

Neurotensin Receptor 1 Determines the Outcome of Non–Small Cell Lung Cancer

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Abstract

Purpose: This study aimed to investigate the role of the neurotensin/neurotensin receptor I (NTSR1) complex in non–small cell lung cancer (NSCLC) progression.

Experimental Design: The expression of neurotensin and NTSR1 was studied by transcriptome analysis and immunohistochemistry in two series of 74 and 139 consecutive patients with pathologic stage I NSCLC adenocarcinoma. The findings were correlated with clinic-pathologic features. Experimental tumors were generated from the malignant human lung carcinoma cell line A459, and a subclone of LNM35, LNM-R. The role of the neurotensin signaling system on tumor growth and metastasis was investigated by small hairpin RNA–mediated silencing of NTSR1 and neurotensin.

Results: Transcriptome analysis carried out in a series of 74 patients showed that the positive regulation of *NTSR1* put it within the top 50 genes related with relapse-free survival. Immunohistochemistry revealed neurotensin- and NTSR1-positive staining in 60.4% and 59.7% of lung adenocarcinomas, respectively. At univariate analysis, NTSR1 expression was strongly associated with worse 5-year overall survival rate ($P = 0.0081$) and relapse-free survival ($P = 0.0024$). Multivariate analysis showed that patients over 65 years of age ($P = 0.0018$) and NTSR1 expression ($P = 0.0034$) were independent negative prognostic factors. Experimental tumor xenografts generated by neurotensin- and NTSR1-silenced human lung cancer cells revealed that neurotensin enhanced primary tumor growth and production of massive nodal metastasis via autocrine and paracrine regulation loops.

Conclusion: NTSR1 expression was identified as a potential new prognostic biomarker for surgically resected stage I lung adenocarcinomas, as NTSR1 activation was shown to participate in lung cancer progression. *Clin Cancer Res*; 16(17); 4401–10. ©2010 AACR.

Lung cancer is the leading cause of cancer-related deaths in the United States and remains the most common malignancy in the world (1–3). Two main histologic categories are recognized: small cell lung cancer and non–small cell lung cancer (NSCLC). NSCLC is usually further divided into three main histologic types: large cell lung carcinoma,

squamous cell carcinoma, and adenocarcinoma. This last represents nowadays the most frequent histologic type in western countries. NSCLC is believed to arise from a multi-step process, with each step associated with genetic and epigenetic alterations, and correlated with tumor aggressiveness. The factors used to define the stage of the disease, to choose the optimal management, and to predict outcome are the size of the primary tumor, the invasion of locoregional nodes, and the presence of distant metastases (4). Nevertheless, a vast disparity in patient outcome is seen within the same stage. Globally, patients with operable lung cancers (stage I–IIIa) have an overall 5-year survival rate of around 40%. The survival rate among those with stage I disease is only 60% to 70%; in a quarter of these patients relapse is local, whereas for the others the disease shows metastatic spread (4–6). The current challenge today is to identify factors that would predict tumor relapse despite curative treatment. In a series of stage IB lung adenocarcinomas, we carried out a tandem DNA copy number and gene expression profile using high-resolution microarrays to establish a robust predictor of clinical outcome

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Translational Relevance

Non-small cell lung cancer is a heterogeneous condition with significant variability in prognosis and in the individual response to treatments. Therefore, identification of patients with high risk of relapse after surgery will enable tailored management in terms of adjuvant treatments and stricter follow-up. We report that the neurotensin/neurotensin receptor 1 (NTSR1) complex is specifically expressed in lung adenocarcinoma. NTSR1 expression was identified as an independent predictive marker of unfavorable outcome in stage I lung adenocarcinoma treated by surgery alone. Experimental data suggest that the neurotensin/NTSR1 complex is an enhancer of lung tumor progression. The neurotensin system is therefore suitable for the development of new therapeutic targets, new applications of already available treatments, and the development of a new diagnostic biomarker ensuing better predictive parameters.

at the early stages of NSCLC (7). Among the first 50 genes upregulated and associated with disease-free survival was the neurotensin receptor 1 (*NTSR1*).

Neurotensin and its cognate receptor (NTSR1) are neuropeptide-receptor complexes frequently deregulated during the neoplastic process. Neurotensin is a 13-amino-acid peptide previously recognized for its distribution along the gastrointestinal tract (8). Typical physiologic functions associated with neurotensin include the stimulation of pancreatic and biliary secretions, inhibition of small bowel and gastric motility, and facilitation of fatty acids translocation (9, 10). The peripheral functions of neurotensin are mediated through its interaction with NTSR1, a high affinity receptor coupled to a Gq/G11 protein. When neurotensin binds to NTSR1, phosphatidylinositols are hydrolyzed leading to Ca²⁺ mobilization and PKC activation.

NTSR1 activation leads to cell proliferation, survival, mobility, and invasiveness in specific cancer cell types via signal transduction through PKC, extracellular signal-regulated kinase 1 and 2, RhoGTPases, NF- κ B, or focal adhesion kinase activation. (11–13). The disruption of the neurotensinergic pathway through a specific antagonist, in experimental tumors from colon, breast, and small cell lung cancer cells, caused a strong reduction in tumor growth (14–16). We had previously shown the presence of a chronic self-activation loop between neurotensin and NTSR1, as one mechanism responsible for the constitutive activation of the mitogen-activated protein kinase mitogenic signaling pathways along with sustained target gene activation (17–20). More recently, NTSR1 expression level was associated with poor prognosis in patients with ductal breast cancer, and similar results have

been found in head and neck squamous cell carcinomas (21, 22).

In this study, we examined the expression of both neurotensin and NTSR1 in two series of consecutive patients undergoing pulmonary lobectomy and nodal dissection for pathologic stage I lung adenocarcinoma by transcriptome analysis and immunohistochemistry, respectively. The expression of NTSR1 was clearly shown to negatively affect the outcome. Experimental tumors were also developed to study the role of the neurotensin system on tumor growth. With this model, we show the aggravating role of the neurotensinergic system in tumor progression.

Materials and Methods

Study from a genome-wide gene expression

We report the results obtained for NTSR1 from a previous large-scale gene expression analysis carried out on 74 homogeneous cases of stage pT₂N₀ lung adenocarcinomas/large cell carcinomas treated by surgery (7). In this study RNA samples were hybridized to the Human U133 Plus 2.0 oligonucleotide arrays (Affymetrix), and 37,771 probes met the quality control criteria and were considered for the analysis (GEO Series accession number GSE10445). We selected transcripts having a high likelihood of being associated with relapse-free survival (RFS) by considering the false discovery rate error as described in Broet et al. (7).

Patients and tissue specimens for neurotensin and NTSR1 immunohistochemistry

Expression of NTSR1 and neurotensin was done in a multicenter study. The clinical files of 139 patients (Table 1) treated by lobectomy and full nodal dissection for pathologic stage I (pT₁N₀, n = 51; pT₂N₀, n = 88) primary lung adenocarcinomas were retrospectively reviewed. They were operated on in three teaching hospitals in Paris, France and Bologna, Italy between January 2001 and March 2003. All patients had macroscopically and microscopically complete resections. None of the patients had preoperative or postoperative chemotherapy or radiotherapy. For all cases histologic slides of primary tumors were obtained by paraffin wax embedded tissues. Standard H&E staining was used to ensure the tumoral character of the specimen, and adjacent sections were obtained for immunohistochemistry. For 23 of 71 patients operated on at the Hôtel-Dieu Hospital, frozen samples of resected tumors were used to detect RNA for neurotensin and NTSR1, after verification of the tumoral character of the samples by frozen sections. As normal tissue, we assessed the lung parenchyma of 26 patients with idiopathic pneumothorax treated by apical resection.

Ethics

The study was carried out according to the Declaration of Helsinki principles and in agreement with the French and Italian laws on biomedical research. The following studies were conducted on tissues obtained from surgical specimens between 2001 and 2003. The experiments reported here were carried out under the current ethical

regulations as defined by the Huriet-Sérusclat Act of December 20, 1988. Under this act, institutional review board approval was not required. Accordingly, patients or next of kin (in case of deceased patients) were specifically asked for verbal informed consent only.

Statistical analysis

Overall survival rates were estimated using the Kaplan-Meier method, and survival curves were compared using the Log-rank test. RFS time was calculated from the date of the patients' surgery until disease-related death, disease recurrence (either local or distant), or last follow-up examination. All available variables potentially influencing survival, namely, age, sex, smoking habit (current and former versus never smoker), presence of vascular or lymphatic or vascular emboli, pT category, and NTSR1 expression, were considered for a multivariate analysis using the Cox proportional hazards model.

For the transcriptome analysis, the prognostic impact of *NTSR1* gene expression changes on RFS was evaluated by calculating the score test statistic derived from the semi-parametric Cox proportional hazards model. The analyses were done with the SPlus software package.

Immunohistochemistry

Immunostaining of neurotensin and NTSR1 was carried out on 4- μ m-thick deparaffinized sections, using the avidin-biotin-peroxidase complex method. Slides were incubated with 10% normal rabbit serum at room temperature for 30 minutes. Neurotensin immunoreactivity was conducted using rabbit antibody directed against neurotensin (1:500; NA1230, Biomol GmbH) for 2 hours. NTSR1 immunoreactivity was detected using a goat polyclonal antibody directed against the human carboxy terminus of the receptor (1:100; C-20, Santa Cruz Biotechnology). All slides were rinsed three times with TBS; sections were incubated with biotinylated secondary antibody (1:200; Vector laboratories, Inc). The antigen-antibody complex was revealed with avidin-biotin-peroxidase complex, according to the manufacturer's instructions for the Vectastain ABC Kit (Vector laboratories, Inc.). Staining was done with diamino-benzidine tetrahydrochlorid. All slides were counterstained with hematoxylin. All specimens were scored by an anatomopathologist (SCB).

Culture procedures

The human lung adenocarcinoma cell lines LNM35 and A459 were grown in RPMI-1640 medium (Invitrogen

Table 1. Clinical characteristics of the multicenter series of patients whose tumors were studied by immunohistochemistry for neurotensin and NTSR1 expression

Lung adenocarcinomas, *n* = 139

Age (years)	64.0 \pm 10.7
Women	34/139 (24.5%)
Tobacco history	
Current smoker	71
Former smoker (stop >2 months)	49
Never smoker	19
pT ₁ disease	51/139 (36.7%)
pT ₂ disease	88/139 (63.3%)
Tumor size (cm), mean \pm SD	3.1 \pm 1.94
N ₀	139/139
Intratumoral or peritumoral neoplastic vascular emboli	34/139 (24.5%)
Intratumoral or peritumoral neoplastic lymphatic emboli	21/139 (15.1%)
NTSR1-positive tumors, <i>n</i> (% of patients)	83/139 (59.7%)
Neurotensin -positive tumors, <i>n</i> (% of patients)	84/139 (60.4%)
NTSR1- and neurotensin-positive tumors, <i>n</i> (% of patients)	54/139 (38.8%)
Lost at follow-up	1/139 (0.7%)
Follow-up in months, mean \pm SD	44.2 \pm 20.9
Deaths during follow-up, <i>n</i> (% of patients)	49/139 (31%)
Perioperative therapies	
Radiotherapy	0/139
Chemotherapy	0/139

Idiopathic pneumothorax, *n* = 26

NTSR1-positive lung parenchyma	0/26
Neurotensin-positive lung parenchyma	0/26
NTSR1- and neurotensin-positive lung parenchyma	0/26

SARL) supplemented with 10% FCS and 2 mmol/L glutamine (23). The LNM35 cell line was subcloned by limiting dilution; clones containing exclusively rounded cells were named LNM-R.

NTSR1 and neurotensin siRNA construction and transfection. Small hairpin RNAs for human NTSR1 (AAGAAGTTCATCAGCGCCATC) and neurotensin (5'-GCAATGTTGACAA-TATACC-3') were prepared using psilencer 3.1-H1 according to the manufacturer's instruction. LNM-R and A549 cells were transfected using the Lipofectamine reagent (20).

Experimental tumors

Xenografts were initiated in nude mice by s.c. injection of 10^6 cells of LNM-R and derivative cell clones, or 10^7 cells of A549 and A549 NTSR1-silenced clones. For tumors generated from a cell mixture, 10^6 cells from each clone were plated together 72 hours prior to injection. Four to six series were done; each series included 5 to 8 mice. The tumor volume was calculated with the ellipsoid formula.

RNA extraction and reverse transcriptase-PCR. The protocols for total RNA extraction, reverse-transcription reaction, and PCR are documented in detail in Souazé et al. (24). Reverse-transcription reaction was done on 2 µg of total RNA using a specific NTSR1 primer (5'-GCTGACGTAGAAGAG-3') or 50 pmol of oligo dT and oligo dN. The PCR amplification was done on a 1:5 v/v of the reverse-transcription reaction using 25 pmol of each primer 5'-CGTGGAGCTGTACAACCTCA-3' and 5'-CAGCCAGCAGACCACAAAGG-3' for NTSR1, and 5'-CAGCTCCTGGAGTCTGTGCT-3' and 5'-GTTGAAAAGCCCTGCTGTGACAGA-3' for neurotensin, 5'-TCAAATGAGATTGTGGAAA-3 and AS 5-AG-ATCATCTCTGCCTGAGTAT-3' for cyclooxygenase-2, 5'-CGGAGTCAACGGATTGGTTCG-3' and 5'-TTCACCACATGGAGAAGGCT-3' for GAPDH, and 1 unit of Taq polymerase.

Neurotensin RIA

One million cells were grown in 60 mm² Petri dishes. After 24 hours media were removed and serum-free media were added to the cells for 24, 48, or 72 hours. Media were collected; centrifuged 5 minutes at 2,000 g, and 5,000 UIK/mL of trasylol was added to the supernatant. RIA was done on 1 mL of lyophilized media according to the procedure developed in Scarceriaux et al. (25).

Results

Genome-wide gene expression study in a population of patients with lung adenocarcinoma/large cell carcinomas

In an attempt to identify a clinical outcome predictor for patients with NSCLC, patterns of genomic alteration and gene expression profiles were analyzed and integrated concerning 74 homogeneous cases of stage Ib pT₂N₀ lung adenocarcinomas/large cell carcinomas (7). In this study, NTSR1 (probe set: 207360_s_at) was the 48th among the 58 selected probes related with RFS. NTSR1 was positively regulated. To correlate the NTSR1 transcription level and

the RFS over time, a log rank test using the third quartile as the cutoff point was applied to determine high-risk subgroups (Fig. 1). High gene expression for NTSR1 was associated with an increased risk of relapse ($P = 0.0005$).

Protein expression of neurotensin and NTSR1, and its impact on the survival of patients with primary lung adenocarcinoma

A neurotensin and NTSR1 expression study was done on a multicenter series of 139 consecutive patients undergoing pulmonary lobectomy and nodal dissection for pathologic stage I lung adenocarcinomas. The patient clinical characteristics are shown in Table 1. NTSR1 staining of cancer cells from patients with primary lung adenocarcinoma was granular, restricted to the cytoplasm, and rarely localized at the cell surface (Fig. 2A, left). Neurotensin labeling was often very intense and always detected throughout the cytosol (Fig. 2A, right). Expression of neurotensin, NTSR1, or both was found in 60.4%, 59.7%, and 38.8% of the cases, respectively. Similar results were found when neurotensin and NTSR1 transcripts were studied (23 patients): 65% of patients expressed neurotensin (Fig. 2B, red dots), 69% expressed NTSR1 (Fig. 2B, green dots), and 43% expressed both markers. No expression of either neurotensin or NTSR1 was observed in the lung parenchyma of 26 patients with idiopathic pneumothorax treated by apical resection (Fig. 2A), suggesting that normal lung tissue does not express neurotensin or NTSR1.

The impact of neurotensin/NTSR1 expression on outcome was assessed on 138 of 139 patients, with a patient lost at follow-up. The overall 5-year survival was 63.5% [95% confidence interval (95% CI), 54.4-71.7%]. With respect to those clinical and pathologic parameters influencing

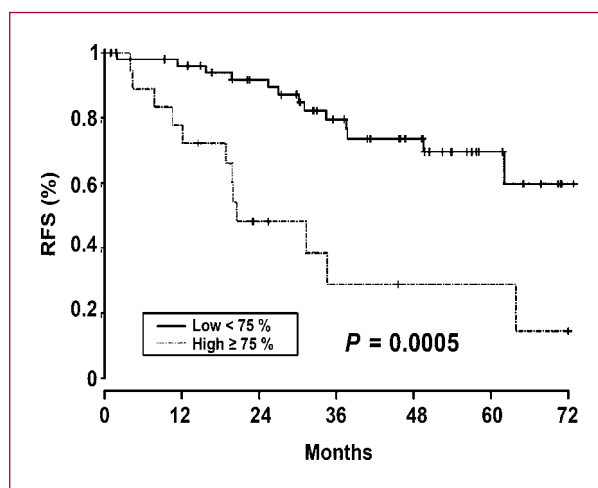


Fig. 1. Relapse-free survival of a study resulting from transcriptome analysis. Large-scale gene expression analysis done on 74 homogeneous cases of stage pT₂N₀ lung adenocarcinomas/large cell carcinomas treated by surgery. Depicted according to the procedure described by Broet et al. (7), the transcriptomic signature of selected positive scores indicates that overexpression increases relapse risk. Shown is the Kaplan-Meier plot according to NTSR1 mRNA levels over (broken line) or below (solid line) the 75th percentile.

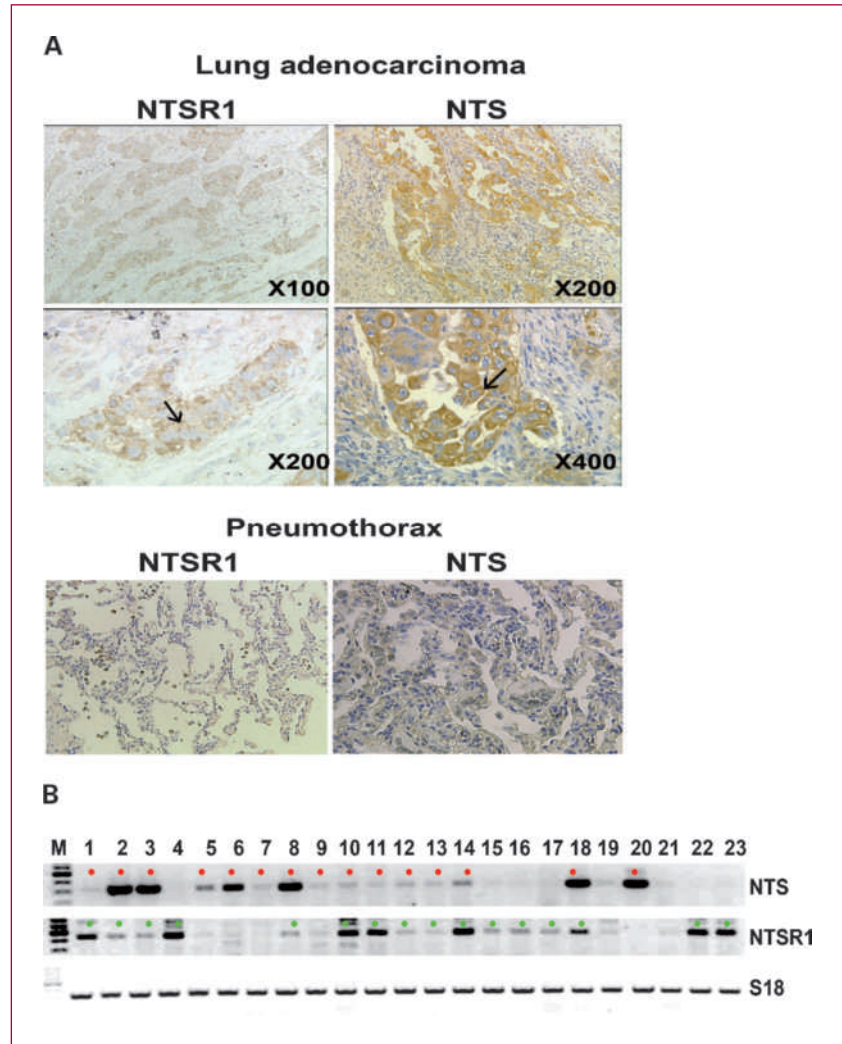


Fig. 2. Neurotensin (NTS) and NTSR1 expression in patients with primary lung adenocarcinomas. A, example of immunohistochemistry for NTSR1 (left) and NTS (right). Top, positive labeling of patients with primary lung adenocarcinomas; bottom, negative labeling of idiopathic pneumothorax at $\times 200$ magnification. B, NTS and NTSR1 transcript analysis on RNA from 23 patients with primary lung adenocarcinomas stage I. M, 100 bp ladder. Patients with red dot or green dot were considered as positive for NTS or NTSR1, respectively.

patient outcome, no difference was observed in survival rates using univariate analysis according to sex, pT parameter, presence of vascular or lymphatic tumoral emboli, or smoking habit (current and former versus never). Five-year survival rates were 72.0% and 57.7% in patients with T₁N₀ and T₂N₀ tumors, respectively ($P = 0.13$). Patients of age 65 years or older had a lower 5-year survival ($P = 0.0028$) as compared with younger patients, at 49.3% (95% CI, 36.2-62.4%) versus 75.9% (95% CI, 64.2-84.7%), respectively. NTSR1 expression, scored as positive (positive staining involving $\geq 10\%$ of tumor cells) or negative (positive staining of $< 10\%$ of tumor cells) was associated with a significantly worse 5-year overall survival [54.6% (95% CI, 42.82-65.86%) versus 76.1% (95% CI, 62.4-85.9%); $P = 0.0081$; Fig. 3A]. The degree of NTSR1 expression also significantly influenced outcome ($P = 0.015$), as patients with none (positive staining of $< 10\%$ of tumor cells), medium (positive staining of $\geq 10\%$ and $< 50\%$ of tumor cells), and strong (positive staining involving $\geq 50\%$ of tumor cells) NTSR1 expression showed 5-year overall survival rates of

76.1% (95% CI, 62.2-86.0%), 56.4% (95% CI, 43.8-68.1%), and 40.5% (95% CI, 15.3-72%), respectively (Fig. 3B). Two independent predictors of worse overall survival were found using multivariate analysis: age ≥ 65 years ($P = 0.0018$) and expression of NTSR1 ($P = 0.0034$).

NTSR1 expression was also associated with a significantly worse 5-year RFS, at 56.5% (95% CI, 45.5-66.8%) versus 79.3% (95% CI, 65.6-88.5%) in patients with tumors expressing or not expressing NTSR1, respectively ($P = 0.0024$; Fig. 3C). The degree of NTSR1 expression also significantly ($P = 0.0076$) influenced outcome, as patients with none, medium, and strong NTSR1 expression showed 5-year RFS of 79.3% (95% CI, 65.6-88.5%), 57.3% (95% CI, 45.6-68.3%), and 50.0% (95% CI, 23.6-76.3%), respectively (Fig. 3D). Two independent predictors of worse RFS were found using multivariate analysis: expression of NTSR1 ($P = 0.00066$) and age ≥ 65 years ($P = 0.00142$). In contrast, neurotensin expression in human tumor did influence neither overall survival nor RFS (Supplementary Fig. S1).

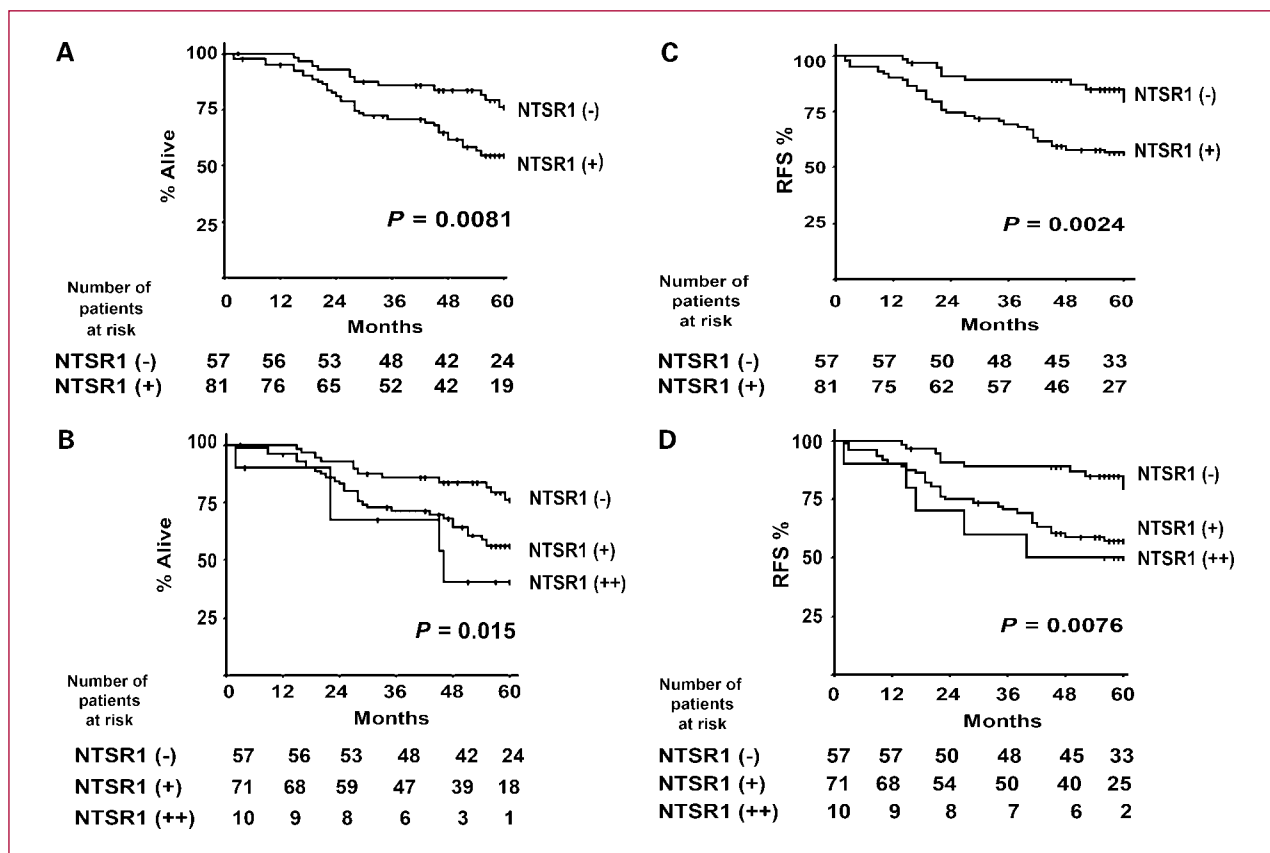


Fig. 3. Overall survival and RFS of patients with stage I adenocarcinoma ($n = 138$) according to NTSR1 expression. A and C, qualitative assessment by immunohistochemistry of NTSR1 expression: (+), positive; (-), negative. B and D, semiquantitative immunohistochemistry: (++), strong expression; (+), medium expression; (-), no expression. The number of patients at risk for each time period is shown below each curve.

Influence of the neurotensin/NTSR1 signaling system on the tumorigenic potential of human lung cancer cells

To evaluate the role of the neurotensin/NTSR1 complex in tumor growth and progression, we used mice xenografted with cancer cells expressing or not expressing the neurotensin/NTSR1 system. We first established xenografts from the adenocarcinoma cell line A549. As shown in Fig. 4A (inset), this cell line expressed both neurotensin and NTSR1. The silencing of NTSR1 induced a 40% reduction in the growth of tumor xenografts in nude mice compared with wild-type cells (Fig. 4A).

Additional evidence of the impact of the neurotensin system on tumor growth derived from LNM-R, a subpopulation of the highly metastatic lung carcinoma cell line LNM35 (23). LNM-R cells express neurotensin and NTSR1 as shown by transcript analysis (Fig. 4B, inset). Experimental lung tumors were generated using LNM-R cells and the silenced derivative clones R-SI NTS and R-SI NTSR1. In the clone R-SI NTS, the transcript encoding neurotensin was completely depleted (Fig. 4B, inset). This result was confirmed by the absence of neurotensin estimated by RIA on the corresponding culture media. In contrast, the

culture medium of LNM-R cells contained 76.4 ± 10.3 , 153.2 ± 25.3 , and 624.3 ± 81.8 fmol/mL of neurotensin corresponding to 14, 48, and 72 hours of culture, respectively. In the clone R-SI NTSR1, the transcript for NTSR1 was depleted, whereas neurotensin transcript levels remained similar to those of LNM-R cells. An example is shown in the inset of Fig. 4B. The level of neurotensin released in the culture media was lower than for LNM-R cells at 22.25 ± 2.4 , 53.4 ± 2.7 , and 140.2 ± 9 fmol/mL corresponding to 14, 48, and 72 hours of culture, respectively.

Using these models, we examined the effect of neurotensin and NTSR1 depletion on the growth of LNM-R xenografts and their neurotensin- and NTSR1-silenced counterparts. Depletion of neurotensin and NTSR1 was accompanied respectively by 35% and 60% decrease of the tumor volume compared with the LNM-R tumor xenografts at day 28 (Fig. 4B). To show the participation of neurotensin in tumor growth enhancement, a mixture of the two silenced clones was seeded at equal density (50/50) and cultured for 3 days, prior to injection into mice. The final tumor volume and weight were similar to those of the parental LNM-R tumor xenografts. Together these results strengthen the tumorigenic consequence of neurotensin

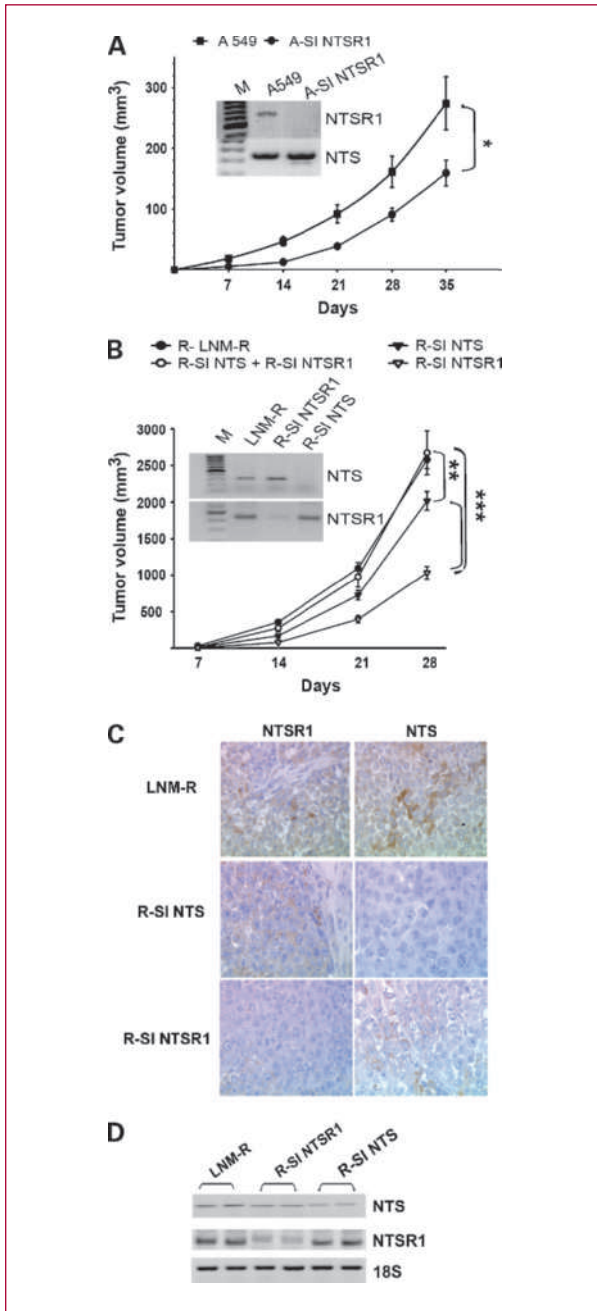


Fig. 4. Tumor growth generated by the human lung adenocarcinoma cell lines. A, A549 cells, or its NTSR1-silenced clone A-SI NTSR1 were injected into 15 mice for each cell line. Inset, NTS and NTSR1 transcript analysis on A549 cells, and A-SI NTSR1 RNA. *, significant differences at $P < 0.05$, using unpaired t test. B, LNM-R, R-SI NTS, R-SI NTSR1, or a 50/50 mixture of R-SI NTS and R-SI NTSR1 cells were injected into 36, 21, 21, and 19 mice, respectively. The ellipsoid formula was used to calculate the volume. Inset, NTS and NTSR1 transcripts analysis on total RNA from LNM-R, R-SI NTSR1, and R-SI NTS. C, typical immunohistochemistry for NTSR1 (left) or NTS (right) for tumors generated from R-SI NTS (top) or R-SI NTSR1 cells (bottom). D, NTS and NTSR1 transcript analysis on tumors from LNM-R, R-SI NTSR1, and R-SI NTS cells. Analysis was done at day 28. Examples of two different tumors are shown. ***, significant differences at $P < 0.001$; **, at $P < 0.01$ using ANOVA and Student-Neuman-Keuls test.

autocrine or paracrine regulation (Fig. 4B). A similar pattern was observed in tumor weight with 4 ± 0.2 , 2.8 ± 0.18 , 1.54 ± 0.17 , and 3.4 ± 0.16 g for LNM-R, R-SI NTS, R-SI NTSR1, and the 50/50 R-SI NTS/R-SI NTSR1 cellular mixture, respectively. Neurotensin and NTSR1 immunohistochemistry and transcript analysis on tumor xenografts shows that neurotensin and NTSR1 silencing constructs were still efficient at 28 days postinjection (Fig. 4C and D).

Influence of the neurotensin/NTSR1 complex on nodal metastasis

The ipsilateral and contralateral regional lymph nodes from mice bearing either LNM-R or the silenced clones for neurotensin and NTSR1 were dissected. The weight of the lymph nodes was 2.5 times smaller when the tumor was not expressing neurotensin or NTSR1 (Fig. 5A). Metastases were scored by an anatomopathologist as negative, micro (metastases < 1 mm), and massive (Fig. 5B). After 28 days, mice bearing tumor R-SI NTS and R-SI NTSR1 xenografts had a smaller percentage of massively invaded lymph nodes (9% and 24%, respectively), as compared with 66% for the LNM-R xenografts ($P < 0.0001$ and $P = 0.0047$, respectively, Fisher's exact test; Fig. 5C). The respective distribution of negative, micro, and massive lymph node metastasis is detailed under the graph in Fig. 5C. The negative and the micro metastasis were combined to form a single set because it covers the global effect of neurotensin/NTSR1 on metastatic process.

Discussion

In this study we found that NTSR1 and neurotensin were not detected in normal pulmonary tissue, but were strongly and frequently expressed in stage I lung adenocarcinomas. Similar results have been seen in colon and breast tissues where NTSR1 is absent in normal epithelial cells but overexpressed in tumors (20, 26). As previously described, one of the possible molecular mechanisms responsible for NTSR1 upregulation is the *Wnt/APC* signaling pathway activation, because of a functional Tcf element within the NTSR1 promoter. In normal human breast epithelial cells, the activation of the *NTSR1* gene was observed by agents causing β -catenin cytosolic accumulation (15). A set of TCF4 regulated genes was recently shown to be associated with metastasis-free survival in patients with lung adenocarcinoma (27). In this study, the transcription factors HOXB9 and LEF1 were identified as mediators of chemotactic invasion. Accordingly, a hyperactive *Wnt/TCF* pathway activity in lung adenocarcinomas is associated with a high rate of relapse at distant sites. As part of *Wnt/TCF* activated genes, *NTSR1* should participate in disease progression and correlation with the worst prognosis.

The availability of useful prognostic factors in NSCLC is made difficult because of the extreme heterogeneity in patient populations in terms of histologic typing, disease staging, type of surgical treatment, and the association with neoadjuvant or adjuvant therapy. For these reasons, we focused our study on patients with stage I primary lung

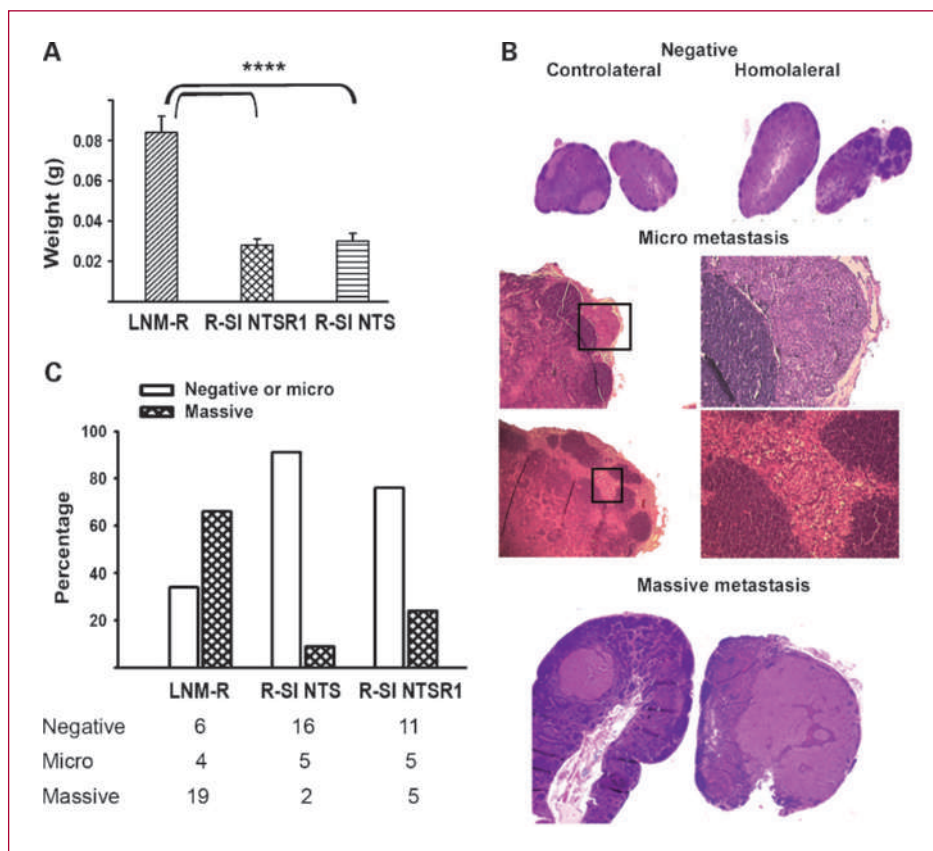


Fig. 5. Lymph node metastasis generated by lung cancer cell lines LNM-R and their subclonal counterparts silenced for NTS and NTSR1. A, ipsilateral lymph node weight at 28 days from mice xenografted with LNM-R, R-SI NTSR1, and R-SI NTS cells. ****, significant differences at $P < 0.001$ using ANOVA and Student-Neuman-Keuls test. B, standard H&E staining done on paraffin section of lymph node embedded tissue. Example is shown for negative (top); micro metastasis, less than 1 mm (middle); and massive metastasis (bottom) of homolateral lymph node. C, for each group, the percentage of ipsilateral lymph nodes with no and micro metastasis or with massive metastasis, from mice xenografted with LNM-R, R-SI NTSR1, and R-SI NTS cells, at 29, 21, and 23 mice, respectively.

adenocarcinoma, treated by surgery only (pulmonary lobectomy and full nodal dissection). This ensured the reliability of the staging and the absence of interfering treatments.

In our clinical series from 138 homogeneous patients with stage I disease, NTSR1 expression was correlated with poor outcome. This result was confirmed by multivariate analysis which showed that among the available clinical and pathologic factors, only the expression of NTSR1 and patient age were independent predictors of worse prognosis. These findings in human lung cancer suggest that NTSR1 is a potential marker and/or a pejorative mediator of lung cancer progression associated with poor prognosis.

In contrast, no correlation was found between neurotensin expression and patient survival. Neurotensin expression in the lung tumor is certainly part of the neoplastic process but is not a discriminative factor of disease progression. The molecular mechanism inducing neurotensin expression in lung neoplasm remains unclear. As it was shown that neurotensin expression can be upregulated by inflammatory process and mediates proinflammatory cytokine release, such as interleukin-6 and interleukin-8 (28, 29), we propose that neurotensin expression is induced and implicated in the pathogenesis of lung cancer due to chronic inflammation and neoplasia in the respiratory epithelium generated by tobacco carcinogens and

metabolites. The evaluation of neurotensin expression in premalignant lesions will settle this hypothesis.

To delineate the biological significance of the neurotensin/NTSR1 system in the progression of human lung tumors, we studied the performance of the neurotensin/NTSR1 complex on tumor growth and progression. Removing neurotensin sensitivity and NTSR1 signaling resulted in a strong reduction of lung cancer cell proliferation and tumor growth in the two experimental models. The silencing of neurotensin was less efficient than NTSR1 silencing in negating the growth of the LNM-R xenografts, suggesting that circulating neurotensin in mice may counteract the depletion of neurotensin induced by RNA interference in cancer cells. Substantiating this argument, we noted that when R-SI NTSR1 cells were injected s.c. into the right flank and R-SI NTS cells in the left flank of the mice, the latter tumor reach the size and weight of the tumors initiated by the corresponding LNM-R parental cells; in contrast, R-SI NTSR1 tumors remained at the same smaller size that was observed in mice bearing only R-SI NTSR1 xenografts (unpublished data). It is likely that neurotensin systemic regulation is implicated in the growth of the tumor xenografts.

Our data support the position that LNM-R cancer cells are driven by an autocrine neurotensin-NTSR1 loop to incur invasive tumor growth and metastasis. LNM-R is a subpopulation of LNM-35 cells selected for their metastatic

properties (23). Within the time period of the experiments, 70% of mice bearing LNM-R xenografts exhibited massive lymph node metastasis. The invalidation of the neurotensin/NTSR1 system in LNM-R cells was associated with less aggressive primary tumor xenografts, and it restricted the metastatic process. Once the cells acquire metastatic potential, the presence of neurotensin in the tumor microenvironment potentiates the emergence of metastasis. When replaced in the context of stage I patients with adenocarcinoma, most of these patients are treated only with surgery as adjuvant treatment, which has not been shown to improve the global outcome (30). In these patients, local and distant tumor recurrence should arise either from active or dormant cancer cells that have spread, both kinds being undetectable with the current diagnostic methods (31). Our experimental model predicts that the autocrine or paracrine loop involved in NTSR1 activation in those patients would enhance tumor progression and recurrence.

Patients within the initial stages of NSCLC are best managed by surgery, with no consensus existing regarding postoperative follow-up measures (32). The identification of patients with poor prognosis, within a determined stage, would be of major importance for the management of patients with NSCLC (33), as the follow-up procedures could be individually tailored. Furthermore, patients with poor prognosis, within a determined stage, could be identified as more suitable candidates for postoperative treatments. Adjuvant chemotherapy has been shown to improve the prognosis of patients with stage II-IIIa resected NSCLC, whereas no benefit has been observed in patients with stage Ia, and the results remain controver-

sial in stage Ib (30). Thus, in patients with stage Ib, the identification of a good prognosis marker would represent an important element permitting the avoidance of adjuvant chemotherapy.

Our data indicate that the neurotensin/NTSR1 system is a good prognostic marker useful to identify, within stage I disease patients, those with a bad prognosis. Furthermore, the role of neurotensin in the growth of experimental tumors would represent a basis for the development of specifically targeted drugs, to be used together with currently available treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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