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Research Article

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Cripto-1 and RUNX2 Expressions in Non-small Cell Lung Cancer, their Roles in its Progression and Patients' Outcome

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Abstract

Background: Non-small cell carcinoma of lung (NSCLC) is the commonest and most lethal lung cancer type; it includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma subtypes. The five-year survival rate in NSCLC patients is still very low although improvements in treatment modalities are still emerging. Hence, new prognostic markers and therapies need to be brought to light aiming to improve patients' outcome. Cripto-1 (CR-1) is one of the family members of epidermal-growth-factor; cripto FRL1 cryptic-(EGF-CFC) is needed for embryogenesis. Runt-related-transcription-factor [RUNX] family members make core-binding factor complex (CBFC) that attach to DNA to stimulate or inhibit many genes transcription, regulate the survival, differentiation and maturation of many tissues. The aim of this work is to detect the clinical significance and prognostic role of CR-1 and RUNX2 expressions in NSCLC using immunohistochemistry.

Method: CR-1 and RUNX2 expressions were evaluated in 59 paraffin blocks sections of NSCLC. The relationship between their level of expressions and patient's prognosis was analyzed.

Results: CR-1 and RUNX2 were highly expressed in NSCLC patients, 59.3% and 67.8%, respectively. There was a significant positive association between their expressions in NSCLC patients (p=0.015). Both markers were significantly correlated with size, grade, stage, site of the tumor within the lung, malignant (pleural and/or pericardial) effusion, presence of distant metastases, ECOG performance status of the patients (p<0.001) and existence of hepatic metastases (p=0.004). Both markers expressions were significantly correlated with poor response to treatment (p<0.001).

After a median follow up of 30 months, mean PFS of NSCLC patients having elevated CR-1 and RUNX2 expressions was shorter (p<0.001). Patients with high RUNX2 expressing have significantly shorter mean OS (p=0.025). High CR-1 expression negatively affected OS but that was not statistically significant (p=0.2).

Conclusion: NSCLC patients with elevated CR-1 and RUNX2 expression values had unfavorable prognosis.

Keywords

Non-small cell lung cancer; CR-1; RUNX2; Progression; Prognosis

Introduction

Globally, lung cancer is the commonest lethal malignancy [1]. Most of them (80-85%) are non-small cell lung carcinoma (NSCLC) [2] and its major subtypes are: adenocarcinoma, squamous cell lung carcinoma and large cell lung carcinoma [3]. The five-year overall-survival-rate in patients having NSCLC has been still very low (15%) although improvements in treatment modalities are still emerging [4]. Low survival rate of patients can be attributed to delay in diagnosis, lack of therapy in adequate time and increasing metastasis risk. Many factors that can assess the prognosis and patient survival have been explored including: ECOG performance status, grade and stage of cancer [5] but were found to be un-satisfactory as the treatment and prognosis of every patient should be individualized. New prognostic markers and therapies need to be discovered to improve outcome of patient by comprehensive studying of the molecular pathogenesis of cancer lung. Cripto-1 (CR-1) is one of the family members of epidermal-growth-factor; cripto FRL1 cryptic (EGF-CFC) that is needed for embryogenesis [6,7]. Recent studies stated that CR-1 also controls the initiation and progression of many human cancers [8]. Previous studies have correlated levels of CR-1 expression with cancer initiation, invasive ability, metastatic potential, and tumor prognosis of many organs [9-11], but researches that studied its expression in NSCLC were insufficient.

The progression from early- to advanced-stage NSCLC is controlled by many genes that were a point of research to understand the initiation and progression of NSCLC. The RUNX family contains 3 members (RUNX1–3), forming the core-binding factor (CBF) complex which attach to DNA, stimulating or inhibiting transcription of many genes [12] which in turn regulate the survival, differentiation and maturation of many tissues [13]. RUNX2 plays an essential role in osteogenesis during embryogenesis [14]. RUNX2 controls the deposition of bone matrix, mainly collagen I, by osteoblasts after birth [15]. RUNX2 may either stimulate or suppress the process of carcinogenesis depending on the type and site of cancer [16], but only few reports focused on the role of RUNX2 in NSCLC.

The aim of this study was to assess the clinical relevance and prognostic role of CR-1 and RUNX2 expression in NSCLC using immunohistochemistry as the prognostic significance of their expression in NSCLC is still unclear.

Patients and Methods

This retrospective study was carried out at Zagazig University Hospitals. The study protocol was approved by the Ethical Committee of Faculty of Medicine, Zagazig University. It comprised 59 previously diagnosed NSCLC patients. Patient's data was collected including: age, gender, smoking condition, tumor size, tumor differentiation, lymph nodal status and pathological stage. Follow up data were obtained from patients' hospital records in Medical Oncology and Clinical oncology and Nuclear Medicine Departments. The 7th TNM staging system for non-small cell lung cancer (NSCLC) was used for pathologic staging [17]. Patients were treated according to their stage either by surgery, chemotherapy (platinum-based chemotherapy), and radiotherapy or combined modalities.

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Expressions of CR-1and RUNX2 were evaluated in 59 paraffin blocks sections of NSCLC that were obtained from Pathology department, Faculty of Medicine, Zagazig University archives in the period from September 2013 to September 2016.

Immunohistochemical staining

Four-unthick paraffin-embedded sections had been deparaffinized then rehydrated. For antigen retrieval, tissues were heated for 10 min in sodium citrate, addition of 3 % hydrogen peroxide blocked the activity of endogenous peroxidase then incubation with polyclonal rabbit anti-RUNX2 antibody ab23981 and monoclonal rabbit anti-CR-1 antibody ab108391 (1:100 dilutions) (Abcam. Cambridge, MA, USA) was done. Colorectal carcinoma was used as positive controls for both CR-1& RUNX2. Secondary antibody was added for half an hour followed by using chromogenic for five minutes. We counterstained the slides with hematoxylin.

Determination of CR-1Expression by Immunohistochemical Assay

The expression of CR-1 was assessed based on the extent of stain (E) (positive cells were graded from 0 to 3: 0<1 %, 1=1-33 %, 2=33-67 %, and 3>67 %) and the intensity of stain (I) (graded from 0-3: 0=none, one=weak stain, two=moderate stain, and three=strong stain). The final score of stain was calculated by the product of extent × intensity, resulting in points zero to nine [18]. We use the cut point of 3 above which is considered high expression.

Determination of RUNX2 Expression by Immunohistochemical Assay

The expression results were done by multiplying intensity of stain by stain area. We calculated stain intensity by the following score: no stain (scored zero), weak stain (scored 1), moderate stain (scored 2), or strong stain (scored 3). We calculated stain areas by the following score: less than 25 percent (scored 1), 25 to 50 percent (scored 2), 50 to 75 percent (scored 3), or more than 75 percent (scored 4) of cancer cells. Final scores of; 0,1, 2,3,4,6,8,9, and 12 were reached [19,20]. We use the cut point of 4 above which is considered high expression

Statistical analysis

We expressed the categorical variables as a number& percentage, but the continuous variables as the mean ± standard deviation & median (range). We calculated Relapse-Free-Survival rate (RFS); the time from treatment finalization to time of recurrence. Overall survival (OS) and RFS rates were calculated in comparison with all clinicopathological features and Immunohistochemical markers. Time-to-mortality distributions were calculated by using of the Kaplan-Meier method, with considering the p-value of less than 0.05 as significant value. Statistics were made by using SPSS 22.0 windows (SPSS Inc., IL, and USA) and windows (MedCalc Software bvba 13, Belgium).

Results

Patient criteria

The clinical data of our patients are summarized in Table 1.

39(66.1%) men and 20 (33.9%) women, aged from (45-77) years (the mean: 62.42 ± 8.45 years), 38 (64.4%) cases were adenocarcinoma, and 21 (35.6) were squamous cell carcinoma.

CR-1 expression and its relation to clinicopathological data of our patients (Tables 2 and 3) (Figure 1)

Increased **CR-1** expression in the cytoplasm was present in thirty-five of fifty-nine (59.3%) patients, and it was positively correlated , significantly, with the presence of; co-morbid conditions, stage, grade of the tumor, distant metastases, lymph node metastases, malignant (pleural and/or pericardial) effusion (p<0.001), liver metastases (p=0.004), brain metastases (0.016), weight loss, age of the patients (p=0.018), site of the tumor within the lung (p=0.030) and performance status (0.005), but we found non- significant correlation with sex, smoking history of the patients, size, histopathological type of the tumor or number of distant metastases.

RUNX2 expression and its relation to clinicopathological data of our patients (Tables 2 and 3) (Figure 2)

Increased nuclear expression of **RUNX2** was demonstrated in forty of fifty-nine (67.8%) patients, and it was significantly positively correlated with the presence of co-morbid conditions, age of the patients, weight loss, stage, size, location of the tumor within the lung, presence of distant metastases, performance status of the patients (p<0.001), grade of the tumor(p=0.003), malignant (pleural and/or pericardial) effusion (p=0.006), liver metastases (p=0.022) and brain metastases (0.045), we found non-significant correlations were found between RUNX2 expression and sex, smoking history of the patients, number of distant metastases or histopathological type of the tumor.

There was a significant positive association between both markers (p=0.015) and both markers together were significantly correlated with size, grade, stage, site of the tumor within the lung, malignant (pleural and/or pericardial) effusion, presence of distant metastases, ECOG performance status of the patients(p<0.001) and presence of liver metastases (p=0.004).

Relations between CR-1 &RUNX2 expressions and patient survival (Tables 4 and 5) (Figure 3)

Both markers expressions were significantly correlated with poor response to treatment (p<0.001). After median follow up of 30 months (range: 5-35 months),the 2year progression free survival (PFS) of all patients was 69.1%, patients with high RUNX2 and/or CR-1 expressions had shorter DFS than those with low expression (p<0.001). OS rate was shorter in patients with high expression of RUNX2 in comparison to those with low expression (p=0.025), also patients with high CR-1 expressing tumors had shorter OS but that was statistically non-significant (p=0.2).

Discussion

There are no specific guidelines for early detection of NSCLC which result in poor prognosis and low survival of patients. Hence, it is extremely important to discover new biomarkers for predicting the progression, metastasis and treatment outcome of NSCLC patients which subsequently lead to improvement in prognosis. In this study, CR-1 expression in NSCLC patients was positively correlated with grade, stage of the tumor, lymph node and distant metastases which proved our hypothesis that CR-1 could have a highly important role in NSCLC Oncogenesis and progression. These results were in agreement with Xu et al. [21] who verified that CR-1 has an essential role in increasing lung cancer cell proliferation and allow early progression. We also found that PFS and OS rates of the patients

Table 1: Clinicopathological features, Immunohistochemical markers and outcome of our patients.

Characteristics	Number	%
Age (year)		
Mean ± SD	62.42 ± 8.45	
Median (Range)	65 (45-77)	
<65 years	26	44.1%
≥ 65 years	33	55.9%
Sex		
Male	39	66.1%
Female	20	33.9%
Comorbidity		
Absent	30	50.8%
Present	29	49.2%
Smoking		
Non smoker	24	40.7%
Smoker	35	59.3%
Performance status		
ECOG 0	26	44.1%
ECOG 1	10	16.9%
ECOG 2	11	18.6%
ECOG 3	12	20.3%
Weight loss		
<10%	36	61%
≥ 10%	23	39%
Histopathological type		
Adenocarcinoma	38	64.4%
Squamous cell carcinoma	21	35.6%
Grade		
Grade I	12	20.3%
Grade II	36	61%
Grade III	11	18.6%
Size		
5-7 cm	18	30.5%
>7 cm	41	69.5%
Site		
Upper lobe	17	28.8%
Middle lobe	24	40.7%
Lower lobe	13	22%
Entire lung	5	8.5%
Malignant pleural/pericardial effusion		
Absent	47	79.7%
Present	12	20.3%
LN metastasis		
Absent	16	27.1%
Present	43	72.9%
Distant metatasis		
Absent	37	62.7%
Present	22	37.3%
Number of metastatic sites		
0-2 sites	55	93.2%
>2sites	4	6.8%
Liver metatasis		
Absent	49	83.1%
Present	10	16.9%

Characteristics	Number	%
Bone metastasis		
Absent	54	91.5%
Present	5	8.5%
Brain metastasis		
Absent	51	86.4%
Present	8	13.6%
Adrenal metastasis		
Absent	55	93.2%
Present	4	6.8%
T		
T2b	13	22%
T3	27	45.8%
T4	19	32.2%
N		
N0	16	27.1%
N1	9	15.3%
N2	14	23.7%
N3	20	33.9%
M		
M0	37	62.7%
M1a	7	11.9%
M1b	15	25.4%
AJCC stage		
Stage IIB	16	27.1%
Stage IIIA	14	23.7%
Stage IIIB	7	11.9%
Stage IV	22	37.3%
RUNX-2		
Low	19	32.2%
High	40	67.8%
Cripto-1		
Low	24	40.7%
High	35	59.3%
RUNX-2/ Cripto-1		
Low/Low	12	20.3%
Low/High	7	11.9%
High/Low	12	20.3%
High/High	28	47.5%
Response to treatment		
PD	10	16.9%
SD	15	25.4%
PR	34	57.6%
Treatment outcome		
PD	10	16.9%
Clinical benefit(SD+PR)	49	83.1%
Follow-up duration		
Mean ± SD	24.05 ± 11.15	
Median (Range)	30 (5-35)	
Outcome		
Progression free	38	64.4%
Progression	21	35.6%
Alive	31	52.5%
Died	28	47.5%

Table 2: Correlation between immune-histochemical expressions of Cripto-1& RUNX-2 with **c**linicopathological features of our patients.

Characteristics	All (N=59)		RUNX-2				p-value	Cripto-1				p-value
			Low (N=19)		High (N=40)			Low (N=24)		High (N=35)		
	No.	(%)	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Age (years)												
Mean ± SD	62.42	± 8.45	62	± 8.14	62.62	± 8.68	0.332•	62.75	± 9.23	63.57	± 7.79	0.278•
Median (Range)	65	(45-77)	63	(45-77)	65	(45-77)		64	(45-74)	65	(46-77)	
<65 years	26	(44.1%)	16	(61.5%)	10	(38.5%)	<0.001‡	15	(57.7%)	11	(42.3%)	0.018‡
≥ 65 years	33	(55.9%)	3	(9.1%)	30	(90.9%)		9	(27.3%)	24	(72.7%)	
Sex												
Male	39	(66.1%)	13	(33.3%)	26	(66.7%)	0.795‡	15	(38.5%)	24	(61.5%)	0.628‡
Female	20	(33.9%)	6	(30%)	14	(70%)		9	(45%)	11	(55%)	
Comorbidity												
Absent	30	(50.8%)	19	(63.3%)	11	(36.7%)	<0.001‡	19	(63.3%)	11	(36.7%)	<0.001‡
Present	29	(49.2%)	0	(0%)	29	(100%)		5	(17.2%)	24	(82.8%)	
Smoking												
Non smoker	24	(40.7%)	10	(41.7%)	14	(58.3%)	0.198‡	10	(41.7%)	14	(58.3%)	0.898‡
Smoker	35	(59.3%)	9	(25.7%)	26	(74.3%)		14	(40%)	21	(60%)	
Performance status												
ECOG 0	26	(44.1%)	18	(69.2%)	8	(30.8%)	<0.001§	15	(57.7%)	11	(42.3%)	0.005§
ECOG 1	10	(16.9%)	1	(10%)	9	(90%)		6	(60%)	4	(40%)	
ECOG 2	11	(18.6%)	0	(0%)	11	(100%)		0	(0%)	11	(100%)	
ECOG 3	12	(20.3%)	0	(0%)	12	(100%)		3	(25%)	9	(75%)	
Weight loss												
<10%	36	(61%)	19	(52.8%)	17	(47.2%)	<0.001‡	19	(52.8%)	17	(47.2%)	0.018‡
≥10%	23	(39%)	0	(0%)	23	(100%)		5	(21.7%)	18	(78.3%)	
Histopathological type												
SCC	38	(64.4%)	12	(31.6%)	26	(68.4%)	0.890‡	18	(47.4%)	20	(52.6%)	0.159‡
Adenocarcinoma	21	(35.6%)	7	(33.3%)	14	(66.7%)		6	(28.6%)	15	(71.4%)	
Grade												
Grade I	12	(20.3%)	9	(75%)	3	(25%)	0.003§	11	(91.7%)	1	(8.3%)	<0.001§
Grade II	36	(61%)	8	(22.2%)	28	(77.8%)		13	(36.1%)	23	(63.9%)	
Grade III	11	(18.6%)	2	(18.2%)	9	(81.8%)		0	(0%)	11	(100%)	
Size												
>5-7 cm	18	(30.5%)	13	(72.2%)	5	(27.8%)	<0.001‡	10	(55.6%)	8	(44.4%)	0.123‡
>7 cm	41	(69.5%)	6	(14.6%)	35	(85.4%)		14	(34.1%)	27	(65.9%)	
Site												
Upper lobe	17	(28.8%)	7	(41.2%)	10	(58.8%)	0.001‡	11	(64.7%)	6	(35.3%)	0.030‡
Middle lobe	24	(40.7%)	3	(12.5%)	21	(87.5%)		7	(29.2%)	17	(70.8%)	
Lower lobe	13	(22%)	9	(69.2%)	4	(30.8%)		6	(46.2%)	7	(53.8%)	
Entire lung	5	(8.5%)	0	(0%)	5	(100%)		0	(0%)	5	(100%)	
Malignant pleural/ pericardial effusion												
Absent	47	(79.7%)	19	(40.4%)	28	(59.6%)	0.006‡	24	(51.1%)	23	(48.9%)	0.001‡
Present	12	(20.3%)	0	(0%)	12	(100%)		0	(0%)	12	(100%)	
LN metastasis												
Absent	16	(27.1%)	8	(50%)	8	(50%)	0.116‡	10	(62.5%)	6	(37.5%)	0.037‡
Present	43	(72.9%)	11	(25.6%)	32	(74.4%)		14	(32.6%)	29	(67.4%)	
Distant metatasis												
Absent	37	(62.7%)	19	(51.4%)	18	(48.6%)	<0.001‡	22	(59.5%)	15	(40.5%)	<0.001‡
Present	22	(37.3%)	0	(0%)	22	(100%)		2	(9.1%)	20	(90.9%)	
Number of metastatic sites												
0-2 sites	55	(93.2%)	19	(51.4%)	36	(65.5%)	0.294‡	24	(43.6%)	31	(56.4%)	0.138‡
>2sites	4	(6.8%)	0	(0%)	4	(100%)		0	(0%)	4	(100%)	

Liver metatasis												
Absent	49	(83.1%)	19	(38.8%)	30	(61.2%)	0.022‡	24	(49%)	25	(51%)	0.004‡
Present	10	(16.9%)	0	(0%)	10	(100%)		0	(0%)	10	(100%)	
Bone metastasis												
Absent	54	(91.5%)	19	(38.8%)	35	(64.8%)	0.165‡	24	(44.4%)	30	(55.6%)	0.073‡
Present	5	(8.5%)	0	(0%)	5	(100%)		0	(0%)	5	(100%)	
Brain metastasis												
Absent	51	(86.4%)	19	(37.3%)	32	(62.7%)	0.045‡	24	(44.4%)	27	(52.9%)	0.016‡
Present	8	(13.6%)	0	(0%)	8	(100%)		0	(0%)	8	(100%)	
Adrenal metastasis												
Absent	55	(93.2%)	19	(34.5%)	36	(65.5%)	0.294‡	24	(44.4%)	31	(56.4%)	0.138‡
Present	4	(6.8%)	0	(0%)	4	(100%)		0	(0%)	4	(100%)	
T												
T2b	13	(22%)	10	(76.9%)	3	(23.1%)	<0.001§	5	(38.5%)	8	(61.5%)	0.215§
T3	27	(45.8%)	7	(25.9%)	20	(74.1%)		15	(55.6%)	12	(44.4%)	
T4	19	(32.2%)	2	(10.5%)	17	(89.5%)		4	(21.1%)	15	(78.9%)	
N												
N0	16	(27.1%)	8	(50%)	8	(50%)	<0.001§	10	(62.5%)	6	(37.5%)	0.001§
N1	9	(15.3%)	7	(77.8%)	2	(22.2%)		6	(66.7%)	3	(33.3%)	
N2	14	(23.7%)	4	(28.6%)	10	(71.4%)		6	(42.9%)	8	(57.1%)	
N3	20	(33.9%)	0	(0%)	20	(100%)		2	(10%)	18	(90%)	
M												
M0	37	(62.7%)	19	(51.4%)	18	(48.6%)	<0.001§	22	(59.5%)	15	(40.5%)	<0.001§
M1a	7	(11.9%)	0	(0%)	7	(100%)		2	(28.6%)	5	(71.4%)	
M1b	15	(25.4%)	0	(0%)	15	(100%)		0	(0%)	15	(100%)	
AJCC stage												
Stage IIB	16	(27.1%)	12	(75%)	4	(25%)	<0.001§	11	(68.8%)	5	(31.3%)	<0.001§
Stage IIIA	14	(23.7%)	7	(50%)	7	(50%)		7	(50%)	7	(50%)	
Stage IIIB	7	(11.9%)	0	(0%)	7	(100%)		4	(57.1%)	3	(42.9%)	
Stage IV	22	(37.3%)	0	(0%)	22	(100%)		2	(9.1%)	20	(90.9%)	
RUNX-2												
Low	19	(32.2%)						12	(63.2%)	7	(36.8%)	0.015‡
High	40	(67.8%)						12	(30%)	28	(70%)	
Cripto-1												
Low	24	(40.7%)	12	(50%)	12	(50%)	0.015‡					
High	35	(59.3%)	7	(20%)	28	(80%)						

Note: Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range).
•Mann Whitney U test; ‡Chi-square test; §Chi-square test for trend; p<0.05 is significant.

having an elevated CR-1 immuno-expression were shorter. Our results showed and explained the promising use of CR-1 as a recent prognostic marker and added a spot light on the relation between its biological function and NSCLC carcinogenesis. Our results were supported by Bianco et al., and Nagaoka et al. [6,7] who showed that CR-1 is EGF-CFC family member that is essential for angiogenesis, cell migration and maintenance of stem cells**.** Sun et al. [22] reported that CR-1 regulated EMT and invasiveness of HCC. Wei et al. [23] demonstrated the role of CR-1 in bladder cancer growth, proliferation, recurrence and metastasis. Moreover, similar results from Wang et al. [24], Zhong et al. [8], Yoon et al. [25] and Wu et al. [26] proved the association between CR-1 over expression, tumor recurrence, lower 5-year survival rates and the histological differentiation in oral squamous cell carcinoma; and the carcinogenesis and progression of nasopharyngeal cancer. Regarding RUNX2 expression in NSCLC, we found that high RUNX2 immuno-expressions were correlated significantly with tumor size, tumor stage and grade, lymph node and distant metastases. PFS and OS rates of NSCLC patients having high RUNX2 immuno-expression were shorter than those with low RUNX2 expression. So we clarify that, RUNX2 elevated expression may be considered a substantial factor in expecting the prognosis of patients having NSCLC, identifying patients with a poor prognosis; hence, may be considered a novel prognostic marker for NSCLC patients. Hong et al. [27], Yang et al. [28], Chua et al. [29] and Tonomoto et al. [30] reported similar results in NSCLC, colon cancer, prostatic cancer and esophageal squamous cell carcinoma, respectively. In tumorigenesis, RUNX2 is a controller of tumor invasion and metastasis, and high expression of RUNX2 is markedly related to metastasis of osteosarcoma [31]. The above mentioned data may prove that RUNX2 up-regulation is linked to the invasiveness of malignant cells [32]. RUNX2 enhances endothelial cell proliferation, invasion, and tube formation and activate vascular endothelial growth factor gene expression; all of which may lead to stimulation of angiogenesis in cancer cells which facilitate their growth and spread [33]. RUNX2 stimulates transcription of osteopontin, which increased metastatic ability in carcinoma cells [34]. RUNX2 increases metastasis of carcinoma mainly by increasing the expression of matrix metalloproteinase [32] and bone sialoprotein [35]**.** RUNX2 can be considered a potent prognostic factor for NSCLC patients through the stimulation of cell proliferation, migration and invasiveness of

Table 3: Correlation between immune-histochemical expressions of both markers together with **c**linicopathological features of our patients.

Characteristics	All (N=59)		RUNX-2/Cripto-1								p-value
			Low/Low (N=12)		Low/High (N=7)		High/Low (N=12)		High/High (N=28)		
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	
Age (years)											
Mean ± SD	62.42	± 8.45	62.83	± 7.86	60.57	± 9.05	58.66	± 10.35	64.32	± 7.44	0.292•
Median (Range)	65	(45-77)	64	(45-74)	60	(49-77)	64	(45-69)	65.50	(46-77)	
<65 years	26	(44.1%)	10	(38.5%)	6	(23.1%)	5	(19.2%)	5	(19.2%)	<0.001‡
≥ 65 years	33	(55.9%)	2	(6.1%)	1	(3%)	7	(21.2%)	23	(69.7%)	
Sex											
Male	39	(66.1%)	7	(17.9%)	6	(15.4%)	8	(20.5%)	18	(45.2%)	0.667‡
Female	20	(33.9%)	5	(25%)	1	(5%)	4	(20%)	10	(50%)	
Comorbidity											
Absent	30	(50.8%)	12	(40%)	7	(23.3%)	7	(23.3%)	4	(13.3%)	<0.001‡
Present	29	(49.2%)	0	(0%)	0	(0%)	5	(17.2%)	24	(82.8%)	
Smoking											
Non smoker	24	(40.7%)	6	(25%)	4	(16.7%)	4	(16.7%)	10	(41.7%)	0.621‡
Smoker	35	(59.3%)	6	(17.1%)	3	(8.6%)	8	(22.9%)	18	(51.4%)	
Performance status											
ECOG 0	26	(44.1%)	11	(42.3%)	7	(26.9%)	4	(15.4%)	4	(15.4%)	<0.001§
ECOG 1	10	(16.9%)	1	(10%)	0	(0%)	5	(50%)	4	(40%)	
ECOG 2	11	(18.6%)	0	(0%)	0	(0%)	0	(0%)	11	(100%)	
ECOG 3	12	(20.3%)	0	(0%)	0	(0%)	3	(25%)	9	(75%)	
Weight loss											
<10%	36	(61%)	12	(33.3%)	7	(19.4%)	7	(19.4%)	10	(27.8%)	<0.001‡
≥10%	23	(39%)	0	(0%)	0	(0%)	5	(21.7%)	18	(78.3%)	
Histopathological type											
SCC	38	(64.4%)	8	(21.1%)	4	(10.5%)	10	(26.3%)	16	(42.1%)	0.439‡
Adenocarcinoma	21	(35.6%)	4	(19%)	3	(14.3%)	2	(9.5%)	12	(57.1%)	
Grade											
Grade I	12	(20.3%)	8	(66.7%)	1	(8.3%)	3	(25%)	0	(0%)	<0.001§
Grade II	36	(61%)	4	(11.1%)	4	(11.1%)	9	(25%)	19	(52.8%)	
Grade III	11	(18.6%)	0	(0%)	2	(18.2%)	0	(0%)	9	(81.8%)	
Size											
>5-7 cm	18	(30.5%)	7	(38.9%)	6	(33.3%)	3	(16.7%)	2	(11.1%)	<0.001‡
>7 cm	41	(69.5%)	5	(12.2%)	1	(2.4%)	9	(22%)	26	(63.4%)	
Site											
Upper lobe	17	(28.8%)	7	(41.2%)	0	(0%)	4	(23.5%)	6	(35.3%)	0.001‡
Middle lobe	24	(40.7%)	0	(0%)	3	(12.5%)	7	(29.2%)	14	(58.3%)	
Lower lobe	13	(22%)	5	(38.5%)	4	(30.8%)	1	(7.7%)	3	(23.1%)	
Entire lung	5	(8.5%)	0	(0%)	0	(0%)	0	(0%)	5	(100%)	
Malignant pleural/ pericardial effusion											
Absent	47	(79.7%)	12	(25.5%)	7	(14.9%)	12	(25.5%)	16	(34%)	0.001‡
Present	12	(20.3%)	0	(0%)	0	(0%)	0	(0%)	12	(100%)	
LN metastasis											
Absent	16	(27.1%)	7	(43.8%)	1	(6.3%)	3	(18.8%)	5	(31.3%)	0.052‡
Present	43	(72.9%)	5	(11.6%)	6	(14%)	9	(20.9%)	23	(53.5%)	
Distant metatasis											
Absent	37	(62.7%)	12	(32.4%)	7	(18.9%)	10	(27%)	8	(21.6%)	<0.001‡
Present	22	(37.3%)	0	(0%)	0	(0%)	2	(9.1%)	20	(90.9%)	
Number of metastatic sites											
0-2 sites	55	(93.2%)	12	(21.8%)	7	(12.7%)	12	(21.8%)	24	(43.6%)	0.191‡
>2sites	4	(6.8%)	0	(0%)	0	(0%)	0	(0%)	4	(100%)	

Liver metatasis											
Absent	49	(83.1%)	12	(24.5%)	7	(14.3%)	12	(24.5%)	18	(36.7%)	0.004‡
Present	10	(16.9%)	0	(0%)	0	(0%)	0	(0%)	10	(100%)	
Bone metastasis											
Absent	54	(91.5%)	12	(22.2%)	7	(13%)	12	(22.2%)	23	(42.6%)	0.109‡
Present	5	(8.5%)	0	(0%)	0	(0%)	0	(0%)	5	(100%)	
Brain metastasis											
Absent	51	(86.4%)	12	(23.5%)	7	(13.7%)	12	(23.5%)	20	(39.2%)	0.017‡
Present	8	(13.6%)	0	(0%)	0	(0%)	0	(0%)	8	(100%)	
Adrenal metastasis											
Absent	55	(93.2%)	12	(21.8%)	7	(12.7%)	12	(21.8%)	24	(43.6%)	0.191‡
Present	4	(6.8%)	0	(0%)	0	(0%)	0	(0%)	4	(100%)	
T											
T2b	13	(22%)	4	(30.8%)	6	(46.2%)	1	(7.7%)	2	(15.4%)	0.001§
T3	27	(45.8%)	7	(25.9%)	0	(0%)	8	(29.6%)	12	(44.4%)	
T4	19	(32.2%)	1	(5.3%)	1	(5.3%)	3	(15.8%)	14	(73.7%)	
N											
N0	16	(27.1%)	7	(43.8%)	1	(6.3%)	3	(18.8%)	5	(31.3%)	<0.001§
N1	9	(15.3%)	4	(44.4%)	3	(33.3%)	2	(22.2%)	0	(0%)	
N2	14	(23.7%)	1	(7.1%)	3	(21.4%)	5	(35.7%)	5	(35.7%)	
N3	20	(33.9%)	0	(0%)	0	(0%)	2	(10%)	18	(90%)	
M											
M0	37	(62.7%)	12	(32.4%)	7	(18.9%)	10	(27%)	8	(21.6%)	<0.001§
M1a	7	(11.9%)	0	(0%)	0	(0%)	2	(28.6%)	5	(71.4%)	
M1b	15	(25.4%)	0	(0%)	0	(0%)	0	(0%)	15	(100%)	
AJCC stage											
Stage IIB	16	(27.1%)	9	(56.3%)	3	(18.8%)	2	(12.5%)	2	(12.5%)	<0.001§
Stage IIIA	14	(23.7%)	3	(21.4%)	4	(28.6%)	4	(28.6%)	3	(21.4%)	
Stage IIIB	7	(11.9%)	0	(0%)	0	(0%)	4	(57.1%)	3	(42.9%)	
Stage IV	22	(37.3%)	0	(0%)	0	(0%)	2	(9.1%)	20	(90.9%)	

Note: • Kraskall Wallis H test; ‡ Chi-square test; § Chi-square test for trend

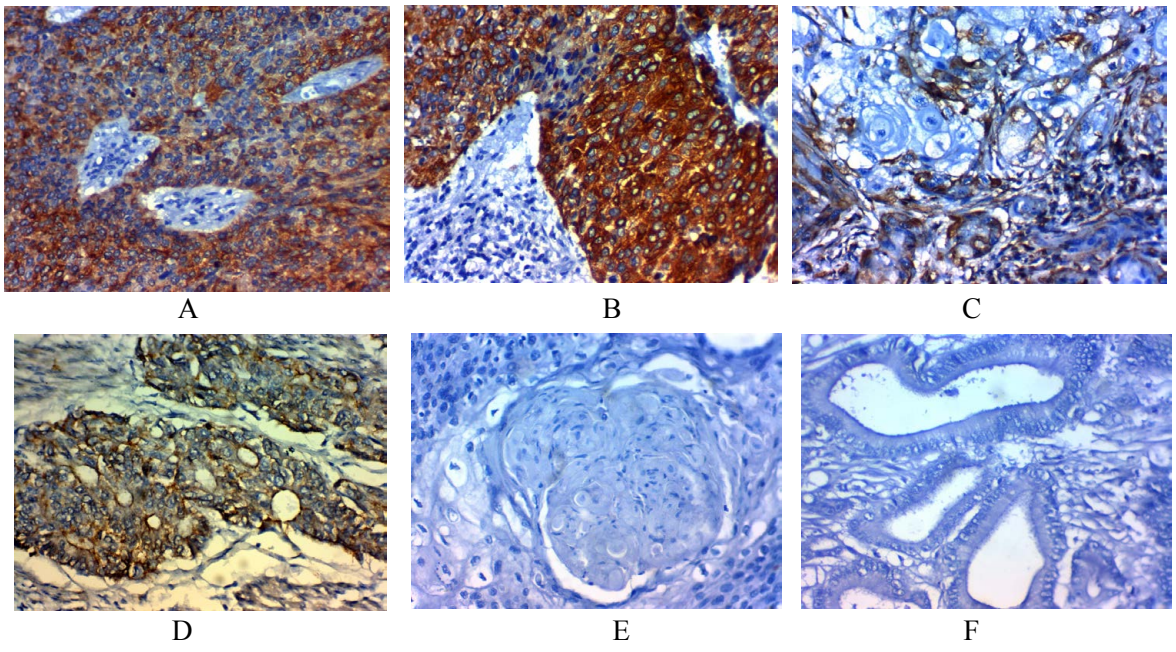

Figure 1: Immunohistochemical expression of Cripto-1(CR1) in non-small cell lung cancer (NSCLC).

*__Note:__ (A) High expression in the cytoplasm of poorly differentiated squamous cell carcinoma x400 (B) High expression in the cytoplasm of poorly differentiated adenocarcinoma x400 (C) Low expression in cytoplasm of moderately differentiated squamous cell carcinoma stage IIx400 (D) Low expression in the cytoplasm of moderately differentiated adenocarcinoma stage IIx400 (E) Negative expression in the cytoplasm of well differentiated squamous cell carcinoma stage IIx400(F) Negative expression in the cytoplasm of well differentiated adeno-carcinoma stage IIx400.

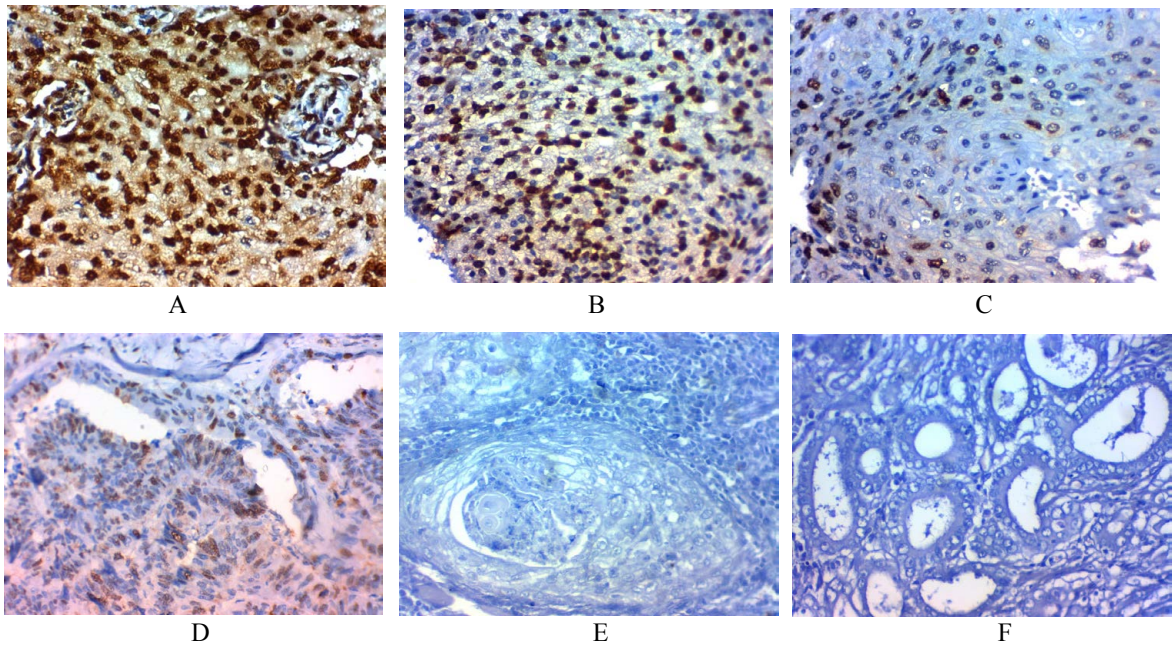

Figure 2: Immunohistochemical expression of RUNX2 in non-small cell lung cancer (NSCLC).

Note: (A) High expression in nucleus of poorly differentiated squamous cell carcinoma stage IVx400 (B) High expression in nucleus of poorly differentiated adenocarcinoma stage IVx400 (C) High expression in nucleus of moderately differentiated squamous cell carcinoma stage IIIx400. (D) High expression in nucleus of moderately differentiated adenocarcinoma stage IIIx400 (E) Low expression in nucleus of well differentiated squamous cell carcinoma stage IIx400 (F) Low expressions in nucleus of well differentiated adenocarcinoma stage IIx400.

Table 4: Correlation between immune-histochemical expressions of Cripto-1& RUNX-2 with outcome of our patients.

Outcome	All (N=59)		RUNX-2				p-value	Cripto-1				p-value
			Low (N=19)		High (N=40)			Low (N=24)		High (N=35)		
	No.	(%)	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Response to treatment												
PD	10	(16.9%)	0	(0%)	10	(25%)	<0.001‡	0	(0%)	10	(28.6%)	0.002‡
SD	15	(25.4%)	0	(0%)	15	(37.5%)		4	(16.7%)	11	(31.4%)	
PR	34	(57.6%)	19	(100%)	15	(37.5%)		20	(83.3%)	14	(40%)	
Treatment outcome												
PD	10	(16.9%)	0	(0%)	10	(25%)	<0.001‡	0	(0%)	10	(28.6%)	0.001‡
Clinical benefit (SD+PR)	49	(83.1%)	19	(100%)	30	(75%)		24	(100%)	25	(71.4%)	
Progression												
Absent	38	(64.4%)	19	(100%)	19	(47.5%)	<0.001‡	23	(95.8%)	15	(42.9%)	<0.001‡
Present	21	(35.6%)	0	(0%)	21	(52.5%)		1	(4.2%)	20	(57.1%)	
PFS												
Mean (month) (95%CI)	26.5 month (23.3-29.7)		32.7 month (31.1-34.3)		22.4 month (18.3-26.5)		<0.001†	34.3 month (32.9-35.7)		21.4 month (16.8-25.9)		<0.001†
HR (95%CI)	--		48.22 (1.26-1843.87)					17.19 (7.29-40.54)				
6 month PFS (%)	84.8%		100%		77.5%			100%		74.3%		
12 month PFS (%)	74.9%		100%		62.5%			100%		57.1%		
24 month PFS (%)	69.1%		100%		54%			95.2%		51.4%		
30 month PFS (%)	62.6%		100%		43.2%			95.2%		42.4%		
Mortality												
Alive	31	(52.5%)	14	(73.7%)	17	(42.5%)	0.025‡	15	(62.5%)	16	(45.7%)	0.205‡
Dead	28	(47.5%)	5	(26.3%)	23	(57.5%)		9	(37.5%)	19	(54.3%)	

OS							
Mean (month) (95%CI)	32.4 month (31.1-33.7)	34.1 month (33.7-34.5)	30.8 month (28.7-33)	0.004†	33.8 month (31.1-34.5)	32 month (30.1-33.9)	0.087†
HR (95%CI)	--	3.58 (1.70-7.56)			1.95 (0.93-4.09)		
6 month PFS (%)	98.3%	100%	90%		100%	88.6%	
12 month PFS (%)	72.9%	100%	60%		95.8%	57.1%	
24 month PFS (%)	62.3%	94.7%	46.8%		74.6%	54%	
30 month PFS (%)	58.6%	89.5%	43.7%		65.2%	54%	

Note: ‡ Chi-square test; † Log rank test; HR: Hazards Ratio; 95%CI: 95% Confidence Interval

Table 5: Correlation between immune-histochemical expressions of both markers together with outcome of our patients.

Outcome	All (N=59)		RUNX-2/Cripto-1								p-value
			Low/Low (N=12)		Low/High (N=7)		High/Low (N=12)		High/High (N=28)		
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	
Response to treatment											
PD	10	(16.9%)	0	(0%)	0	(0%)	0	(0%)	10	(35.7%)	<0.001§
SD	15	(25.4%)	0	(0%)	0	(0%)	4	(33.3%)	11	(39.3%)	
PR	34	(57.6%)	12	(100%)	7	(100%)	8	(66.7%)	7	(25%)	
Progression											
Absent	38	(64.4%)	12	(100%)	7	(100%)	11	(91.7%)	8	(28.6%)	<0.001§
Present	21	(35.6%)	0	(0%)	0	(0%)	1	(8.3%)	20	(71.4%)	
PFS											
Mean (month) (95%CI)	26.5 month (23.3-9.7)		32.5 month (29.9-35.1)		33 month (31.8-34.2)		33.3 month (30.3-36.4)		17.9 month (13.1-22.9)		<0.001†
6 month PFS (%)	84.8%		100%		100%		100%		67.9%		
12 month PFS (%)	74.9%		100%		100%		100%		46.4%		
24 month PFS (%)	69.1%		100%		100%		88.9%		39.3%		
30 month PFS (%)	62.6%		100%		100%		88.9%		27.5%		
Mortality											
Absent	31	(52.5%)	9	(75%)	5	(71.4%)	6	(50%)	11	(39.3%)	0.023§
Present	28	(47.5%)	3	(25%)	2	(28.6%)	6	(50%)	17	(60.7%)	
OS											
Mean (month) (95%CI)	32.4 month (31.1-33.7)		34.4 month (33.9-34.8)		33.6 month (32.8-34.4)		30.3 month (26.6-34)		31.1 month (28.3-33.9)		0.026†
6 month PFS (%)	98.3%		100%		100%		100%		85.7%		
12 month PFS (%)	72.9%		100%		100%		91.7%		46.4%		
24 month PFS (%)	62.3%		91.7%		100%		57.1%		42.2%		
30 month PFS (%)	58.6%		83.3%		100%		45.7%		42.2%		

Note: § Chi-square test for trend; † Log rank test; 95%CI: 95% Confidence Interval;

malignant tissue; therefore, RUNX2 plays important roles in the growth and metastasis of cancer cells through wide spectrums of its biological functions.

In this study, we found a significant positive association between the expression of both CR-1 and RUNX2 in NSCLC cases (p=0.015), and together they have a significant effect on the performance status, PFS, OS and subsequently the prognosis of NSCLC patients.

These results indicated that CR-1 could serve as a feasible prognostic biomarker of NSCLC. RUNX2 plays an important role in the tumorigenesis and progression of NSCLC; hence both markers together may provide a chance for discovering recent-therapeutic targets, as well as prognostic markers in NSCLC. Further studies are recommended on large number of cases of NSCLC and other types of cancers to clarify the precise molecular functions of these markers and the value of using them together in assessment of NSCLC prognosis and as therapeutic targets.

Previous studies have investigated a panel of prognostic markers for NSCLC but most of them were investigated as serum markers with low sensitivity and specificity conveying conflicting results; hence, our study assessed the tissue expressions of both Cripto-1 and RUNX2 using immunohistochemistry which demonstrated more sensitive and specific results for assessment of NSCLC prognosis [36-39].

Summary

Our research confirmed that CR-1 and RUNX2 high expressions had an essential role in tumor aggression and poor NSCLC patients' prognosis. Also, their levels appear to be an important predictor for NSCLC patient's survival. Nonetheless, further studies are needed to elucidate the mechanisms by which both markers facilitate NSCLC development and progression and to address whether one of them or both together could be used as targets for therapeutic approaches.

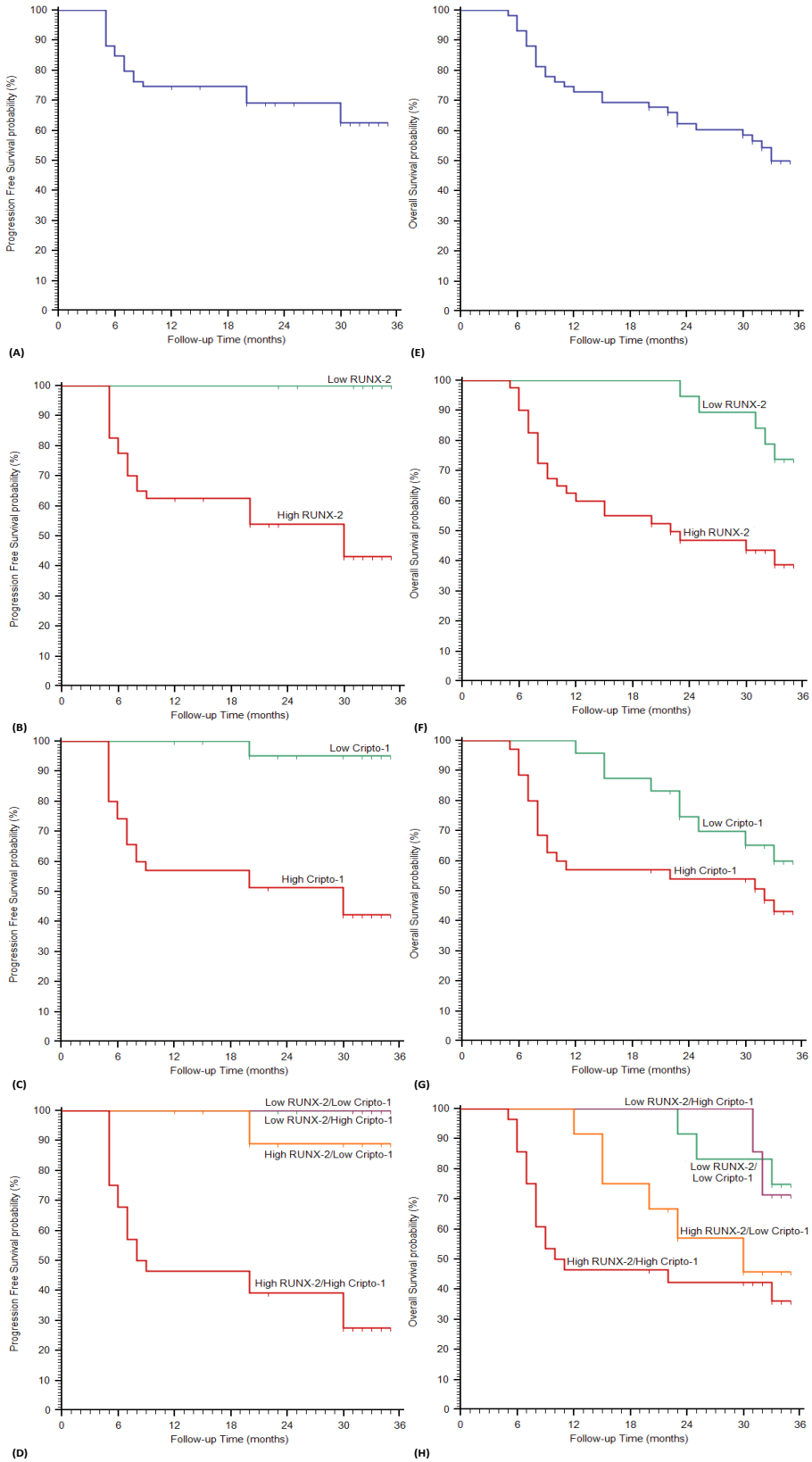

Figure 3: Kaplan Meier Survival plots; Left Panel: Progression Free Survival (PFS); Right Panel: Overall Survival.

'**Note:** (A) & (E): All studied NSCLC patients; (B) & (F) Stratified by RUNX-2 IHC staining; (C) & (G) Stratified by Cripto-1 IHC staining; (D) & (H) Stratified by RUNX-2/Cripto-1 IHC staining.

Limitations of our Work

- Small number of patients.
- Assessment of Cripto-1& RUNX2 expression was only by immunohistochemistry without any assessment of serum levels or genetic analysis by ISH or real time-PCR.

Recommendations

- Cripto-1 and RUNX2 together may provide a chance for discovering recent-therapeutic targets as well as prognostic markers in NSCLC.
- Further studies are recommended on large number of cases of NSCLC and other types of cancers to clarify the precise molecular functions of these markers and the values of using them together in assessment of NSCLC prognosis, and as therapeutic targets.
- To use different methods of assessment of both markers; serum levels or gene analysis.
- to compare between both markers expression with levels of CEA, TPA, SCC-Ag, CYFRA 21-1, ferritin, CA19-9, CA50, CA242, H-K-N-ras mutations and p53 mutation seem which were previously used as the most specific biomarkers in N-SCLC.

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