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High cripto-1 and low miR-205 expression levels as prognostic markers in early stage non-small cell lung cancer

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Abstract

Objectives: Cripto-1 (CR-1) plays a critical role in the activation of SMAD, SRC, and epithelial-to-mesenchymal transition (EMT) pathways and has been shown to be prognostic in several cancer types. In addition, we showed that CR-1 renders EGFR-mutated NSCLC cells resistant to EGFR-TKI through the activation of SRC and EMT via miR-205 downregulation. This study aimed to investigate the correlation between expression of CR-1 and miR-205 and prognosis of NSCLC patients with or without EGFR mutations.

Materials and methods: A total of 265 patients with stage I (AJCC 6th edition) radically resected NSCLC were tested for CR-1 expression and EGFR mutations by immunohistochemistry and miR-205 expression via qPCR assay.

Results: CR-1 expression was evaluated with immunohistochemistry using a tissue microarray on 265 T1-2N0 surgical NSCLC samples. Of the 265 tumors, 250 (94%) expressed various levels of CR-1. A significant inverse correlation was identified between expression of miR-205 and CR-1. NSCLC patients (T1N0, n = 106) with high CR-1 expression had worse prognosis (shorter recurrence-free survival, p = .045) than those with low CR-1 expression. A similar trend was observed in NSCLC patients with normal preoperative carcinoembryonic antigen (CEA) levels (serum CEA levels < 5ng/ml; n = 179; p = .085); however, no significant correlation was found between CR-1 expression and survival rate in the T2N0 or high CEA groups. In addition, NSCLC

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Conflict of interests

None declared.

patients with low miR-205 expression (n = 126) had poorer prognosis in terms of recurrence than those with high miR-205 expression (n = 127; p = .001).

Conclusion: High CR-1 expression is correlated with poor prognosis in NSCLC with low tumor burden and may be used to select high-risk patients for adjuvant chemotherapy in early NSCLC. Moreover, low miR-205 expression likely related to high CR-1 expression could be a prognostic marker for patients with NSCLC.

Keywords

Cripto-1; miR-205; NSCLC; Prognostic marker

1. Introduction

Lung cancer is the leading cause of cancer-related death, accounting for approximately 29% of all cases [1]; approximately 85% of lung cancer cases are non-small cell lung cancer (NSCLC). NSCLCs are composed of several different subtypes, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Surgical resection is the treatment of choice for early-stage NSCLC [2], but unfortunately, only approximately 20%–25% of patients are radically resectable. Even when diagnosed at early stage of the disease, more than half of the patients experience recurrence. Adjuvant chemotherapy is standard for patients with stage II and III, is controversial for stage IB and is not recommended in stage IA. The most important prognostic factor for survival, in resected patients, is the disease stage albeit rather imprecise [3]. A plethora of potential biological markers have been described, but none have been demonstrated to be more useful and reproducible than pathological staging [4].

The human Cripto-1 (CR-1) gene, initially isolated from human teratocarcinoma cDNA, encodes a 188-amino acid glycosylphosphatidylinositol-linked glycoprotein. This gene is a member of the epidermal growth factor-cripto FRL1 cryptic protein family [5] and is involved in the activation of several signaling pathways during embryonic development and cellular transformation [6]. Although CR-1 was identified as a marker for embryonic stem cells and is absent from adult tissues, high CR-1 gene expression was detected in several human tumors including gastric, pancreatic, colorectal, gall bladder, breast, endometrial, cervical, ovarian, bladder, lung (NSCLC), and testicular cancer [7–13]. In addition, CR-1 was shown to be an oncogene in various tumors, and *in vitro* as well as *in vivo* experiments demonstrated that CR-1 promotes cellular proliferation and epithelial-to-mesenchymal transition (EMT) by binding to ALK4/7 to activate the SMAD2/3/4 and Glypican-1/SRC pathway [14–19]. Both EMT and SRC activation are known markers of EGFR-TKI resistance [21–24] and associated with poor prognosis in NSCLC patients [25,26]. We recently demonstrated that CR-1 expression is associated with intrinsic EGFR-tyrosine kinase inhibitor (TKI) resistance in advanced NSCLC patients and that this occurs via SRC activation, but not EMT [20].

In addition, microRNAs have recently been shown to control genes related to chemotherapy resistance and EGFR-TKIs [27,28]. In particular, microRNA-205 (miR-205) was downregulated in TGF-β-induced EMT cells [29], and ZEB1/ZEB2 [29] and SRC are

known targets of miR-205 [30]. These two target genes are known to promote drug resistance via EMT signaling. Furthermore, miR-205 downregulation has been previously shown to confer resistance to chemotherapy-induced apoptosis in prostate cancer cells [31]. In our previous study, we also demonstrated that miR-205 expression is reduced by CR-1 and causes intrinsic EGFR-TKI resistance in NSCLC cells harboring EGFR-sensitive mutations [20]. CR-1 can also activate SRC and ZEB1 to promote EMT via miR-205 downregulation. While miR-205 depletion induced erlotinib resistance, miR-205 overexpression inhibited CR-1-dependent ZEB1 and SRC activation, thereby restoring erlotinib sensitivity [20].

CR-1 was shown to be a poor prognostic marker in gastric, nasopharyngeal and breast cancer [32–34]. Recently, CR-1 was shown to be associated with disease stage [35,36] and poor prognosis in NSCLC patients [36]. However, these studies included a large number of patients with late stage NSCLC.

In the present study, we examined miR-205 expression with quantitative RT-PCR in relation to CR-1 in 265 very early stage NSCLC patients.

2. Materials and methods

2.1. Tissue samples

CR-1 expression was determined by immunohistochemistry in formalin-fixed, paraffin-embedded surgical specimens from 265 patients with early stage radically resected NSCLC. All tumor specimens were obtained from a pathological stage I cohort that underwent complete surgical resection between 1998 and 2007 without neoadjuvant treatment at the Yonsei Cancer Center in Korea. Tumor staging was performed according to TNM staging revised in 2002 by the American Joint Cancer Committee [37]. Tissue microarray (TMA) blocks were generated with punctures of the areas which consisted of > 80% of tumor cells in each tumor sample.

2.2. Immunohistochemistry

Serial sections that were 4 μm thick were mounted on glass slides coated with 10% polylysine. The sections were dewaxed in xylene and rehydrated in graded ethanol. The endogenous peroxidase activity was blocked with an immersion in 0.3% methanolic peroxide for 40 min. Immunoreactivity was enhanced by microwaving the tissue sections for 10 min in 0.1 M citrate buffer. Immunostaining was performed using the avidin–biotin–peroxidase complex method, and antigen–antibody reactions were visualized with chromogen diaminobenzidine. The CR-1 antibody and EGFR mutation (Exon 19 del and L858R) antibodies for immunohistochemistry were purchased from Rockland (Limerick, PA, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively. Stained tissues were scored by a pathologist (M. Raffeld, NCI) according to the overall staining intensity using a four-point (0–4) scoring system of the tumor cell staining intensity. A score of 0 indicated no staining of tumor. Scores of 1 and 2 corresponded to trace and weak staining, respectively, while scores of 3 and 4 corresponded to moderate and strong staining,

respectively. Low CR-1 expression was defined as immunohistochemistry scores of 0–2 and high CR-1 expression to scores of 3–4 (Supplementary Table 1).

The low CR-1 expression was defined as immunohistochemistry scores of 0–2 and high CR-1 expression as immunohistochemistry scores of 3–4.

2.3. CEA detection method

The serum CEA level was measured using an automated immunoanalyzer systems and chemiluminescent immunoassay (ADVIA Centaur, Bayer Healthcare LLC Diagnostic Division, NY, USA).

2.4. Quantitative RT-PCR

Quantitative RT-PCR was performed on RNA isolated from NSCLC patient samples. The total RNA was extracted using an RNeasy FFPE Kit and RNA later RNA stabilization reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed on a LightCycler with SYBR Green system (Roche, Pleasanton, CA, USA) using 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). TaqMan ® MicroRNA Assays (miR-205: has-miR-205-5p, microRNA endogenous control: RNU6B) were purchased from Invitrogen (Bartlesville, OK, USA).

2.5. Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS, Chicago, IL, USA). Student's *t*-test was used to compare continuous clinical variables. All p values were two tailed and p values $< .05$ were regarded as significant. Recurrence-free survival (RFS) was defined as the time from curative surgery to NSCLC recurrence or the last date at which the patient was known to be free of recurrence (censoring time). Overall survival (OS) was defined as the time from curative surgery to death or the date at which the patient was last confirmed to be alive (censoring time). Disease-specific OS (DSOS) was defined as OS where only deaths due to disease were considered as events. Kaplan-Meier plots were used to estimate patient survival rates. Comparisons of the survival curves were assessed using a log-rank test, and $p < .05$ was considered to be significant. A multivariate analysis for the prognostic factors was performed using the Cox proportional hazards regression model.

3. Results

3.1. CR-1 is expressed in NSCLC tumors

Previous reports established that CR-1 is expressed in various tumors, but not in normal tissues [11,38]. We evaluated CR-1 expression by immunohistochemistry using a TMA containing 265 T1-2 N0 surgical NSCLC samples. Patient characteristics are described in Table 1 and Table 2. The majority of the patients had adenocarcinoma histology (57%). Approximately 57% of the 265 NSCLC patients were never-smokers. Using a validated

CR-1-specific antibody (Fig. 1A), we found that 250 (94.34%) of the 265 tumors expressed CR-1 at various levels (Fig. 1B and C).

3.2. High CR-1 expression correlates with poor prognosis only in early NSCLC

Expression of cripto-1 was rather homogeneous and therefore a scoring system of intensity was used rather than percentage of tumor cells positive. Staining of cripto-1 was mainly cytosolic. Two different cut-off values were explored for CR-1 expression with immunohistochemistry. Scores of 0–2 vs. 3–4 were found to better predict recurrence (Suppl. Table 1). There was no association between levels of CR-1 expression and major patient characteristics as presented in Table 1. Supplementary Table 2 shows the results of the univariate analysis for survival according to the major patient characteristics listed in Table 1 and expression of cripto-1 and miR-205. The T status is clearly the most important prognostic factor in this analysis.

NSCLC patients with high CR-1 expression (n = 97) had shorter RFS than those (n = 168) with low CR-1 expression (Fig. 2A; p = .154), but this did not reach statistical significance, and there was no difference in DSOS (Suppl. Fig.1A). NSCLC patients with T1 tumors and with high CR-1 expression (n = 41) had significantly shorter RFS (Fig. 2B; p = .045) and also shorter disease-specific OS than those (n = 65) with low CR-1 expression (Fig. 2C; p = .077). In particular, in multivariate analysis of DSOS, high CR-1 expression remained a significant poor prognostic factor (Suppl. Table 3). A similar trend was observed in NSCLC patients with normal preoperative CEA levels (serum CEA level < 5 ng/ml; n = 179; p = .085; Fig. 2D and E). There was a significant difference in RFS and DSOS between T1 and T2 tumors (Suppl. Fig. 1B and C) and a significant difference only in RFS in favor of normal CEA vs elevated CEA (Suppl. Fig. 2A and B). In patients with T2 tumors (Suppl. Fig. 1D and E) or in those with high CEA levels (Suppl. Fig. 2C and D), no significant difference in RFS or DSOS was found between high and low CR-1 expression. These findings suggest that CR-1 expression is prognostic only in early stages of the disease (Suppl. Table 5).

3.3. Overall and recurrence-free survival of CR-1-expressing NSCLC patients according to EGFR mutations

We identified 38 patients (14.34%) with an EGFR mutation, as detected by immunohistochemistry using mutant specific antibodies, out of the total 265 patients including 34 patients (22.5%) with adenocarcinoma and 4 patients (3.5%) with other histologies using EGFR-mutant-specific antibodies (Fig. 3A, Suppl. Table 4). Supplementary Table 4 also presents the common sensitive EGFR mutations assayed with immunohistochemistry using our TMA (n = 265). An exon 19 deletion was observed in 13 cases (4.9%) and L858R mutation in 25 cases (9.4%). Most mutations detected by IHC were present in adenocarcinomas (34/38) and there was no difference in presence of EGFR mutations between CR-1 high and low groups in the whole population or in adenocarcinoma histology only. There was no difference in the survival rate between EGFR mutant and wild type cases in the whole patient population as well as adenocarcinoma only (Fig. 3B). There was no statistically different survival outcome between CR-1 high and low in either EGFR mutant or wild type cases (Fig. 3C and D).

3.4. Low miR-205 and high CR-1 expression is associated with poor prognosis in NSCLC patients

qRT-PCR was performed to determine the differential expression of miR-205 in the 265 T1-2N0 surgical NSCLC samples. As shown in Fig. 4A, a significant inverse correlation between miR-205 and CR-1 expressions was identified ($R2 = 0.450$; $p < .0001$), in which miR-205 was significantly downregulated in the samples with high CR-1 expression and was upregulated in samples with low CR-1 expression. No correlation was found between EGFR mutations and miR-205 expression (Suppl. Fig.6). Female gender, age less than 65 years, and adenocarcinoma histology are significantly correlated with low miR-205 (Table 2). For survival analysis, miR-205 expression was divided into high (≥ median value) and low expression (< median value) groups. NSCLC patients with low miR-205 expression (n = 126) exhibited poorer prognosis regarding recurrence than those with high miR-205 expression (n = 127) ($p = .001$; Fig. 4B). In OS analysis, miR-205 low group showed a trend to poorer survival without statistical significance (Fig. 4C, Table 2).

4. Discussion

CR-1 is a member of the EGF–CFC family and it is required for the regulation of embryonic development and plays a central role in EMT as well as in stem cell maintenance [39,40]. High CR-1 expression is associated with tumor transformation, tumor metastasis, and poor prognosis in various tumors [33,34,41,42]. Recently, a group in China demonstrated that CR-1 expression with immunohistochemistry is prognostic in heterogeneous populations of NSCLC patients. In these studies, the authors demonstrated that higher CR-1 expression was present in more advanced cancers and that poor survival was associated with higher CR-1 expression levels [36]. In addition, this group also revealed that soluble CR-1 in the serum was a prognostic indicator [43]. These previous reports and our study were performed using East Asians populations, known to have a high incidence of EGFR mutations. However, no information regarding the EGFR status was present in these Chinese reports.

In this study, we investigated CR-1 expression in patients with NSCLC and its correlation with survival in a large cohort of patients with early stage NSCLC (stage I according to the 6th AJCC classification), without lymph node involvement. In [20] and we confirmed that CR-1 expression determined by immunohistochemistry was significantly higher in NSCLC tissues than in normal lung tissues, similar to previous findings in other cancer types [11,34,41,42]. These results advocate the previous studies that CR-1 acts as an oncogene in NSCLC.

We also assessed the CR-1 expression in NSCLC tumors with immunohistochemistry and analyzed its correlation to clinicopathological characteristics. In the Kaplan–Meier survival analysis, RFS of patients with high CR-1 expression was significantly shorter than that of patients with low CR-1 expression. In particular, CR-1 strongly correlated with the poor prognosis of stage T1 NSCLC patients with low CEA levels. This finding suggests that CR-1 is a more reliable and useful prognosis marker during the early stage of NSCLC. Moreover, the recurrence rate was moderately higher in the high CR-1 patient cohort than in the low CR-1 patient cohort.

In the histology subset analysis, the recurrence rates of patients with adenocarcinoma and squamous cell carcinoma increased two-fold for high CR-1 expression in all NSCLC patients. In particular, high CR-1-expressing patients exhibited a two-to three-fold higher recurrence rate in early NSCLC patients with stage T1 adenocarcinoma. The recurrence rate was not different in the late NSCLC patients with stage T2 adenocarcinoma. These analyses suggest that CR-1 may play an important role in recurrence as well as in the survival of early NSCLC patients with adenocarcinoma.

We confirmed the use of antibodies for EGFR mutant detection (exon 19 del and L858R). EGFR mutant, NSCLC patients with high CR-1 expression were associated with poorer survival compared with patients with low CR-1 expression. However, this requires confirmation in future studies due to the low number of patients included in the present study. In our previous study, we proved that CR-1 induces miR-205 downregulation, which then promotes EGFR-TKI resistance via SRC activation as demonstrated *in vitro, in vivo*, as well as in patient samples.

As no previous reports have linked CR-1 to miR-205, we found a significant inverse correlation between miR-205 and CR-1 expressions in 17 NSCLC patient samples [20]. We did not detect a difference of miR-205 expression level between EGFR wild-type and EGFR mutated patients.

In the current study, we confirmed that miR-205 is significantly downregulated in samples with high CR-1 expression and upregulated in samples with low CR-1 expression using 253 NSCLC patient samples ($R2 = 0.450$, $p < .0001$). Moreover, NSCLC patients with low miR-205 expression were associated with poorer prognosis than those with high miR-205 expression. Several miRNAs are known to be potential serum markers of various tumors, including NSCLC [27,28,44,45]. Moreover, miR-205 has recently emerged as a crucial serum marker in breast cancer [46], prostate cancer [47], NSCLC [48], SCLC [49], and glioma [50]. Therefore, lower miR-205 expression associated with CR-1 expression may be a useful marker for survival in patients with early stage NSCLC.

5. Conclusions

This study demonstrates that CR-1 is highly expressed in resected NSCLC and supports the use of CR-1 as a negative prognostic marker in early NSCLC. It is also the first report that presents a possible link between CR-1 and miR205 in patients with NSCLC. Low miR-205 expression in conjunction with high CR-1 expression could be a potential prognostic marker for patients with NSCLC. Further studies are needed to define the mechanisms by which CR-1 regulates miRNA in NSCLC. In addition, our study is the first to present CR-1 expression and EGFR status in a large-scale population with NSCLC: no difference in survival rate was however observed in CR-1-expressing NSCLC patients with EGFR wild type or mutant EGFR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.lungcan.2017.12.010.

Abbreviations:

CR-1	cripto-1
RFS	recurrence-free survival
DSOS	disease-specific overall survival
OS	overall survival
CEA	carcinoembryonic antigen

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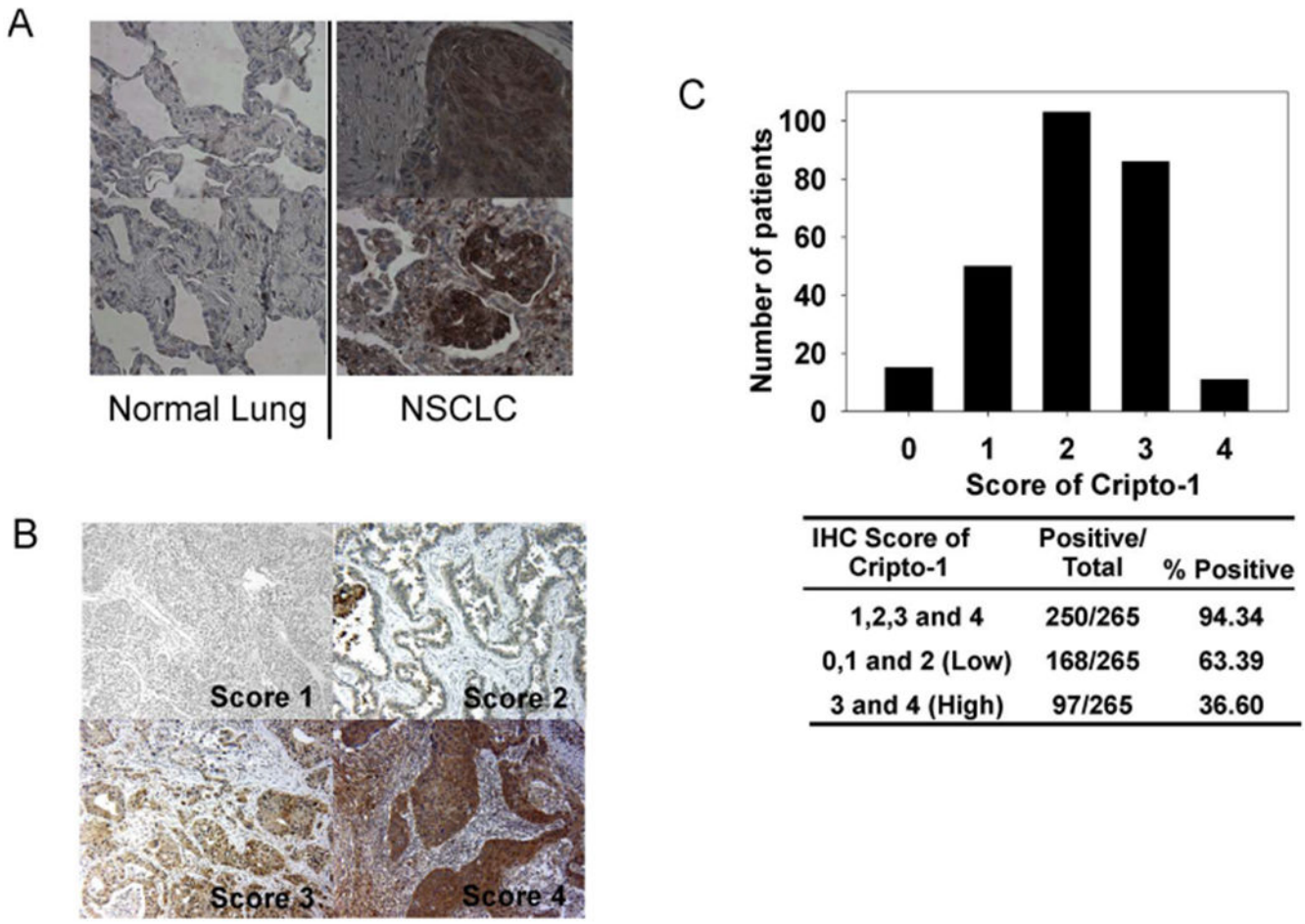

IHC Score of Cripto-1	Positive/ Total	% Positive
1,2,3 and 4	250/265	94.34
0,1 and 2 (Low)	168/265	63.39
3 and 4 (High)	97/265	36.60

Fig. 1.

Cripto-1 is expressed in NCSLC tumors. (A) Cripto-1 immunohistochemical staining of human normal lung tissue and NSCLC patient tissue. (B) Representative Cripto-1 immunohistochemistry images according to the associated score, Original magnification, 400X. (C) Distribution of Cripto-1 immunohistochemistry staining in 265 NSCLC patients. Cripto-1 expression levels according to the immunohistochemistry scores (score: 0–4) by a pathologist (M. Raffeld) are shown. All stained patients are divided into groups of low (score: 0–2) and high (score: 3–4) Cripto-1 expression.

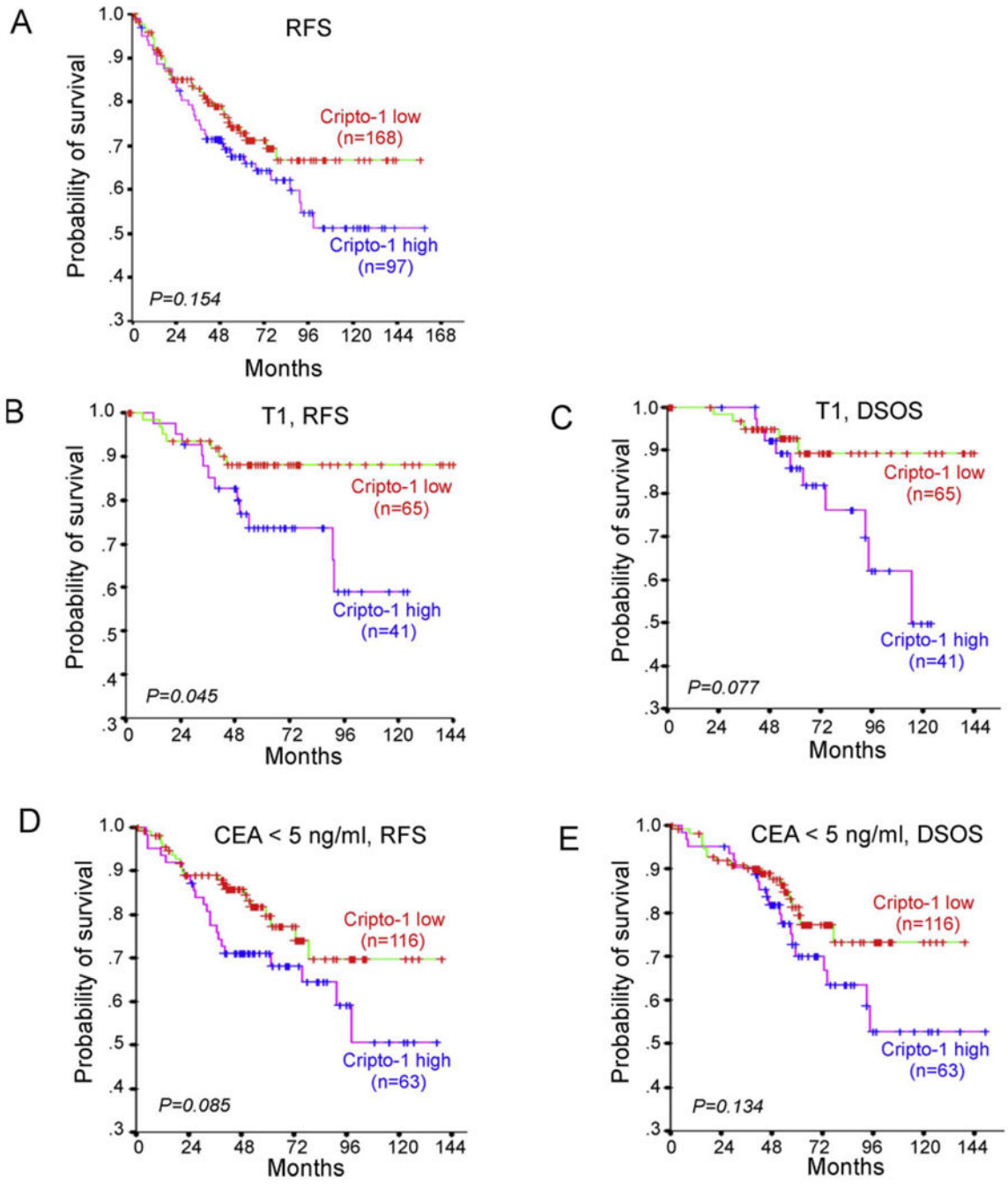

Fig. 2.

High Cripto-1 expression correlates with poor prognosis in patients with early NSCLC. (A) RFS according to the Cripto-1 expression level in 265 NSCLC patients. (B) RFS and (C) DSOS according to the Cripto-1 expression level in 106 NSCLC stage T1 patients. (D) RFS and (E) DSOS according to the Cripto-1 expression level in 179 NSCLC patients with low CEA level (< 5 ng/ml). RFS, recurrence-free survival; DSOS, disease-specific overall Survival; CEA, carcinoembryonic antigen.

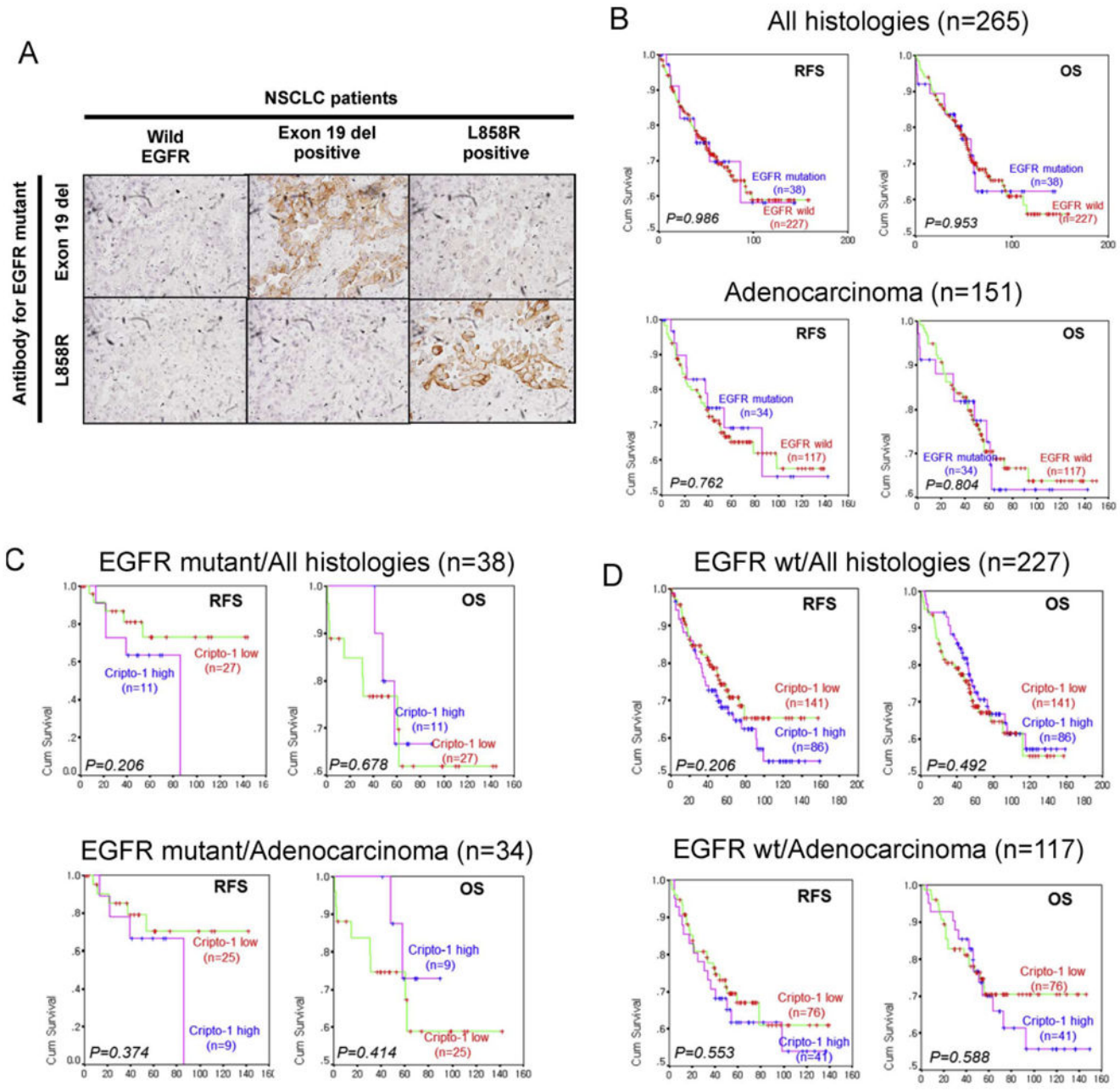

Fig. 3.

The OS and RFS of Cripto-1-expressing NSCLC patients according to the presence of an EGFR mutation. (A) Representative EGFR mutant immunohistochemistry images according to the mutant species exon 19 del and L858R. Original magnification, 400X. (B) RFS and OS according to the EGFR status; EGFR mutations vs. EGFR wild type. RFS and OS according to Cripto-1 expression in NSCLC-harboring EGFR mutants (C) and EGFR wild type (D). RFS, recurrence-free survival; OS, overall survival.

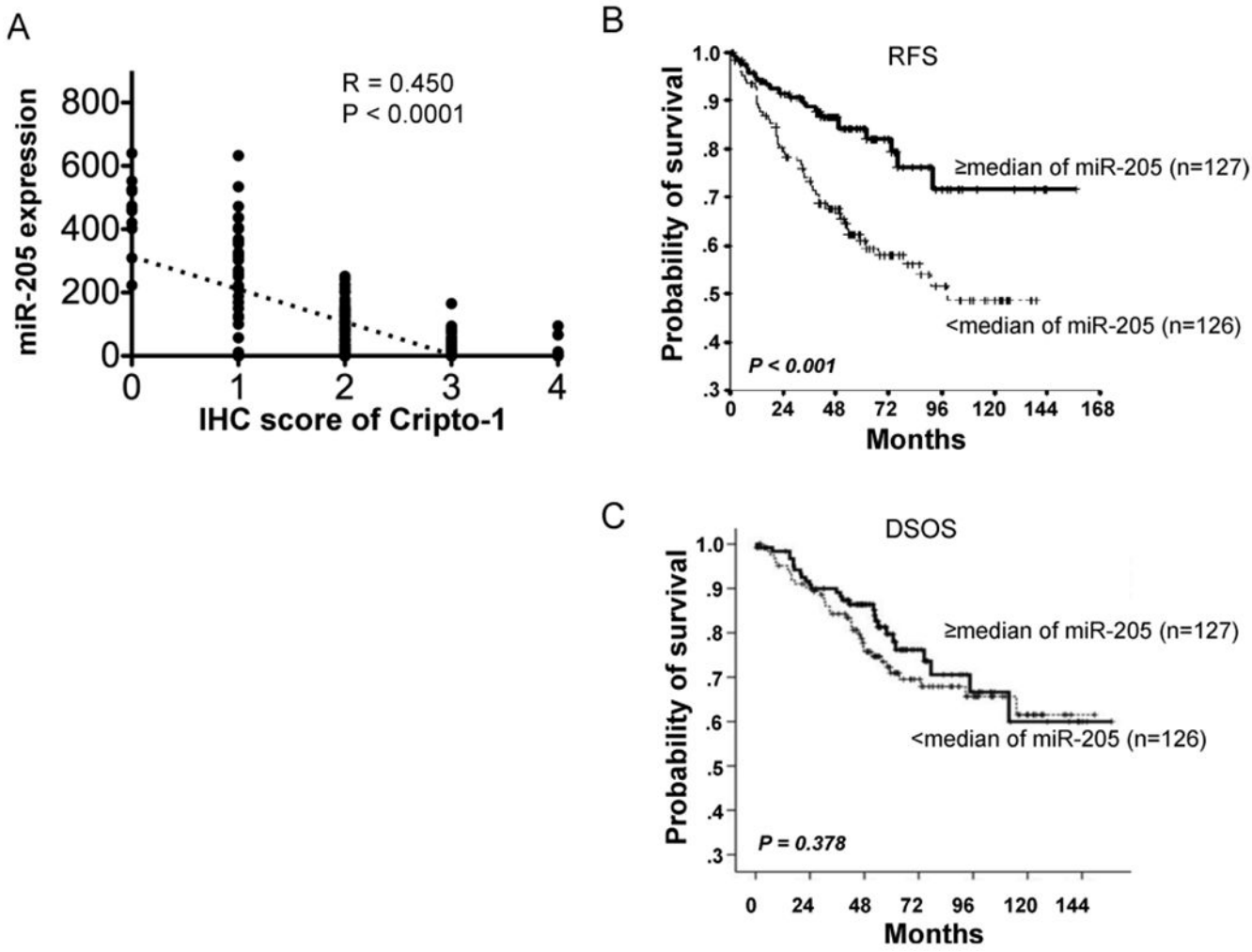

Fig. 4.

Low miR-205 induced by CR-1 expression is associated with poor prognosis in patients with NSCLC. (A) An inverse correlation between Cripto-1 (immunohistochemistry) and miR-205 expression (qPCR) in NSCLC patient samples. The graph illustrates a nonlinear regression analysis of Cripto-1 protein and miR-205 expression in 265 NSCLC patient samples. (B) RFS and (C) DSOS according to miR-205 expression in 263 NSCLC patient samples. All patients were divided into groups of low (< median, n = 126) and high (> median, n = 127) miR-205 expression by qPCR. RFS, recurrence-free survival; DSOS, disease-specific overall survival.

Table 1

Cripto-1 expression and patient characteristics.

	Cripto-1 low		Cripto-1 high		
	N = 168	**(%)**	**N = 97**	**(%)**	**P value**
Sex					
Female	63	(37.5)	33	(34.0)	0.570
male	105	(62.5)	64	(66.0)	
Age					
< 65 yrs	83	(49.4)	56	(57.7)	0.191
≥ 65 yrs	85	(50.6)	41	(42.3)	
Histology					
Adenocarcinoma	101	(60.1)	50	(51.5)	0.324
Squamous cell carcinoma	56	(33.3)	41	(42.3)	
Large cell carcinoma	9	(5.4)	3	(3.1)	
Adenosquamous carcinoma	1	(0.6)	1	(1.0)	
Mucoepidermoid carcinoma	1	(0.6)	2	(2.1)	
Pathologic stage (AJCC 6th)					
Stage IA (T1N0)	65	(38.7)	41	(42.3)	0.567
Stage IB (T2N0)	103	(61.3)	56	(57.7)	
Tumor size, cm (AJCC 7th)					
< 2 (T1a; stage IA)	35	(20.8)	23	(23.7)	0.199
> 2-3 (T1b; stage IA)	45	(26.8)	28	(28.9)	
> 3-5 (T2a; stage IB)	65	(38.7)	30	(30.9)	
> 5-7 (T2b; stage IIA)	13	(7.7)	14	(14.4)	
> 7 (T3; stage IIB)	10	(6.0)	2	(2.1)	
Preoperative CEA*					
< 5 ng/ml	116	(80.6)	63	(76.8)	0.507
≥ 5 ng/ml	28	(19.4)	19	(23.2)	
Smoking status					
Never-smoker	99	(58.9)	51	(52.6)	0.146
Current smoker	44	(26.2)	36	(37.1)	
Ex-smoker	25	(14.9)	10	(10.3)	
Smoking amount					
Never-smoker	99	(58.9)	51	(52.6)	0.102
Light smoker (< 40 py)	24	(14.3)	24	(24.7)	
Heavy smoker (≥ 40 py)	45	(26.8)	22	(22.7)	

Table 2

Correlations with covariates (miR-205 low vs. miR-205 high).

	MiR-205 low		MiR-205 high		
	N = 126	(%)	N = 127	(%)	P value
Sex					
Female	53	(42.1)	37	(29.1)	0.032
male	73	(57.9)	90	(70.9)	
Age					
< 65 yrs	75	(59.5)	59	(46.5)	0.037
≥ 65 yrs	51	(40.5)	68	(53.5)	
Histology					
Adenocarcinoma	94	(74.6)	47	(37.0)	< 0.001
Squamous cell carcinoma	24	(19.0)	72	(56.7)	
Large cell carcinoma	6	(4.8)	5	(3.9)	
Adenosquamous carcinoma	1	(0.8)	2	(1.6)	
Mucoepidermoid carcinoma	1	(0.8)	1	(0.8)	
Pathologic stage (AJCC 6th)					
Stage IA (T1N0)	51	(40.5)	51	(40.2)	0.959
Stage IB (T2N0)	75	(59.5)	76	(59.8)	
Tumor size, cm (AJCC 7th)					
0 ~ < 2 (T1a; stage IA)	28	(22.2)	28	(22.0)	0.940
2 ~ < 3 (T1b; stage IA)	36	(28.6)	33	(26.0)	
3 ~ < 5 (T2a; stage IB)	43	(34.1)	47	(37.0)	
5 ~ < 7 (T2b; stage IIA)	12	(9.5)	14	(11.0)	
7 ~ (T3; stage IIB)	7	(5.6)	5	(3.9)	
Preoperative CEA*					
< 5 ng/ml	86	(75.4)	84	(82.4)	0.215
≥ 5 ng/ml	28	(24.6)	18	(17.6)	
Smoking status					
Never-smoker	77	(61.1)	65	(51.2)	0.302
Current smoker	34	(27.0)	42	(33.1)	
Ex-smoker	15	(11.9)	20	(15.7)	
Smoking amount					
Never-smoker	77	(61.1)	65	(51.2)	0.139
Light smoker (< 40 py)	23	(18.3)	22	(17.3)	
Heavy smoker (≥40 py)	26	(20.6)	40	(31.5)	