

FADD protein release mirrors the development and aggressiveness of human non-small cell lung cancer

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BACKGROUND: The need to unfold the underlying mechanisms of lung cancer aggressiveness, the deadliest cancer in the world, is of prime importance. Because Fas-associated death domain protein (FADD) is the key adaptor molecule transmitting the apoptotic signal delivered by death receptors, we studied the presence and correlation of intra- and extracellular FADD protein with development and aggressiveness of non-small cell lung cancer (NSCLC).

METHODS: Fifty NSCLC patients were enrolled in this prospective study. Intracellular FADD was detected in patients' tissue by immunohistochemistry. Tumours and distant non-tumoural lung biopsies were cultured through trans-well membrane in order to analyse extracellular FADD. Correlation between different clinical/histological parameters with level/localisation of FADD protein has been investigated.

RESULTS: Fas-associated death domain protein could be specifically downregulated in tumoural cells and FADD loss correlated with the presence of extracellular FADD. Indeed, human NSCLC released FADD protein, and tumoural samples released significantly more FADD than non-tumoural (NT) tissue ($P=0.000003$). The release of FADD by both tumoural and NT tissue increased significantly with the cancer stage, and was correlated with both early and late steps of the metastasis process.

CONCLUSION: The release of FADD by human NSCLC could be a new marker of poor prognosis as it correlates positively with both tumour progression and aggressiveness.

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Lung cancer is the deadliest cancer in the world. Non-small cell lung cancer (NSCLC), which includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC), represent ~80% of the lung cancer. The usual therapy of NSCLC consists of surgical resection of the tumour possibly preceded or followed by chemotherapy or radiotherapy. Nevertheless, most patients have poor clinical outcome in spite of these treatments, in view of the tumour–node–metastasis (TNM; T = primary tumour, N = regional lymph nodes, M = distant metastasis) status (Mountain, 1997) of the patients on the day of surgery. Although the 5-year post-surgery survival rate is >70% for T1N0M0 patients, it decreases to 10–30% when lymph nodes are invaded by tumour cells (N1–N2), and declines to <5% in distant metastasis (M1). For these reasons, the need to unfold the underlying mechanisms of lung cancer aggressiveness is of prime importance.

It has been observed that functional FasL was expressed in most resected NSCLC cells (Niehans *et al*, 1997). Thus, NSCLC might

have developed Fas-mediated apoptosis resistance mechanisms. Because FADD (Fas-associated death domain protein) is the key adaptor molecule transducing the apoptotic signal mediated by all death receptors (DRs) of the TNF receptor superfamily (Chinnaiyan *et al*, 1996; Kuang *et al*, 2000), its regulation of expression was a good candidate. Indeed, the absence of FADD can confer numerous advantages to tumour cells including multiple resistance to DRs and some anticancer drugs (Micheau *et al*, 1997), acquired ability to coexpress DRs and ligands, and a proliferative advantage (Tourneur *et al*, 2003). Furthermore, we previously confirmed that FADD can also act as a tumour suppressor (Newton *et al*, 2000; Tourneur *et al*, 2005) by showing that (1) FADD protein could be lost *in vivo* in mouse thyroid adenoma/ADC cells and human acute myeloid leukaemia cells, (2) Fas signalling, in absence of FADD, led to an accelerated growth of thyrocytes, and (3) low level or absence of FADD in leukaemia cells at diagnosis was of poor prognosis in patients' chemotherapy response (Tourneur *et al*, 2003; Tourneur *et al*, 2004).

The FADD protein is located both in the cytoplasm and nucleus of most cells (Gomez-Angelats and Cidlowski, 2003; Screaton *et al*, 2003; Tourneur *et al*, 2009). Recently, we and others ascertained a new localisation site and regulatory mechanism for the protein (Tourneur *et al*, 2008), (Kriebardis *et al*, 2008). We demonstrated that FADD could be held within plasma membrane-derived

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microvesicles, and rapidly released in the extracellular compartment following shedding of these vesicles. Moreover, we showed that adenosine receptors (AR) are implicated in this process. We showed that treatment with A₃AR inhibitor but not A₁AR, A_{2a}AR, and A_{2b}AR inhibitors triggers FADD release from mouse lung (Tourneur *et al*, 2008), demonstrating that in the lung FADD secretion is a regulated process.

Herein, we tested the hypothesis that FADD release could occur during lung cancer and be a useful marker in NSCLC. To do so, we analysed a prospective cohort of 50 NSCLC patients.

PATIENTS AND METHODS

Patients

Human primary lung tumour biopsies from 50 successive NSCLC patients were collected at the Hôtel-Dieu hospital and the Institut Mutualiste Montsouris between April 2008 and 2009, during the course of complete surgical resection of their tumours (stages I–III), including multilevel lymph node sampling or lymphadenectomy. For each patient, distant lung tissue (taken at 8–10 cm from the primary tumour) was used as non-tumoural (NT) tissue. Early metastatic invasion was defined by the presence of blood and/or lymphatic vascular emboli (BVE/LVE). The TNM stage and BVE/LVE status of the tumours were determined from the histopathological reports obtained at the time of resection. Histopathological and clinical findings were scored according to staging system of the American Joint Committee for Cancer Staging and End Results Reporting, and the TNM staging system of the Union Internationale Contre le Cancer (Mountain, 1997). The main clinical and pathological features of the patients are presented in Table 1. All patients' identities were coded to protect confidentiality. Consent was obtained from all patients and the study was approved by the local ethical committee (Agreement 2007-A00845-48).

Table 1 Patients characteristics

Category	Subcategory	All patients with NSCLC (n = 50)
Surgery, n	HDH	28
	IMM	22
Sex, n (%)	Male	32 (64%)
	Female	18 (36%)
Age, year	Mean	63, 4
	Range	44–82
Smoking history, n	Never smoker	21
	Passive smoker	1
	Smoker	28 (56%)
Preoperative chemotherapy, n (%)	Pack/year, n	40 (mean) 5–>80 (range)
Histological type, n (%)	Yes	8 (16%)
	ADC	29 (58%)
	SCC	13 (26%)
Stage, n (%)	LCC	8 (16%)
	I	19 (38%)
	IA	6
	IB	13
	II	11 (22%)
	IIA	0
	IIB	11
	III	20 (40%)
IIIA	15	
IIIB	5	
	IV	0

Abbreviations: NSCLC = non-small cell lung cancer; ADC = adenocarcinoma; SCC = squamous cell carcinoma; LCC = large cell carcinoma; HDH = Hôtel-Dieu Hospital; IMM = Institut Mutualiste Montsouris; pTNM = pathologic TNM stage. Neoadjuvant chemotherapy was based on platinum salt regimens.

Immunohistochemistry

Immunostaining with FADD antibody was performed on paraffin sections of lung tumour and distant non-tumour tissues. After antigen retrieval, FADD antibody (mouse monoclonal anti-hFADD (clone 64A6, Abcam, Cambridge, UK), 1:50 dilution) was applied and revealed with a streptavidin-biotin-peroxidase kit (BIOSSPA, Abcys, Paris, France). Peroxidase activity was revealed using diaminobenzidine (Dako Cytomation, Glostrup, Denmark). Tissue sections were then counterstained with Harris haematoxylin (Sigma-Aldrich chimie SARL, Saint Quentin Fallavier, France), and slides were mounted with Glycergel Mounting Medium (Dako Cytomation) and visualised by light microscopy (Axiovert II, Leica, Solms, Germany). Blinded examination of sections was done by an experienced lung pathologist (DD).

In vitro culture of human lung biopsies

All tumours and distant normal lung tissues were obtained immediately at the time of surgery and transported on ice to the laboratory in RPMI 1640 medium. Lung biopsies were stored at 4 °C and treated 1 day after surgery, after we have checked that storage did not modify the FADD release process. Small lung samples (1–5 mm³) were incubated in 200 µl PBS medium in the upper side of a trans-well membrane with 0.4 µm pores (BD353495, Becton Dickinson, Franklin Lakes, NJ, USA) to avoid red blood cells contamination. Lower side of the trans-well initially contained 100 µl PBS medium. After 1 h incubation at room temperature, culture mediums were collected in the lower side of the membrane and their volume measured. Lung culture mediums were kept at –20 °C until FADD detection was performed. One hour-incubated lung samples were removed and kept at –20 °C until protein extraction was performed.

Protein extraction and dosage

Total cytoplasmic proteins were extracted from lung samples using lysis buffer (10 mM Tris-HCl, 50 mM NaCl pH 7.4, 1% Triton X100, containing a cocktail of protease inhibitors) and tissue homogeniser MP FastPrep-24 (MP Biomedicals SARL, Illkirch, France). Protein extract volume was measured and sample concentration was determined using the Bradford protein assay. We then calculated the total cytoplasmic proteins quantity in each lung sample.

Fas-associated death domain protein-specific quantitative sandwich ELISA

To detect soluble FADD, 2.5 µl of PBS containing 1% Tween 20 were added to 50 µl of human lung culture mediums that were then heated for 5 min at 100 °C. The ELISA analysis was conducted using anti-hFADD antibody clone 125–140 (5 µg ml⁻¹ in PBS, Calbiochem-Novabiochem Corp., La Jolla, CA, USA) for coating, PBS 2% BSA for blocking of non-specific binding sites, anti-hFADD antibody clone A66-2 (1 µg ml⁻¹ in PBS/0.05% Tween 20/2% BSA; BD Pharmingen, San Diego, CA, USA) for detection, and peroxidase-conjugated anti-mouse IgG1 antibody (1:8000 in PBS/0.05% Tween 20/2% BSA; Caltag, Burlingame, CA, USA) for revelation. The colorimetric reaction was revealed by adding 3,3',5,5'-tetramethyl-benzidine. Reaction was stopped with 2N sulphuric acid and the plates were read at 450 nm with a Titertek multiscan spectrophotometer (MR5000, Dynatec, Mettman, Germany). Recombinant human FADD protein (Sigma-Aldrich) was used to perform standard range. As lung biopsies' size varied among the patients, for each lung sample the released FADD protein quantity (in ng) was reported to the total cytoplasm proteins quantity contained within the sample (in mg), which reflected the biopsy size.

Statistical analysis

Statistical analyses were performed by using the Wilcoxon signed-rank test and Mann–Whitney test for paired and unpaired data, respectively. Alternatively, the unpaired Student’s *t*-test could be used. *P* values of <0.05 were considered to indicate statistical significance. Correlation analyses using the Spearman method were performed with the *R* statistical software.

RESULTS

Fas-associated death domain protein loss by human NSCLC

We investigated FADD protein expression in NSCLC by immunohistochemistry. In distant NT (*n* = 6) lung tissue, FADD protein expression was restricted to bronchial epithelial cells and was never found in normal alveolar epithelial cells (pneumocytes) (Figure 1A). Similarly, in the tumour (T, *n* = 24) area, high and low FADD expression was observed in bronchial and alveolar cells, respectively (Table 2). We found a variable patient-dependent FADD protein expression in tumoural cells (Table 2 and Figure 1B), with sometimes coexistence of both FADD-positive and -negative tumoural cells within the same T area. In such cases,

FADD was mainly expressed in tumoural cells located at the periphery of the tumour nests (data not shown). Whereas tumoural cells from stage I patients expressed FADD, the expression in tumoural cells from stage II patients was very low to barely detectable (Table 2). However, once the cancer has fully fixed itself (stage III), FADD expression did not decrease anymore. In all cases, we detected FADD expression exclusively in cytoplasm (Figure 1C).

Tumour samples are heterogeneous tissues containing tumoural cells, potentially hyperplastic cells, normal lung cells and immune cells. In light of that, we investigated the release’s potential of different areas of a same tumour biopsy. Distant NT tissue that is postulated to contain only normal lung cells was used as control. We detected the FADD protein in the culture medium from both T and NT NSCLC biopsies (Figure 2A). These results argued for FADD release by human cells *in vitro*. As expected, FADD release by NT tissues was quite homogeneous for different samples from the same biopsy (mean s.d. = 7.4 ± 2.9 ng FADD per mg proteins in the tissue (PT), Figure 2A). In contrast, the amount of FADD released by different areas from the same T biopsy varied (mean s.d. = 12.3 ± 3.8 ng mg⁻¹ PT, Figure 2A).

We then investigated FADD release by T and NT tissues from a 50 NSCLC patients prospective cohort. We detected extracellular FADD protein in the culture medium from both T and NT NSCLC biopsies and showed that T tissue released significantly more FADD than distant NT tissue (Figure 2B, *P* = 0.000003). Results obtained by ELISA were confirmed by western blot analysis (Supplementary Figures 1–3). We observed no correlation between

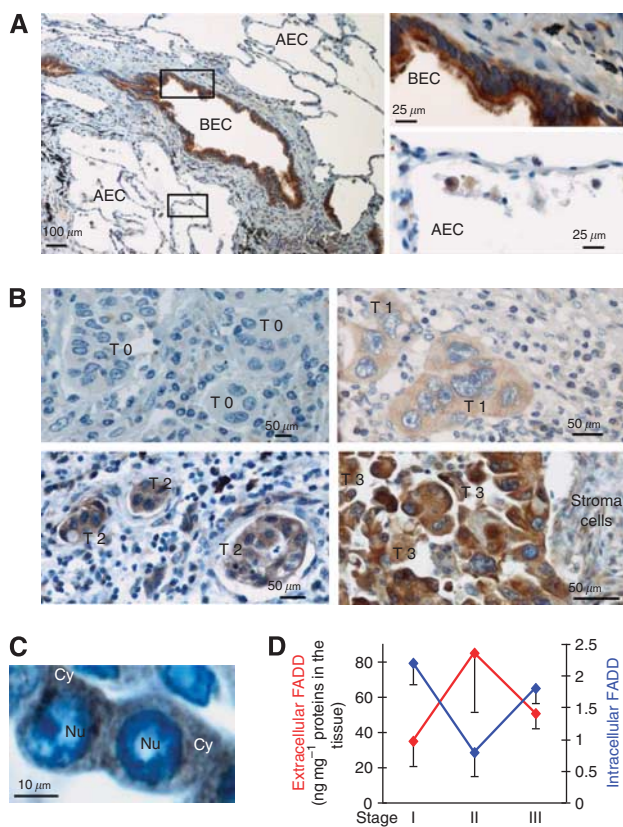


Figure 1 Loss of FADD in *ex vivo* NSCLC can result from FADD release. (A–C) Fas-associated death domain protein immunohistochemistry analysis of NT (A) and T tissues (B and C). T0, neg; T1, +; T2, ++; T3, + + +. A, alveolar; B, bronchial; EC, epithelial cells; Cy, cytoplasm; Nu, nucleus; T, tumour. (D) According to the patients’ cancer stage, *in vitro* T biopsies’ FADD release (extracellular FADD) determined by ELISA and *ex vivo* tumour cells’ intracellular FADD expression determined by IHC were inversely correlated (*n* = 24 patients, all from the Hôtel-Dieu). Data represented mean ± s.e.m. Intracellular FADD expression concerns tumour cells only (not normal epithelial cells). Stage I, II, III: *n* = 5, 6, and 13, respectively.

Table 2 Loss of FADD in *ex vivo* NSCLC

Patients	Stage	Tumour biopsies intracellular FADD expression ^a		
		Tumour	Alveolar EC	Bronchial EC
35	I	3	np	np
31	I	3	I	3
77	I	1.5	0 and I near T	3
76	I	2	0.5	3
69	I	1.5	0	3
Mean stage I		2.2	0.5	3
44	II	0	0 and I Hyp	3
70	II	0.5	0	np
30 ^b	II	3/ 0 LNM	2/ 0	3/ 2
55 ^b	II	0.5/ np	0/ np	2/ 3
50	II	0.5	0	np
48 ^b	II	1/ np	0.5/ 0	1/ 1
Mean stage II		0.8	0.4	2.1
37	III	2	0	np
33	III	3	I	3
49	III	2	0	3
86 ^b	III	3/3	0.5/ np	2/ 3
83	III	0 and 2 VE	0.5	3
82 ^b	III	2/ np	2/ 0	2.5/ 2
68 ^b	III	2/ 2	np/ np	np/ np
61 ^b	III	1.5/ np	0/ 0	3/ 1
87	III	np	0	3
88 ^b	III	np/ 2	2/ np	3/ 3
59 ^b	III	np/ 0.5	0 and 2 Hyp/ I	3/ 3
46	III	I	1.5	2
28 ^b	III	1/ np	0/ 0	np/ np
Mean stage III		1.8	0.6	2.6

Abbreviations: EC = epithelial cells; FADD = Fas-associated death domain protein; np = structure not present in the section; Hyp = hyperplasia; LNM = lymph node metastasis; VE = vascular emboli; T = tumour. Correlation between patients’ cancer stage and FADD expression in T area determined by IHC. All patients were treated at the Hôtel-Dieu hospital. ^a0, neg; 0.5, ±; 1, +; 1.5, +/ + +; 2, + +; 2.5, + +/ + + +; 3, + + +. ^bIndicates that two samples of a same biopsy were embedded in distinct paraffin blocks that were sectioned and analysed.

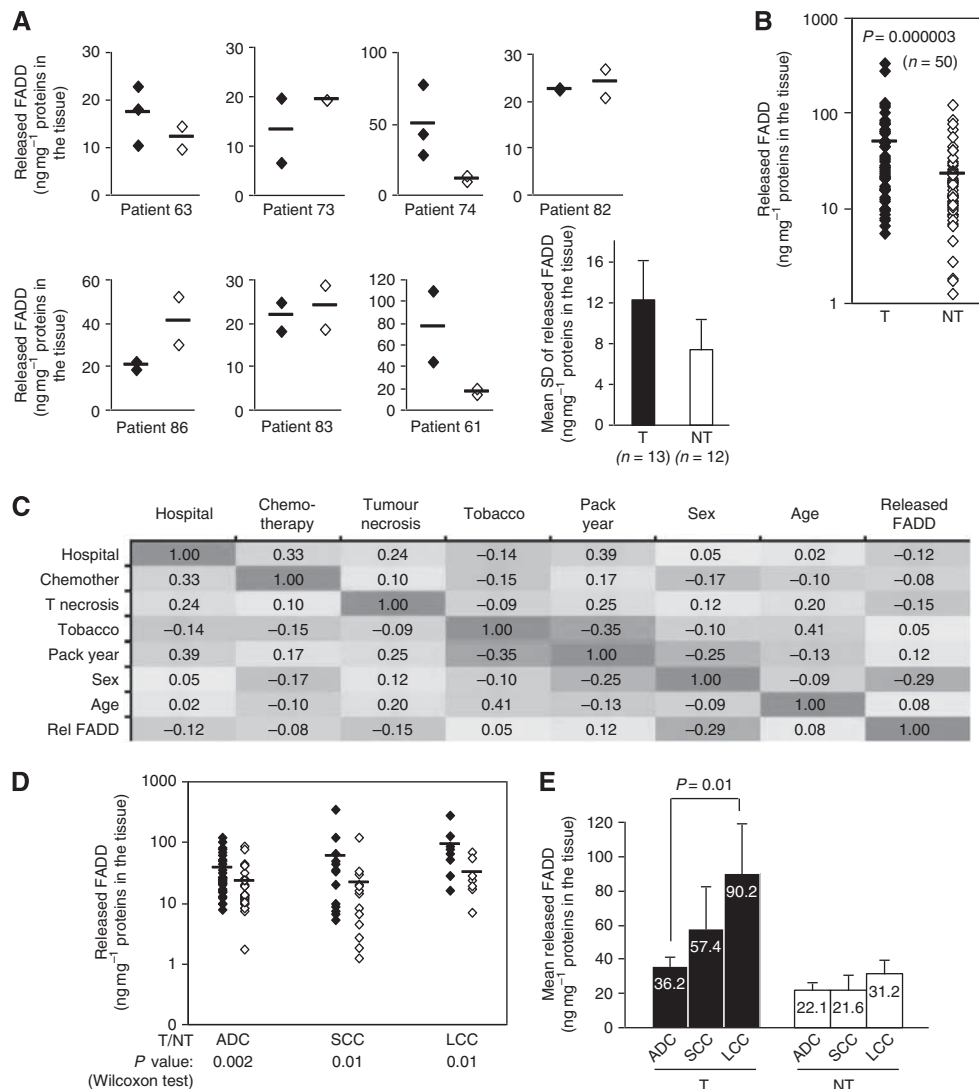


Figure 2 Fas-associated death domain protein is released by human NSCLC. The amount of FADD released by T (black) and by distant NT (white) biopsies *in vitro* was determined by ELISA. **(A)** Fas-associated death domain protein release by different samples (diamond) of the same biopsy is shown for seven patients. Bars represent the mean value for each patient. Histogram represents the mean s.d. of FADD release for T and NT patients. **(B)** Fas-associated death domain protein release by T and NT biopsies from 50 NSCLC patients. When several samples of a same biopsy were tested, we used the samples' mean value. Each diamond represents a patient. Bars represent the mean value for the entire cohort. *P* values were determined using the Wilcoxon signed-rank test. Range for T and NT biopsies were from 5.3 to 339.7 and 1.2 to 122.5 ng mg⁻¹ PT, respectively. **(C)** Spearman's correlation. **(D and E)** Fas-associated death domain protein release depends on the NSCLC histological type. Levels of released FADD determined in Figure 2B were reanalysed after the 50 NSCLC patients were classified according to their histological type. Each diamond represents a patient biopsy (T in black, NT in white). Bars represent the mean value **(D)**. Histograms represent the mean released FADD value \pm s.e.m. *P* values were determined using the Mann-Whitney test **(E)**.

FADD release by lung biopsies and patient's hospital, sex, age, chemotherapy, tobacco consumption, or tumour necrosis state (Spearman's correlation test, Figure 2C).

Presence of extracellular FADD in human NSCLC culture depends on the histological type

We investigated whether FADD release differed according to histological type. We observed that T biopsies released significantly more FADD than NT biopsies, and this independently of the histological type (Figure 2D). The NT biopsies from ADC, SCC, and LCC patients released similar level of FADD (Figure 2E). Despite being a morphologically and clinically heterogeneous disease, ADC was the NSCLC with the most reproducible extracellular FADD level, demonstrating that the differentiation

state of ADC did not influence the FADD release process (Figure 2D). Moreover, T ADC was the histological type releasing significantly the least FADD, whereas T LCC was the one releasing the most (*P* = 0.01, Figure 2E).

Presence of extracellular FADD in human NSCLC culture is correlated with patients' cancer stage

To investigate whether FADD release could be involved in tumour progression and aggressiveness, we looked for a correlation between extracellular FADD level and patients' cancer stage. We showed that FADD release by both T and NT biopsies increased with the cancer stage (Figure 3A and B), suggesting that extracellular FADD could be a poor prognostic marker. Fas-associated death domain protein increase was significant for

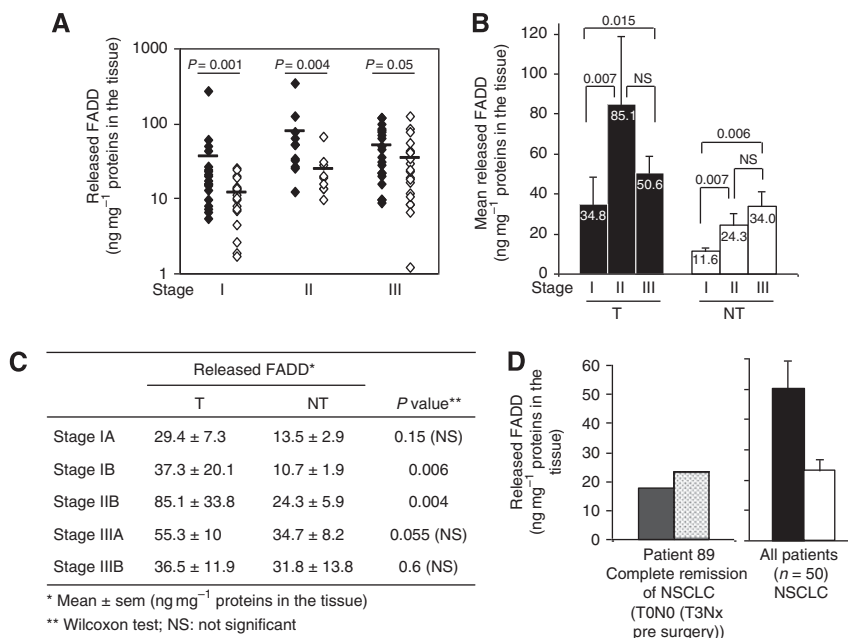


Figure 3 Fas-associated death domain protein release is correlated with cancer development. (A–C) Fas-associated death domain protein release is correlated with patients' cancer stage. Levels of released FADD determined in Figure 2B were reanalysed after the 50 NSCLC patients were classified according to their cancer stage. Each diamond represents a patient biopsy (T in black, NT in white). Bars represent the mean value. P values were determined using the Wilcoxon signed-rank test (A). Histograms represent the mean released FADD value ± s.e.m. P values were determined using the Mann–Whitney test (B). (D) Patient 89 is a 54-year-old smoking (60 pack per year) man. Surgical resection of brain metastasis was performed in 2007, followed by 2 years of radiotherapy and chemotherapy. Fas-associated death domain protein release by remitted tumour (dark grey) and adjacent parenchyma (light grey) biopsies following 1-h *in vitro* culture was determined by ELISA, and was compared with the mean level of FADD released by the 50 NSCLC T (black) and NT (white) biopsies. Abbreviation: NS = not significant.

T biopsies between stages I/II ($P = 0.007$), and I/III ($P = 0.015$), but was no more significant between stages II/III ($P = 0.4$) (Figure 3B). Moreover, the highest level of FADD was released by stage II T biopsies, which corresponds to the stage where the lowest cellular FADD expression was observed (Figure 1D).

We found that distant lung tissue, which was classified as non-tumour tissue, acquired the ability to release high level of FADD during the course of tumour progression (Figure 3A and B). Confirming this data, we observed that although stage I T tissue released significantly more FADD than distant NT tissue ($P = 0.001$), the difference between T and NT released FADD level decreased with cancer progression (Figure 3A). These results were confirmed using the ISSLC stage grouping of the TNM subsets (Figure 3C). Furthermore, we observed that lung biopsies from patient 89 who had achieved complete remission following radiotherapy and chemotherapy, released low level of FADD (Figure 3D). Altogether, these results hinted that FADD release increased during tumour development, accordingly to the cancer stage, and returned to the basal level following complete remission.

Presence of extracellular FADD in human NSCLC culture is correlated with early and late steps of the metastasis process

Early steps of the metastasis process include blood and lymphatic vascular emboli that can arise within the tumour or at its periphery. The T biopsies from patients with lung vascular emboli released a higher level of FADD than those of unaffected counterparts (Figure 4A, $P = 0.04$). Whatever its type (BVE/LVE) and localisation (intra/peri-tumour), the emboli was always correlated with an increased amount of extracellular FADD (Supplementary Tables 1 and 2). Distant NT biopsies from NSCLC patients with lung emboli released amount of FADD equivalent to T biopsies from patients without emboli (T (A) vs NT (P)). These

results confirmed that invasive/aggressive tumour (i.e., able to form distant metastasis) affected distant 'non-tumour' lung tissue that behaved like T tissue regarding FADD release. These results suggested that FADD release by T/NT biopsies and vascular emboli formation are positively correlated.

Late steps of the metastasis process include the formation of regional lymph node metastasis (N status). Tumour tissue released more FADD than distant NT tissue. The T/NT difference was even more significant for N0 patients who did not have lymph node metastasis (Figure 4B, $P = 0.01$). However, the difference between T and NT release waned until becoming insignificant for patients with regional lymph node metastasis (Figure 4B, N1 and N2). In addition, a relevantly higher level of FADD was released by T biopsies from patients with lymph node metastasis (N+) than patients without (N0), and the same result was observed for NT biopsies ($P = 0.03$ and $P = 0.02$, respectively) (Figure 4C). These results demonstrated that increased aggressiveness of the tumour, characterised by regional lymph node metastasis, was correlated with a strong increase in the ability of T as well as distant NT tissues to release FADD.

DISCUSSION

The overall data, obtained in a prospective cohort of patients, showed that FADD release by both T and NT biopsies was positively correlated with the patients' cancer stage, the early and late steps of the metastasis process, and the histological type (Figure 2D, E, 3 and 4). Moreover, the data described herein brings an explanation for intracellular FADD loss by human NSCLC tissues that could result from FADD release. As patient's TNM stage, presence of tumour vascular emboli, lymph node metastasis, and histological type are poor prognostic factors, the ability of lung tissue to release FADD appeared as a poor prognostic marker.

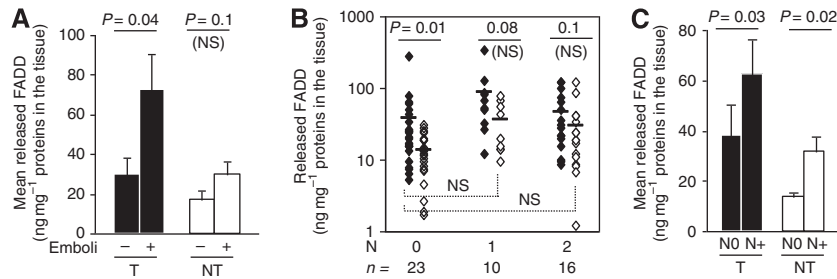


Figure 4 Fas-associated death domain protein release is correlated with cancer aggressiveness. (A–C) Fas-associated death domain protein release by human NSCLC is positively correlated with the presence of vascular emboli and the lymph node invasion. (A) For 26 patients of the cohort, the level of released FADD is correlated with the presence (+, $n=22$) or absence (–, $n=4$) of vascular emboli. For other patients, histopathological report gave no information on the presence or absence of emboli. (B and C) Levels of released FADD determined in Figure 2B were reanalysed after the 50 NSCLC patients were classified according to their lymph node invasion status. Each diamond represents a patient biopsy (T in black, NT in white). Bars represent the mean value. n represents the number of patients; one patient with N3 NSCLC is not represented. P values were determined using the Wilcoxon signed-rank test (B). Histograms represent the mean released FADD value \pm s.e.m. of biopsies from N0 ($n=23$) and N+ ($n=26$) patients (C). P values were determined using the Mann–Whitney test (A, C). Abbreviation: NS = not significant.

As we performed a prospective study, all patients were alive at the time of the study. Consequently, at the present time we could not correlate presence of extracellular FADD and patients' overall survival.

We first investigated FADD protein expression in NSCLC and found a variable cancer stage-dependent FADD protein expression in tumoural cells, with a very low expression in tumoural cells from stage II patients. In lung ADC, FADD protein phosphorylation and nuclear localisation have been associated with aggressive cancer and poor prognosis (Bhojani *et al*, 2005; Chen *et al*, 2005). Therefore, it is likely that different mechanisms might be involved to regulate FADD expression or activity in NSCLC. Overexpression of phosphorylated nuclear FADD in cancer cells could lead to NF- κ B activation and higher proliferation rate (Bhojani *et al*, 2005; Chen *et al*, 2005). However, we showed that, alternatively, FADD could be underexpressed in cancer cells cytoplasm, thus conferring survival advantages through DRs-induced apoptosis resistance. Moreover, the very low expression observed in stage II cancer cells suggested that downregulation of FADD could be a mechanism contributing to disease development, probably more importantly during early (from I to II) than late (from II to III) stages of cancer development.

Several mechanisms could explain the presence of extracellular FADD in cultured NSCLC. We primarily hypothesised that FADD release was the result of cells dying within a tumour. However, the release of FADD started as soon as 5 min of biopsies' incubation (Supplementary Figure 4), and *in vitro* incubation of lung biopsies never exceeded 1 h, which did not seem sufficient to induce a massive cell death. In accordance with this assumption, and independently of the level of FADD released, we observed only a slight activation of caspase 8 during FADD release, and no LDH (which is released upon cell lysis) could be detected in the culture medium from 1-h incubated NSCLC tumour biopsies (Supplementary Figure 5). Moreover, FADD release was not correlated with the necrosis state of the tumour (Figure 2C). Then, considering the results previously obtained in mouse normal lung (Tourneur *et al*, 2008), we hypothesised that FADD release from human NSCLC could occur by the means of secretion. In accordance with this hypothesis, we found that FADD could be specifically lost in tumoural cells (Table 2), and the highest level of FADD was released by stage II' T biopsies, which corresponds to the stage where the lowest cellular FADD expression was observed (Figure 1D). These data showed that, in tumour tissues, released and cellular FADD amounts were negatively correlated, suggesting that the release of FADD could much more be a mechanism accounting for cytoplasmic FADD loss than any type of cell death affecting specifically and in a stage-dependent manner the lung cancer cells.

Considering the key role played by FADD in signal transduction (Newton *et al*, 1998; Yeh *et al*, 1998; Zhang *et al*, 1998; Newton *et al*, 2000; Beisner *et al*, 2003; Hua *et al*, 2003; Screaton *et al*, 2003; Balachandran *et al*, 2004; Ma *et al*, 2004; Pyo *et al*, 2005; Tourneur *et al*, 2005; Bell *et al*, 2008; Tourneur and Chiocchia, 2010), the latter's release could modulate several essential biological processes. The significant higher level of FADD released by T tissue compared with NT tissue suggests that FADD release could be a regulated process preferentially occurring in pathological conditions, and thus potentially contributing to cancer. These results obtained in human NSCLC are in accordance with those obtained in mice showing that FADD secretion does not occur spontaneously but rather in a controlled manner in the lung (Tourneur *et al*, 2008). The fact that human biopsies are not taken from healthy lungs but at a distance from the tumour could explain the discrepancy between the lack of secretion by normal mouse tissue and the observed release by human NT lungs. One other possibility is a differential regulation of such process in humans and mice. It is also possible that the tumour environment either influences, modifies, or even enables the NT tissue to release FADD. The presence of hyperplastic cells observed in some NT biopsies (not shown) could support the latter hypothesis. Data showing that FADD release by NT biopsies evolved similarly to FADD release by T biopsies, although the level of released FADD was lower, seemed appealing in that sense (Figures 3 and 4). In light of these data, we must heed the bias existing in using NT tissues as control tissues. Moreover, it opens new intriguing questions regarding the consequence of FADD protein release, both for the releasing (survival/growth advantage?) and the surrounding (apoptosis induction/malignant transformation?) cells.

The observation that FADD release by lung biopsies increased with the cancer stage led to the assumption that FADD release may contribute to tumour progression. As the highest level of FADD was released by stage II' T biopsies, FADD release may be more important during early (from I to II) than late (from II to III) stages of cancer development, confirming data obtained with cellular FADD. Undoubtedly, intracellular FADD loss resulting from FADD release could confer survival advantages to tumoural cells by multiple resistance to DRs and simultaneously to immune cells. Moreover, acquired ability to coexpress DRs and death ligands could mount the tumour 'counter-attack' (O'Connell *et al*, 1996; Niehans *et al*, 1997; Buzyn *et al*, 1999). More recently, FADD has been implicated in autophagy (Pyo *et al*, 2005; Bell *et al*, 2008). It has been shown that FADD deficiency abrogated autophagic cell death in HeLa cells and promoted hyperautophagy in T cells. Thus, it might be hypothesised that release of FADD leading to intracellular FADD loss could enhance autophagy in NSCLC cells. As autophagy could promote metastasis development by

protecting stressed tumour cells as they travel through the vasculature (Kenific *et al*, 2010), it is possible that FADD release could participate to metastasis formation in NSCLC. Consequently, FADD release by tumoural cells could contribute to disease development and blocking FADD release could probably help to inhibit cancer progression. The very low 5-year overall survival of patients (14%) despite existing treatments stress the prime importance of understanding the molecular mechanisms underlying FADD release, as involved molecules could constitute potential therapeutic targets that could improve health benefits.

The abovementioned ability of T and distant NT biopsies to release FADD seemed to be poorly correlated with an increase in tumour size (T status, Supplementary Figure 6), but instead was correlated with the potential of tumoural cells to invade the lymph nodes (N status) (Figure 4B and C). This is undeniably of importance as the NSCLC patients' 5-year survival rate dramatically decreases to 10–30% when lymph nodes are invaded by the tumour (N+ patients). By the same way, FADD release by T/NT biopsies and vascular emboli formation are positively correlated (Figure 4A). This result was interesting as the presence of intratumour, peritumour, or both neoplastic vascular emboli was linked to declined 5-year survival of NSCLC patients (24% vs 43% in absence of emboli) (Alifano *et al*, 2009). Our data pointed out

that the level of released FADD by T biopsies and the patients 5-year survival rate were inversely correlated, confirming that FADD release may have an important role in tumour aggressiveness and may be used as a poor prognostic marker.

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Conflict of interest

The authors declare no conflict of interest.

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