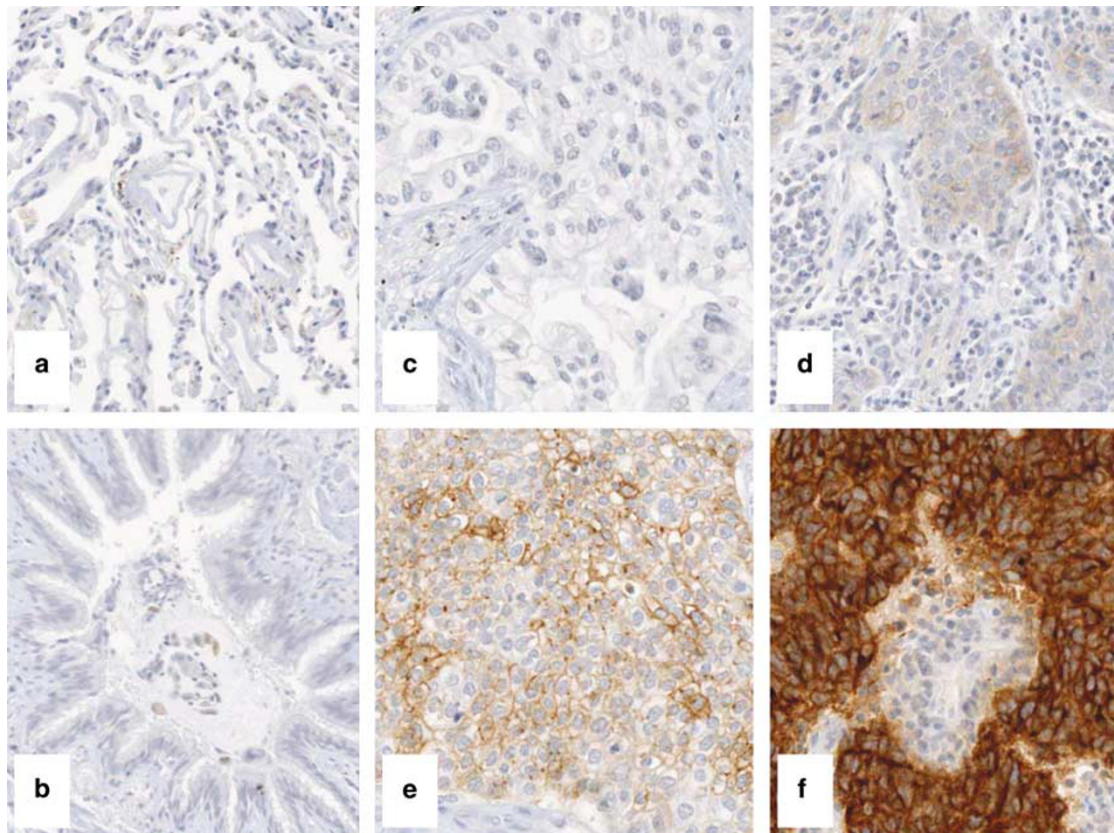

Figure 1 Immunohistochemistry results for glypican-3. Immunohistochemical staining for glypican-3 of normal lung tissue was negative both in the pneumocytes (**a**) and in the bronchiolar respiratory epithelium (**b**). Immunostain for glypican-3 resulted in a membranous and cytoplasmic staining pattern. Staining intensity was scored from 0 to 3 (**c–f**).

Table 2 Primer sequences

Gene	Forward	Reverse
ACTB	TCCTAAAAGCCACCCCACTTCT	GGGAGAGGACTGGGCCATT
B2M	GATTGCTGTCTCCATGTTTGATGT	AAGTTGCCAGCCCTCCTAGAG
BAT1	AGGGCTTGGCTATCACATTTGT	GCGATCCTGCACATCATTGA
TBP	GGGCATTATTTGTGCACTGAGA	TAGCAGCACGGTATGAGCAACT
GPC3	CAGCTCCTGAGAACCATGTCTATG	CCCTTCCTCATCCAGGTTTTT

ization and standardization of data were accomplished using the geometric mean of the expression levels of four housekeeping genes *ACTB* (actin, β), *B2M* (β-2-microglobulin), *TBP* (TATA box-binding protein) and *BAT1* (HLA-B-associated transcript 1). The normalization method has recently been published.[25]

Detection of RAS Mutations

Allele-specific oligonucleotide hybridization was used to detect mutations on codons 12, 13 and 61 of *HRAS, KRAS* and *NRAS*, as described previously.[26]

Statistical Analysis

The Spearman correlation coefficient was calculated between the intensity of glypican-3 immunostaining and the percent of positively stained tumor cells. Since this correlation was very high, glypican-3 protein expression level was expressed as the percent of positive cells and was dichotomized at 10% (<10 *vs* ≥ 10). The association between categorical variables was analyzed using the χ^2 or Fisher's exact tests. Wilcoxon rank-sum test was used to investigate the association between the amount of smoking and glypican-3 mRNA expression with categorical variables. A linear regression model was built to test the effect of both the amount of smoking and histology, and their interaction with glypican-3 mRNA expression levels. The amount of smoking was expressed in log-transformed pack years, where one 'pack year' is defined as one pack of cigarettes smoked/day for 1 year. A Cox proportional hazards model was used to study the association between the covariates and survival (time elapsed from surgery to death), disease-free survival (time elapsed from surgery to relapse or death) and time to relapse (time elapsed from surgery to relapse).

Results

There were 97 cases that had glypican-3 immunohistochemistry scores from two or more cores. The glypican-3 intensity and percent of positively stained cells were highly correlated ($r = 0.99$), which

led us to decide that further analysis would be based only on the percent of cells stained positively. The three patient cohorts (immunohistochemistry, mRNA and those in both) showed similar characteristics (Table 1). For patients in both immunohistochemistry and mRNA cohorts, glypican-3 protein expression correlated well with mRNA levels (median mRNA $= -0.5$ in immunohistochemistry-negative *vs* 2.23 in immunohistochemistry-positive tumors; $P = 0.00029$).

None of the normal lung cores stained positively for glypican-3, and no staining was observed in bronchiolar respiratory epithelium (Figure 1a and b). However, 22 (23%) of lung carcinoma samples stained positively for glypican-3, with significantly higher expression levels in squamous cell carcinoma compared with in adenocarcinoma (positive staining in 55% of squamous cell carcinoma *vs* 8% of adenocarcinoma; $P < 0.0001$) (Figure 2a). Likewise, high expression of glypican-3 mRNA was associated with squamous cell carcinoma compared with adenocarcinoma ($P < 0.0001$) (Figure 2b).

In the entire cohort (where either immunohistochemistry or mRNA was measured, $n = 182$) *RAS* mutation status was not tested in 17 patients, and mutations were identified in 49 of the remaining 165 patients (30%). All but one mutation occurred in codon 12 of *KRAS* (Table 3). The mutation rate was similar among men and women (20/70 or 29% in women and 29/95 or 31% in men) and mutations were highly associated with adenocarcinoma (42/101 = 42% in adenocarcinoma *vs* 6/57 = 11% in squamous cell carcinoma; $P < 0.0001$). Fewer cases stained positively for glypican-3 in the presence of *RAS* mutations than in the wild type, but this difference was not significant (15 *vs* 25%; $P = 0.38$) (Figure 3a). However, the presence of *RAS* mutations was strongly associated with lower glypican-3 mRNA levels ($P < 0.0001$) (Figure 3b). In multivariate analysis, association of glypican-3 mRNA levels with *RAS* mutation weakened but remained significant ($P = 0.012$), whereas association of glypican-3 expression with squamous cell carcinoma remained highly significant ($P < 0.0001$).

Twenty-six percent of smokers had positive glypican-3 staining compared with only 12% among non-smokers, but this difference was not statistically significant ($P = 0.34$) (Figure 4a). However, we found a significant difference ($P = 0.013$) between the amounts that patients smoked in negative
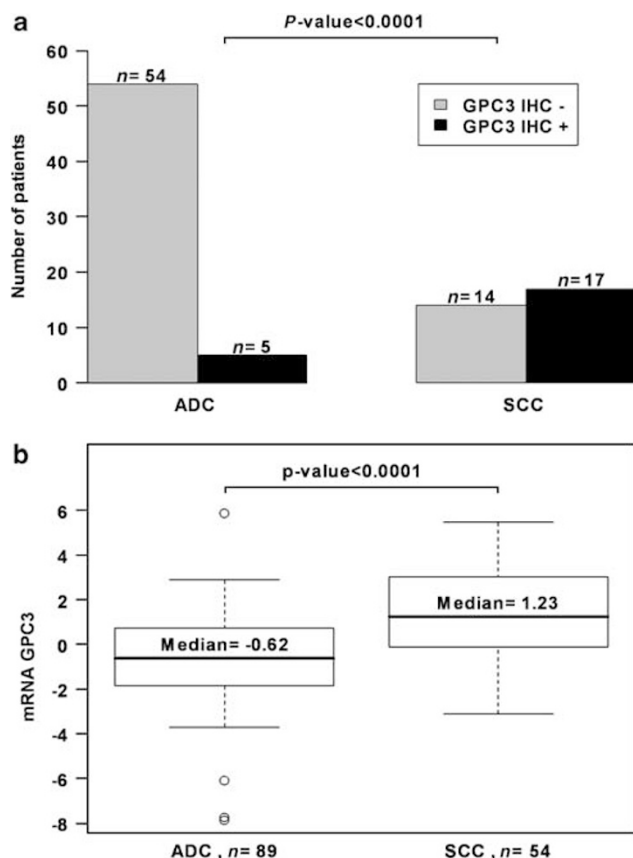
Figure 2 Glypican-3 protein and mRNA levels are associated with histology. (**a**) Glypican-3 immunostaining showed significant predilection for squamous cell carcinoma compared with adenocarcinoma. (**b**) Similarly, high expression of glypican-3 mRNA was associated with squamous cell carcinoma compared with adenocarcinoma. Data are presented in a box plot, where the upper limit of the rectangle is the third quartile and the bottom limit is the first quartile. Thus, the rectangle limits show the interquartile range (IQR) and half of the data are within these limits. The lines extending from the rectangle are 1.5* IQR.

Table 3 Summary of mutations on *RAS* family genes

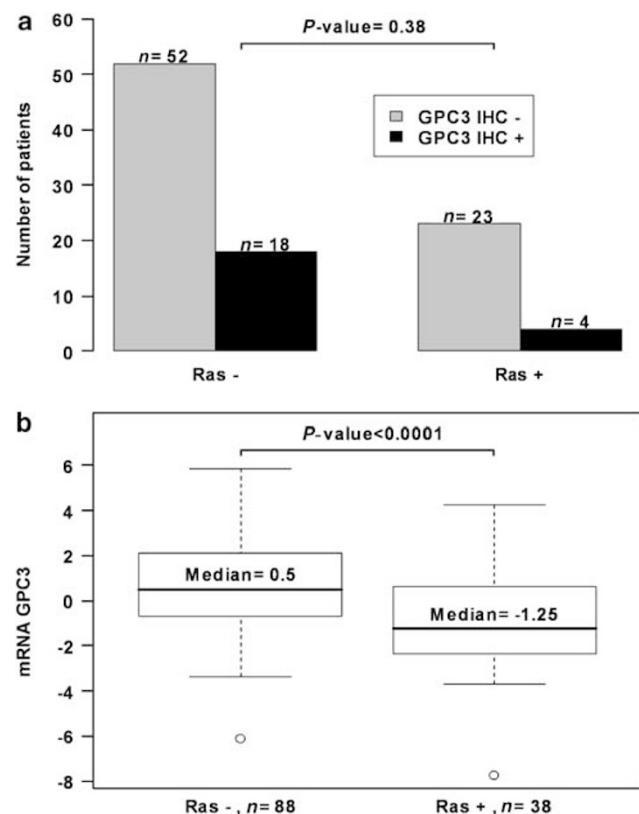|	Number of patients
Patients in either immunohistochemistry or mRNA cohorts	182
Patients not tested	17
Patients tested	165
All identified mutations	49 (30%)
KRAS	
Codon 12 (wild type is GGT)	23
–TGT	11
–GTT	5
–GAT	5
–GCT	2
–AGT	2
–CGT	1
HRAS (codon 13; CGT)	1

Figure 3 Glypican-3 protein and mRNA levels are associated with *RAS* mutations. (**a**) Fewer cases stained positively for glypican-3 in the presence of an *RAS* mutation than with wild-type *RAS*, but this difference was not significant. (**b**) The presence of *RAS* mutations was significantly associated with lower glypican-3 mRNA level. Data are presented in a box plot.

compared with in positive glypican-3 cases (median pack-year = 20 *vs* 45, respectively) (Figure 4b). There was no difference in glypican-3 mRNA expression levels between non-smokers and smokers ($P = 0.61$). However, there was a significant interaction between histology and both smoking status ($P = 0.0009$) and amount of smoking ($P = 0.0025$) with respect to their effect on glypican-3 mRNA levels. In adenocarcinoma, smokers had significantly lower glypican-3 mRNA levels than non-smokers ($P = 0.013$) (Figure 5a). In contrast, squamous cell carcinoma patients who smoked had significantly higher levels of glypican-3 expression than non-smokers ($P = 0.03$) (Figure 5b). Consistent with this observation, glypican-3 mRNA levels decreased with increased smoking in adenocarcinoma patients, whereas it increased in squamous cell carcinoma (Figure 5c). Similar analysis at the protein level, which segregated adenocarcinoma and squamous cell carcinoma patients, was limited

to 53 patients with known smoking load, and was not significant.

There was no association of glypican-3 staining with gender, age or stage, nor was it associated with patient outcome. Among the clinical factors, only stage (I *vs* II/III) was significant for overall survival
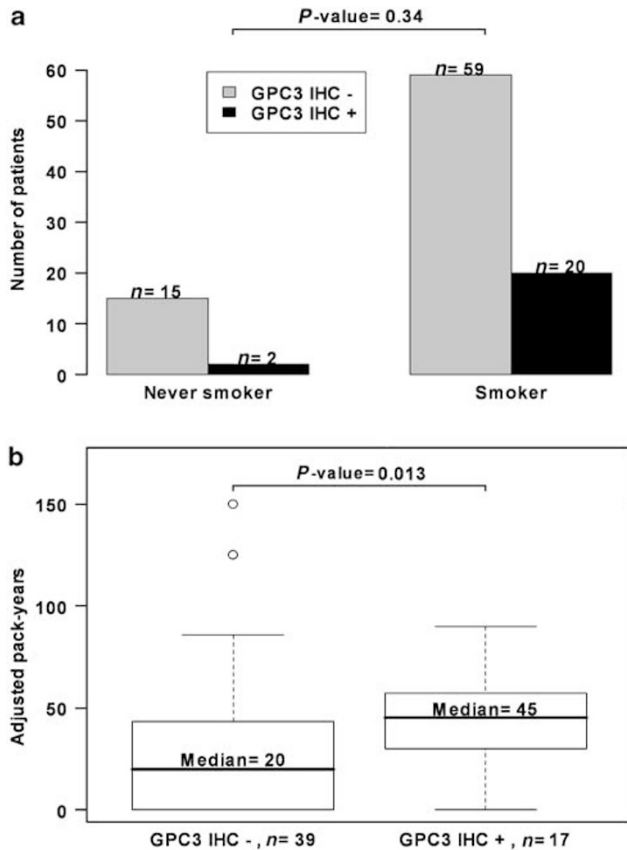
Figure 4 Glypican-3 immunostaining is associated with smoking. (**a**) Among smokers, 26% had positive glypican-3 staining compared with only 12% of non-smokers, but this difference was not statistically significant. (**b**) Significant difference between the amount of smoking in patients with negative compared with positive glypican-3 staining was observed. Data are presented in a box plot.

(HR = 2.0, 95% CI, 1.2–3.2; $P = 0.0097$), disease-free survival (HR = 1.7, 95% CI, 1.1–2.6; $P = 0.0213$) and time to relapse (HR = 1.7, 95% CI, 1.1–2.8; $P = 0.0229$). Likewise, in survival analysis of adenocarcinoma cases only, neither glypican-3 immunohistochemistry nor glypican-3 mRNA levels had prognostic significance, whereas stage remained a significant factor.

Discussion

Our study of glypican-3 expression in lung cancer resulted in new observations, which partially contradict previous reports. At the protein level, we detected overexpression of glypican-3 in tumor compared with normal lung tissue. Glypican-3 expression was highly associated with squamous cell carcinoma and the presence of wild-type *RAS* (mRNA level). The association of glypican-3 mRNA levels with smoking depended on the histology and load: in adenocarcinoma, glypican-3 expression decreased with smoking, whereas it increased in squamous cell carcinoma. These findings suggest that glypican-3 may behave as an oncogene in squamous cell carcinoma.

Kim *et al*[22] have suggested that glypican-3 is a potential lung tumor suppressor whose expression is downregulated by smoking, relaying on a series of experiments in small cohorts of adenocarcinoma. They started with gene expression profiling of 11 lung adenocarcinoma and their paired normal tissue,[27] and validated their findings by quantitative RT-PCR of the original samples and additional 11 adenocarcinoma and 17 normal lung samples. Additional validation was accomplished *in silico* using expression data from a large publicly available lung cancer microarray database,[28] which included mostly adenocarcinomas. Kim *et al* also performed some studies on lung cancer cell lines, but again chose primarily adenocarcinoma cell lines. Next, they reported that protein expression was also undetectable in lung tumors by the monoclonal anti-glypican-3 antibody 8H5; however, the number and histological classifications of the cases examined were not specified. They only observed positive immunohistochemical staining in the normal lung localized to the apical cell membrane surface of the respiratory bronchiolar epithelium.

These results do not correlate with our findings, which showed lack of expression of glypican-3 protein in normal lung tissue, including bronchiolar respiratory epithelium, whereas in squamous cell carcinoma, it was expressed both at the protein and mRNA levels. However, in view of the small cohorts used by Kim *et al*,[22] and especially due to the histology selection bias (lack of squamous cell carcinoma where glypican-3 is mainly expressed), these differences can be easily explained. Our evaluation of glypican-3 expression in normal tissue focused on pneumocytes rather than bronchiolar respiratory epithelium, and showed no expression, as was also observed by Kim *et al*. In contrast, we did not detect positive staining in bronchiolar respiratory epithelium, possibly due to differences in antibody or inter-laboratory variability.

We were unable to compare directly the level of glypican-3 mRNA in paired normal lung and tumor specimens due to lack of tissue samples. However, in view of the good correlation we found between glypican-3 protein and mRNA levels, one would expect to find higher levels of glypican-3 mRNA in cancerous compared with normal lung. The fact that previous studies[22,28] observed a reduction in glypican-3 mRNA in lung carcinoma compared with normal tissue may reflect the existence of post-translational regulation of the glypican-3 protein in normal lung that is inactivated in the latter.

The observed prevalence of *RAS* mutations (30%) and its association with adenocarcinoma are in agreement with previous studies.[29] We also found strong associations between wild-type *RAS* and high glypican-3 expression, both at the protein and mRNA levels. However, the weakening of this association in multivariate analysis suggests that
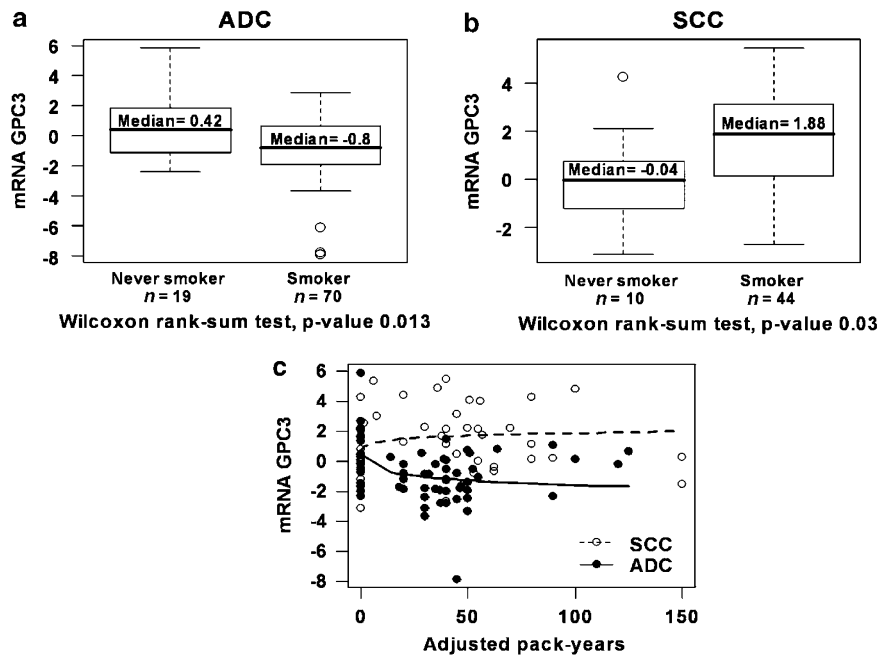
Figure 5 Glypican-3 mRNA expression levels are associated with smoking. (**a**) Adenocarcinoma smokers had significantly lower glypican-3 mRNA levels than never smokers. (**b**) Squamous cell carcinoma smokers had significantly higher glypican-3 mRNA expression levels (adenocarcinoma to squamous cell carcinoma interaction $P = 0.0009$). (**c**) Correlating smoking load with glypican-3 mRNA levels showed reduction in glypican-3 expression with increased smoking in adenocarcinoma, which is in contrast to an increase in glypican-3 expression in squamous cell carcinoma (adenocarcinoma to squamous cell carcinoma interaction $P = 0.0025$).

the glypican-3–*RAS* wild-type association may be due to the strong association between both glypican-3 and wild-type *RAS* with squamous cell carcinoma.

In our analysis, only stage had prognostic value, which is consistent with numerous previous studies.[30,31] In a meta-analysis of three microarray studies,[28,32,33] Parmigiani *et al*[23] found that high expression of glypican-3 was a predictor of better survival in lung adenocarcinoma, although glypican-3 was not associated with patient outcome in any of these three studies. Likewise, we did not find glypican-3 expression to be of prognostic significance.

We found differential association of glypican-3 expression and smoking in adenocarcinoma and squamous cell carcinoma at the mRNA level. Whereas smoking was associated with lower glypican-3 expression in adenocarcinoma, higher glypican-3 levels were detected in smokers with squamous cell carcinoma and this association also correlated with smoking load. Even though the immunohistochemistry cohort included almost twice as many adenocarcinoma as squamous cell carcinoma, the relationship between glypican-3 levels and smoking primarily reflected squamous cell carcinoma characteristics.

Cigarette smoking is the principal causal agent of lung cancer and multiple mechanisms by which smoking is carcinogenic exist, creating a range of lung neoplasms differing in their characteristics.[27,34,35] Differential association between adeno-carcinoma and squamous cell carcinoma with regards to smoking exists not just with glypican-3 expression but also with the presence of *RAS* mutations, as the causal association between smoking, *RAS* mutations and the development of a neoplasm is well established in lung adenocarcinoma but not squamous cell carcinoma.[34–36] In addition, glypican-3 itself has a complex expression pattern: in some tumors (prototyped by hepatocellular carcinoma[3,15,37] and testicular germ-cell tumors[19]), it behaves as an oncofetal protein that is overexpressed in the tumor compared with in the normal tissue, whereas in others (breast carcinoma,[2,14] ovarian carcinoma[12] and mesothelioma[13]), it is silenced and behaves as a potential tumor-suppressor gene. In our cohort of lung cancer patients, glypican-3 appeared to function as a tumor-suppressor gene in adenocarcinoma, as previously suggested,[22] whereas it behaved as an oncofetal protein in squamous cell carcinoma. In both adenocarcinoma and squamous cell carcinoma, glypican-3 expression was highly correlated with cigarette smoking. Whether glypican-3, the oncofetal protein in lung squamous cell carcinoma, also contributes to tumor progression and functions as an oncogene is yet to be explored. Further study is warranted to test this hypothesis, and should include a comparison of glypican-3 expression in a large cohort of paired tumor and normal tissues, and functional studies on a variety of lung cancer cell lines. Regardless, it is clear that glypican-3

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expression in lung carcinoma is variable and more complex than initially thought.

Glypican-3 can be detected in the serum, as it may be secreted from the tumor cells.[15,38] It has already been shown to be a reliable serum marker of hepatocellular carcinoma, which can be used for early detection of hepatocellular carcinoma patients by blood screening.[15,38,39] In this study, we have found a strong association between lung squamous cell carcinoma, glypican-3 expression and smoking. Additional studies are required to examine the association between glypican-3 immunohistochemistry in lung squamous cell carcinoma and its level in the serum. Provided that such an association exists, glypican-3 may be a promising candidate marker for early detection of lung squamous cell carcinoma by blood screening, especially among the high-risk group of smokers.

In conclusion, we found that glypican-3 is over-expressed at the protein level in lung carcinoma compared with normal lung tissue, is more likely found in squamous cell carcinoma at both the protein and mRNA levels, and is associated with wild-type *RAS* at the mRNA level. The predilections of glypican-3 expression for squamous cell carcinoma and its differential association with smoking in adenocarcinoma *vs* squamous cell carcinoma make glypican-3 a possible tumor marker and a potential oncogene in lung squamous cell carcinoma.

Acknowledgements

Conflict of interest

None of the co-authors have any conflict of interest to declare.

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