Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in Scienze Biomediche Integrate e Bioetica.

XXXI ciclo a.a. 2015-2016

Title

Expression of HIF-1α in advanced non small cell lung cancer, comparison between patients with bone metastasis and without bone metastasis, the influence of smoking habit : a retrospective analysis over two years of recruitment.

Aldo Pezzuto

Coordinatore
Prof Paolo Pozzilli

Tutore
Prof. Giuseppe Tonini

20 Marzo 2019

TITLE

Expression of HIF-1α in advanced non small cell lung cancer, comparison between patients with bone metastasis and without bone metastasis, the influence of smoking habit : a retrospective analysis over two years of recruitment.

Abstract

Background: HIF-1 is a transcription factor that allows cells to adapt to hypoxia. It consists of two forms HIF-1α and HIF-1β. It is able upon activation, in hypoxia condition, to foster several oncogenes and proteins which in turn are involved in carcinogenesis, cell invasion and migration.

Aim: to verify if HIF-1α tissue expression is associated with lung cancer bone metastasis process and evaluate its influence on prognosis.

Methods a retrospective analysis was carried out on samples deriving from bronchial biopsy and CT-guided trans-thoracic needle biopsy.

The data collected concerned advanced NSCLC patients and included age, staging, detailed histotype, pack-year, comorbidities.

Detection of HIF-1 expression was performed by the use of a murine specific antibody. A comparison was carried out between group with visceral metastases and group having also bone metastases.

Results

A total of 146 patients with mean age of about 70 were considered, the prevalent histotype was adenocarcinoma(67.1%). Former smokers accounted for 58.9%. The mean pack-year was 36.4. CT-guided trans-thoracic biopsy was the main source of the specimens.

The population was subdivided in two groups based on the presence or absence of bone metastasis and the comparison showed non-significant differences about ECOG PS, age, cardiovascular comorbidities. Significant differences were detected about pack-year($p=0.02$), time to progression(TTP)($p=0.001$) and COPD comorbidity($p=0.04$).

The Kaplan-Meier method with Log-rank test applied on survival analysis comparing the subgroups showed a longer TTP in patients with visceral metastases with HR of 1.3 and p =0.14.

The sample available for immunohistochemistry detection of HIF-1 α consisted of 61 patients. A higher intensity of expression along with higher positive cells percentage was recorded in the group with bone metastases. The main variable affecting HIF expression was the presence of bone metastasis(p=0.01), whereas histotype seems to influence the TTP(p=0.04).The product of intensity for percentage of positive cells was significantly higher in the group with bone metastases (p=0.02).

Conclusions

Patients affected by NSCLC IV stage with both visceral and bone metastases have lower survival than those with only visceral metastases.

The presence of bone metastasis is tightly linked with the expression of HIF-1α.

The intensity combined with the percentage of positive expression is higher in patients with bone metastasis than in patients without it, suggesting a role of HIF-1α in cancer progression .

Key words: HIF-1 expression, Lung cancer, bone metastases, time to progression

INTRODUCTION

Hypoxia is a frequent occurrence that could be found in roughly 50% of solid tumors owing to high proliferation rate of cancer cells along with altered vascularization(1). The hypoxic tumor microenvironment influences both the early and late stage of the disease. The transcription factor hypoxia-inducible factor-1alpha (HIF-1) is a protein ubiquitously expressed and notably produced by tumor cells in hypoxia condition . It is a heterodimer helix-loop protein with a carboxy- and amino-terminus consisting of form α and β , it is the master regulator of oxygen homeostasis that binds to hypoxia responsive element (HRE) on target genes. The form β is constitutively expressed within the nucleus, by contrast the form α is expressed in oxygen-dependent manner and present in the cytoplasm(1).

HIF-1α is associated with cell activation, metastasis, and resistance to chemotherapy(2,3) . In normoxia conditions the aforementioned factor is located in the cytoplasm and hydroxylation of proline residues occurs by means of hydroxylase enzymes (EGLN) that in turn allows von Hippel Lindau tumor suppressor(VHL) to bind to HIF and elicit its degradation by the ubiquitin proteasome system (4). Conversely in hypoxic conditions, HIF-1α is unable to bind to the VHL protein in the cytoplasm, escapes decomposition and enters the nucleus. In the nucleus, it combines with HIF-1β to form the HIF-1 stable complex, which binds to DNA and acts as a transcription factor. HIF-1 brings about the activation of genes involved in angiogenesis, glycolysis, cancer proliferation and other associated pathways (5).

HIF-1 is indeed involved in neo-angiogenesis and in bone metastasis mechanisms, and it's able to elicit the expression of growth factors such as VEGF. In-vitro and animal model showed that hypoxia , frequently associated with lung disease and HIF-1 expression contribute both to bone loss and as a consequence they foster the development of bone metastases(6,14)

There is a strong link between hypoxia, HIF-1 expression and smoking habit (7). In a rat model for COPD, using exposure to LPS and cigarette smoke it was shown that expression of hypoxia inducible factor 1a gene was increased(7,28)

Indeed, the oncogenic role of cigarette smoking recognizes an important factor such as benzopyrene, an aromatic policyclic hydrocarbon (PAH) able to activate the receptor for the epidermal growth factor receptor (EGFR) and then cell proliferation (8).

Cigarette smoke is also responsible of dysfunction of bone metabolism through several mechanisms such as intestinal calcium absorption and sex hormone production. It also favors bone metastases through the activation of several growth factors, transcriptional factors, oncogene activation and inhibition of apoptosis (9,10). It is responsible of about 80% of lung cancer development.

We know that lung cancer is the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) encompasses about 80 % of all lung malignancies. More than half of NSCLC patients are diagnosed when tumor is at a late stage (III B and IV) and the only option is systemic chemotherapy(11,12).

However, 5-year survival rate of these patients remains below 10 % in patients without activating EGFR or ALK mutation(13).

Tumor metastasis is a major challenge issue , responsible for cancer cell death.

It is a very frequent occurrence in lung cancer, both on visceral and bone sites(14).

Our search was focused on the potential prognostic role of hypoxia-related HIF-1α in bone metastatic non small cell lung cancer.

AIM OF THE STUDY

The HIF-1α expression was already studied in vitro and in humans in lung cancer and it is associated with poor prognosis.

The aim of the present manuscript is to better understand the association between HIF-1 α and lung cancer bone metastasis and its influence on prognosis.

Hypothesis: HIF-1α is expressed higher in patients affected by lung cancer with bone metastases than in patients without it.

Primary endpoint : to determine the expression of HIF-1α in patients suffering from metastatic non small cell lung cancer comparing group with bone metastases with group without bone metastases .

Secondary endpoint: to correlate the expression of HIF with smoking status, bone metastasis and prognosis. To determine differences in terms of time to progression (TTP) between the two groups, with and without bone metastasis.

METHODS

From November 2015 to December 2016 we conducted a retrospective analysis of 146 patients who underwent bronchoscopy or CT-guided trans-thoracic biopsy for lung mass, presenting a primary lung cancer. The inclusion criteria were: Patients age≥18 years former or current smoker with advanced lung cancer. Histological confirmation of lung cancer with detailed histotype has been considered. The exclusion criteria was lung cancer stage I-II-IIIA.

The study was approved by Campus Bio-Medico Ethic Committee.

TARGET POPULATION:

SAMPLE SIZE and population

The sample size was determined by comparison of proportions with 80% power to detect HIF- 1 α and the α value set at 0.05 of significance. At least overall 50 patients had to be recruited for this purpose.

The initial study was conducted on a sample of 146 patients suffering from advanced lung cancer current or former smoker referred to the clinic. Two subgroups were identified with or without bone metastases.

Ninety-five were the total population with proven positive histology obtained by trans-thoracic CT guided biopsies. Sixty-one of 95 cases had tumor blocks available for immunohistochemical analysis for HIF1α expression.

MEASUREMENTS AND PARAMETERS

Demographic and functional baseline parameters

General demographic characteristics were collected and reported such as gender, age, smoking habit (never, previously, current),body mass index (BMI), smoking index risk defined as pack / year to distinguish heavy and light smokers.
The historical and functional parameters collected were the following:
performance status, comorbidities, staging by CT scan total body , FDG-PET/CT and optionally skeletal scintigraphy.
The histology tissue used for biological parameters detection comes from lung and bronchial biopsies.

Techniques for histology specimen

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections of bronchoscopic or computer tomography-guided needle specimens. Representative paraffin blocks were cut into 3 µm sections that were mounted onto coated slides.
Afterwards, the sections were dewaxed by xylene and ethyl alcohol and rehydrated. All tissue sections slides were heated in citrate buffer solution at pH 6.0 for 40 minutes at 97°C, and then rinsed with H2O2 for 30 minutes.
Hence the sections were incubated with mouse monoclonal antibody anti‑HIF‑1α (clone H1 alpha 67; cat. no. NB100‑105; Novus Biologicals, Littleton, CO, USA; 1:50) for 2 hours at room temperature.
After washing, the slides were incubated with secondary antibody conjugated with 2nd generation visualization kit which is suitable for both rabbit and mouse primary antibodies (DAKO) , and the binding was displayed by 3-3' diaminobenzine tetrahydrochloride after 30 minutes.
The staining results were scored semiquantitatively as intensity: negative 0, mild 1, moderate 2 and high 3 and as percentage of positive cells. A histoscore was generated multiplying the intensity value (score 0-3) by the percentage of cells.

Statistical Analysis

The Fisher's exact test was applied for categorical variables. The Mann Whitney test was applied for continuous variables do not following a normal distribution, to detect differences between groups.

Logistic regression method was performed to detect the variables affecting HIF expression concerning percentage and intensity. A Kaplan-Meier analysis with log-rank test was used to compare the TTP of the subgroups.

A cox-proportional hazard regression analysis was performed to highlight which variables may affect the TTP as outcome.

The SPSS 24.0 statistical software package was used for analysis(Inc,Chicago,IL,USA). Data were expressed as mean ± standard deviation and median plus interquartile range as appropriate, with significance level set at $p<0.05$.

RESULTS

The total sample was 146 among which 95 were obtained by trans-thoracic biopsies, 21 from bronchial biopsies and 30 from trans-bronchial needle aspiration (TBNA).

Sixty-one histology samples were eventually available for HIF detection .

Table 1 summarizes the characteristics of the sample overall: 146 patients

Mean age was 70.1±9.9, range 35-88, the ECOG PS was 1 for roughly 73% of patients.

The histotype frequency was the following:

19.8% squamous , adenocarcinoma 67.1%, large cell carcinoma 13.1%

Fifty-seven patients (39.1%) presented with visceral and bone metastases, the remaining 89(60.9%) reported only visceral ones.

The main comorbidity was COPD present in 74(50.6%) of patients.

The smoking status was the following: mean pack-year 36.4, former smokers were 86(58.9%), current smokes were 38(26%), non smokers only 22(15%).

A comparison between patients having or not bone metastases is displayed in Table 2 including only trans-thoracic biopsy samples (95 patients).

Pack-year was higher in patients with visceral metastases than those with bone and visceral and the difference was significant (p=0.02) as well as the difference in terms of TTP (p=0.001), median 6 months for visceral metastatic group 1 versus 5 for bone metastatic group and COPD comorbidity(p=0.04). The other comparisons including age, ECOG PS, heart comorbidity, EGFR mutation frequency were not significant.

In figure 1 it is depicted the difference between groups in terms of TTP curves showing a HR 1.3 with IC 0.8-2.2; the data were not significant but there was a trend of better course of disease in group without bone metastases with a longer time to progression.

The influence of some variables on HIF-1 α expression is represented in table 3, highlighting that bone metastasis significantly affects the expression of the above.

Thus the HIF expression is eight time higher in presence of bone metastases(p=0.01). The other parameters did not affect significantly HIF-1 α expression .

In table 4 the influence of considered parameters was analyzed in a multivariate model by cox-proportional regression method. The histotype(adenocarcinoma/squamous) was shown to be the only variable affecting significantly the time to progression, increasing the probability of time to progression at six month detection(p=0.04).

The difference concerning HIF-1α expression intensity multiplied by percentage(histoscore) and cells positive percentage are displayed in table 5 (61 samples available). A significant value was recorded about the difference in histoscore between groups (p=0.02) . The histoscore was indeed higher in group harboring bone metastases. A difference was also found concerning the percentage of positive cells though without a significant value.

In figure 2 the difference between the groups about the histoscore of HIF-1α is depicted , whereas in figure 3 it is represented the difference between subgroups about percentage of positive cells.

The staining for HIF-1 α is represented in figure 4 where we can find a negative expression in figure A , a mild and high expression in B and C respectively .

DISCUSSION

In the present study we have analyzed a sample of patients affected by metastatic lung cancer.

As we know from the literature the finding of metastases is associated with a high mortality rate.

We focused on the comparison between subjects with both bone and visceral metastases and subjects with only visceral metastases.

Some differences regarding age, ECOG and comorbidities were detected although not significant .

The time to progression analysis displayed a significant difference with a better survival in the group with visceral metastases than the second group. The log rank test displayed a difference with a trend of significance.

We have also found that some variables may affect the HIF expression, among which the presence of bone metastases as expected. Those data are consistently with scientific literature.

As we know, indeed the transcription factor HIF-1α seems to play a role in the differentiation of osteoblasts and osteoclasts (15). In fact, co-culture of monocytes with stromal cells including osteoblasts, fibroblasts, and cancer cells has revealed that hypoxia stimulates local production of pro-osteoclastogenic cytokines including receptor activator of nuclear factor kappa B ligand (RANKL), vascular endothelial growth factor (VEGF), and at the same time it inhibits the production of osteoprotegerin (OPG), a soluble decoy receptor for RANKL that inhibits osteoclast formation and activity(16,17). Furthermore, in vitro and in vivo studies demonstrated that adaptation to hypoxia is a critical step in tumor progression and it is partially regulated by HIF-1α. Hypoxia and HIF-1α have been recognized to be responsible of

enhanced osteolytic bone metastases in MDA-MB-231 breast cancer cell lines , thus causing a poor prognoses coupled to increased patient mortality(16) .

We also found a poor prognosis associated with bone metastasis which is linked in turn with HIF-1α expression.

Furthermore, we know from the literature that HIF-1α when activated is able to stimulate the expression of such growth factors and glycolytic enzymes thereby affecting energy production in human cancer cells (18). Glucose is the main source for osteoclast to foster bone resorption. Many glycolytic enzymes are induced by HIF-1α, such as pyruvate dehydrogenase kinase-1(PDK-1), hexokinase, glucose transporter (GLUT-1). Activation of genes encoding GLUT-1 and GLUT-2 was reported to implement cancer cell metabolism favoring proliferation as well as the induction of mitochondrial reactive oxygen species (ROS) (19). HIF-1 induces upregulation of GLUT-1 in lung cancer cells, it was already reported(20). Those findings supported our data suggesting that hypoxia is a frequent occurrence in bone metastatic tissue and promotes by itself the tumor cell proliferation and migration by the induction of several genes.

Various are the mechanisms responsible of HIF-induced metastasis and tumor progression including the epithelial-mesenchymal transition pathway genes(EMT). The EMT process is characterized by changes in cell morphology and cell-matrix adhesion with loss of E-cadherin and overexpression of fibronectin and vimentin fostering the detachment of cells (21).

Moving to the role of smoke, studies in vitro on lung cancer A549 cells suggest that HIF-1 is nicotine- induced by means of mithocondrial reactive oxygen species(ROS)(22,23).

The present study highlighted a relationship between pack-year and therefore smoking habit and HIF expression.

According to a in vitro and human study the prevalent histotype associated with high HIF expression was squamous cell carcinoma(24).

Conversely in the present study we found that adenocarcinoma was the prevalent histotype influencing time to progression . The histoscore which encompasses the full significance of HIF expression was very higher in the group with bone metastases worsening the prognosis.

COPD is associated with metastatic lung cancer even though there is no significant relationship of the latter with the expression of HIF-1α. As a result inflammatory stimuli could foster the progression of the disease.

HIF-1α high staining has already been associated with a poor prognosis in small cell lung cancer , accordingly our study in bone metastatic NSCLC confirm its poor prognostic value(25).

An explanation of a reduced progression free survival, though non-significant, in group with bone metastases compared with that of visceral ones may be that HIF-1α being overexpressed in bone microenvironment induced tumor resistance by the influence on residential and immunity cells(26,4). Additionally, HIF- 1signaling can promote resistance to T cell–mediated killing by increasing the expression of programmed death–ligand 1 (PD-L1) on tumor cells and by increasing CTLA-4 expression on CD8+ T cells. HIF-1 also mediates stabilization of NF-kB and as a result it regulates the expression of anti-apoptotic factors such as Bax, Bcl-2 upon treatment with chemotherapeutic agents and mediates suppression of p53(26,27).

CONCLUSIONS

The study displays that HIF-1α expression is tightly linked with the advanced disease and bone metastases occurrence. Patients with bone metastasis have a worse time to progression than patients without it and the prevalent histotype adenocarcinoma could influence it. Smoking habit index such as pack-year and COPD are frequently associated with lung cancer though they not significantly influence the outcome. Our findings support HIF-1α as a potential biomarker of bone metastasis risk development and thus it is an adverse prognostic factor in lung cancer.

References

1- Soni S, Padwad Y. HIF-1 in cancer therapy: two decade long story of a transcription factor. Acta Oncol 2017;56(4):503-15.

2- Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. Cancer Res 2007;67(2):563–572.

3- Muz B, De la Puente P, Azab F et al. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia 2015;3:83–92.

4- Rankin E and Giaccia AJ. Hypoxic control of metastasis.Science . 2016; 352(6282):175–180.

5- Ke Q, Costa M. Hypoxia-inducible factor-1 [HIF-1]. Molec Pharmacol 2006;70(5):1469-80.

6- Bendinelli P, Maroni P, Matteucci E, et al.Cell and Signal Components of the Microenvironment of Bone Metastasis are affected by hypoxia.Int J Mol Sci. 2016;17(5):706-16.

7- Dajio H, Hoshino Y, Kai S et al.Cigarette smoke reversibly activates hypoxia-inducible factor 1 in a reactive oxygen species dependent manner. Nature Scientific Reports 2016;6: 34424-34436 .

8- Zhou B, Heeschen C, Sievers R, et al. Second hand smoke stimulates tumor angiogenesis and growth. Cancer Cell 2003; 4(3):191-6.

9- Tonini G; D'Onofrio L; Dell'Aquila E; Pezzuto A. New molecular insights in tobacco-induced lung cancer. Future Oncol. 2013;9(5):649-655.

10- Yoon Y, Maalouf M, Sakhaee K.The effects of smoking on bone metabolism. Osteoporosis 2012 ;23(8):2081-92.

11- Wang X. Lung cancer and metastases new opportunities and challeges. Cancer metastasis Rev 2015;34(2):169-71.

12- Siegel R, Naishadham D, Jemal A. Cancer statistics. CA Cancer J Clin. 2012;62(1):10–29.

13- Hoffman PC, Mauer AM, Vokes EE. Lung cancer. Lancet. 2000;355(9202):479–485.

14- Tamura T, Kurishima K, Nakazawa K et al. Specific organ metastases and survival in metastatic non-small-cell lung cancer. Mol Clin Oncol. 2015 ; 3(1): 217–221.

15- Knowles HJ. Hypoxic regulation of osteoclast differentiation and bone resorption activity. Hypoxia 2015 ;11(3):73-82.

16- Hiraga T et al. Hypoxia and hypoxia-inducible factor-1 expression enhance osteolytic bone metastases of breast cancer. Cancer Res 2007;67(9):4157-63

17- Guise A, Mohammed K, Clines G et al. Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. Clin Cancer Res 2006; 12(20 Pt 2):6213s-6216s.

18- Suzuki N, Gradin K, Poellinger L. Regulation of hypoxia-inducible gene expression after HIF activation. Exp Cell Res. 2017;356(2):182-186

19- Semenza G.HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev. 2010 ; 20(1): 51-6.

20- Fan R, How W, Zhao Y et al.Overexpression of HPV16 E6/E7 mediated HIF-1 α upregulation of GLUT-1 expression in lung cancer cells.Tumor Biol 2016;37(4)4655-63.

21- Yang MH, Wu KJ.TWIST activation by hypoxia inducible factor-1 (HIF-1): implications in metastasis and development. Cell Cycle. 2008;7(14):2090-6.

22- Schaal , Chellappan SP. Nicotine mediated cell proliferation and tumor progression in smoking related cancers Mol cancer Res 2014;12(1):14-23.

23- Guo L, Li L, Wang W. Mithocondrial reactive oxygen species mediate nicotine-induced hypoxia inducible factor-1 α expression in human non

small cell lung cancer cells. Byochimica et Byophisica acta 2012;1822:852-861.

24- Takasaki C, Kobayashi M, Ishibashi H. Expression of hypoxia-inducible factor-1α affects tumor proliferation and antiapoptosis in surgically resected lung cancer. Molec and Clin Oncol 2016; 5: 295-300.

25- Lin C, Liu T, Lee M, et al. Independent prognostic value of hypoxia-inducible factor 1-alpha expression in small cell lung cancer. Int J Med Science 2017;14(8):785-90.

26- Pezzuto A, Carico E. Role of HIF-1 in Cancer Progression: Novel Insights. A Review. Curr Molec Medicine 2018;18(6):343-51.

27- Rohwer N, Cramer T.Hypoxia-mediated drug resistance:novel insights on the functional interaction of HIFs and cell death pathways. Drug Resist Updat. 2011 Jun;14(3):191-201.

.

TABLE 1: Overall sample including cytologic and histologic

Total 146 TTNA+TBNA	
Age	
mean±SD	70.1±9.9
range	35-88
Gender M/F	74/72
Histotype	
squamous	29(19.8%)
Nsclc+large cell	19(13.1%)
Adenocarcinoma	98(67.1%)
PS ECOG 1	106(72.6%)
COPD	74(50.6%)
bone Metastasis +visceral	57(39.1%)
visceral	89(60.9%)
Smoking status	
former smoker	86(58.9%)
current smokers	38(26.0%)
non smokers	22(15.0%)
Sample:bronchial biopsy	21(14.0%)
Trans-thoracic biopsy	95(65.0%)
TBNA	30(20.0%)
Main Comorbidities:hypertension	45(30.8%)
mean pack year smoking	36.4±13.7

TABLE 2 Comparison between groups: 95 histology specimens

	BONE PLUS VISCERAL METASTASES	VISCERAL METASTASES	p
PACK-YEAR	27.5(25.0-35.0)	37.5(30.0-50.0)	0.02°
AGE	72.0 (68.0-80.0)	73.0(67.0-77.5)	0.28°
ECOG PS	1.0(1.0-2.0)	1.0(0.8-1.5)	0.10°
FURTHER CANCER%	8.2	17.0	0.04°°
TTP	5.0(4.0-7.0)	6.0(4.0-9.7)	0.001°
Adenocarcinoma %	76.0	75.0	0.14°°
HEART COMORBIDITY%	24.0	45.0	0.06°°
COPD%	27.0	51.0	0.04°°
EGFR MUTATION%	8.0	11.0	0.15°°

Mann-Whitney test° and Fisher's exact test°°
Values are expressed as median and interquartile range (IQR) for Mann-Whitney test

TABLE 3 Influence of variables on HIF expression

	OR	CI	p
Pack-Year	0.96	0.91-1.05	0.50
Age	0.95	0.85-1.04	0.23
ECOG PS	2.93	0.62-14.21	0.18
Bone metastasis	8.04	1.42-44.10	0.01
Histotype	0.94	0.81-7.82	0.11

Logistic Regression analysis

TABLE 4 Influence of variables on TTP

Covariate	HR	CI	p
Pack-Year	0.99	0.96-1.02	0.64
Age	1.00	0.94-1.06	0.88
ECOG PS	0.82	0.42-1.60	0.57
COPD	2.58	0.81-8.25	0.11
Histo-type	1.80	0.97-3.32	0.04

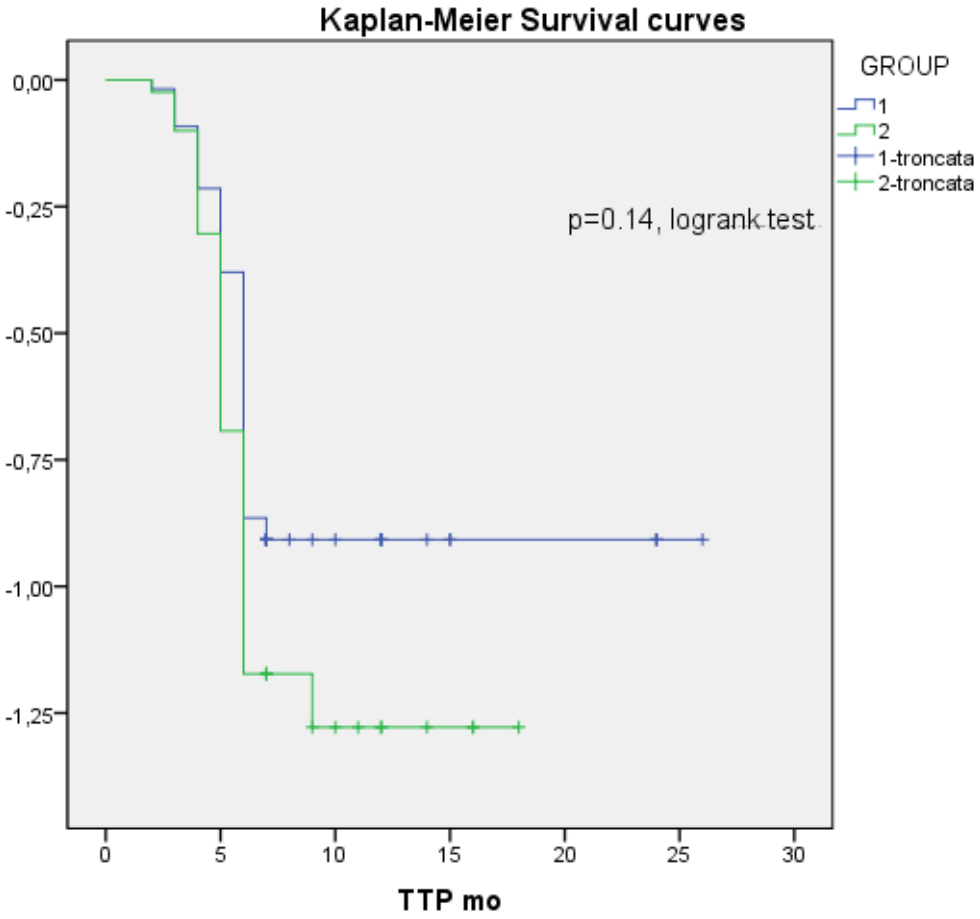
Cox proportional- hazard regression analysis

Table 5.HIF expression, difference between subgroups

	VISCERAL METASTASES	ASSOCIATED BONE METASTASES	p
Intensity x percentage	20(10.0-37.5)	50 (20-109.0)	0.02°
Percentage of posit cell	35.6± 24.7	46.8± 26.7	0.12°°

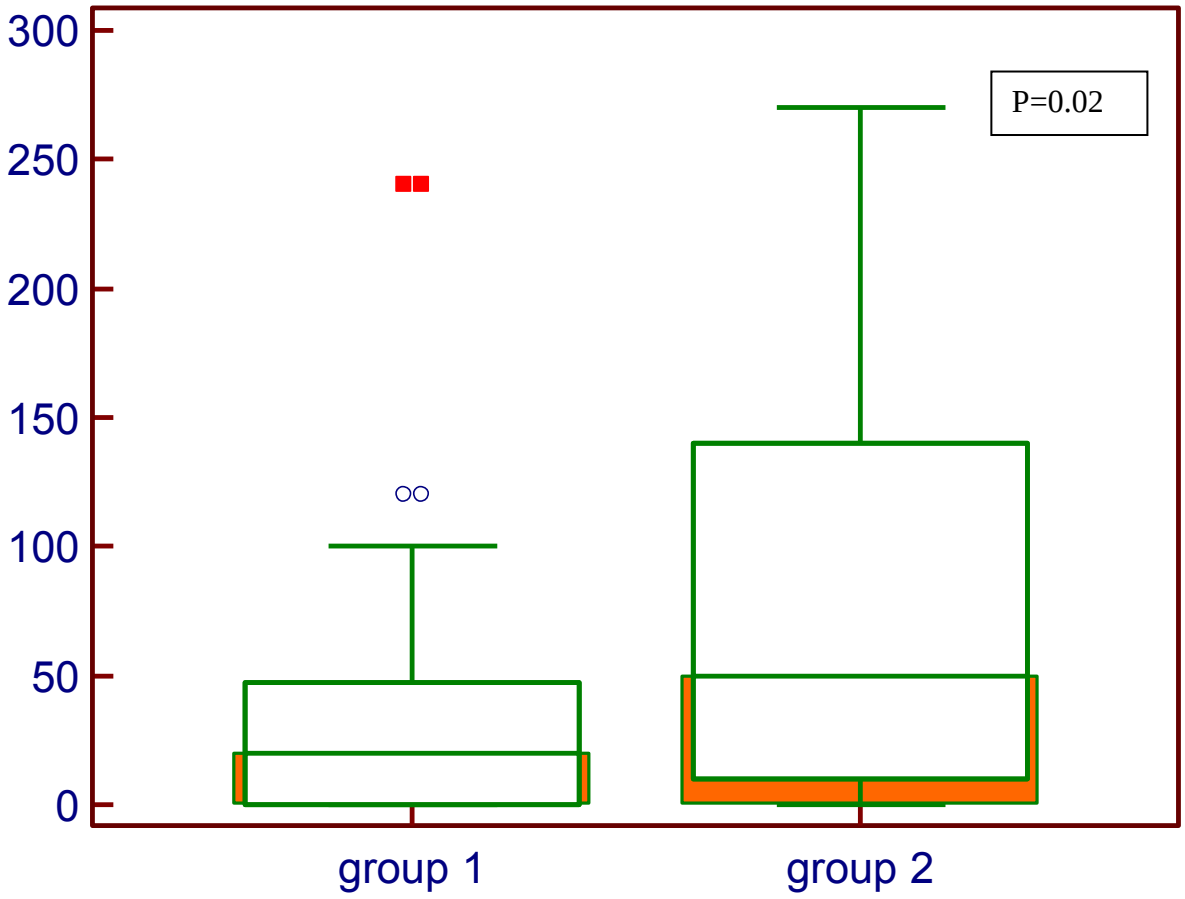
Mann-Whitney U test° Values expressed as median and interquartile range
Fisher's test values expressed as mean and SD°°
Intensity: mild +, moderate ++, high +++

Figure 1. Kaplan-Meier survival curve



HR: 1.3. 95% CI: 0-8-2.2
Group 1: without bone metastases
Group 2: with bone metastases

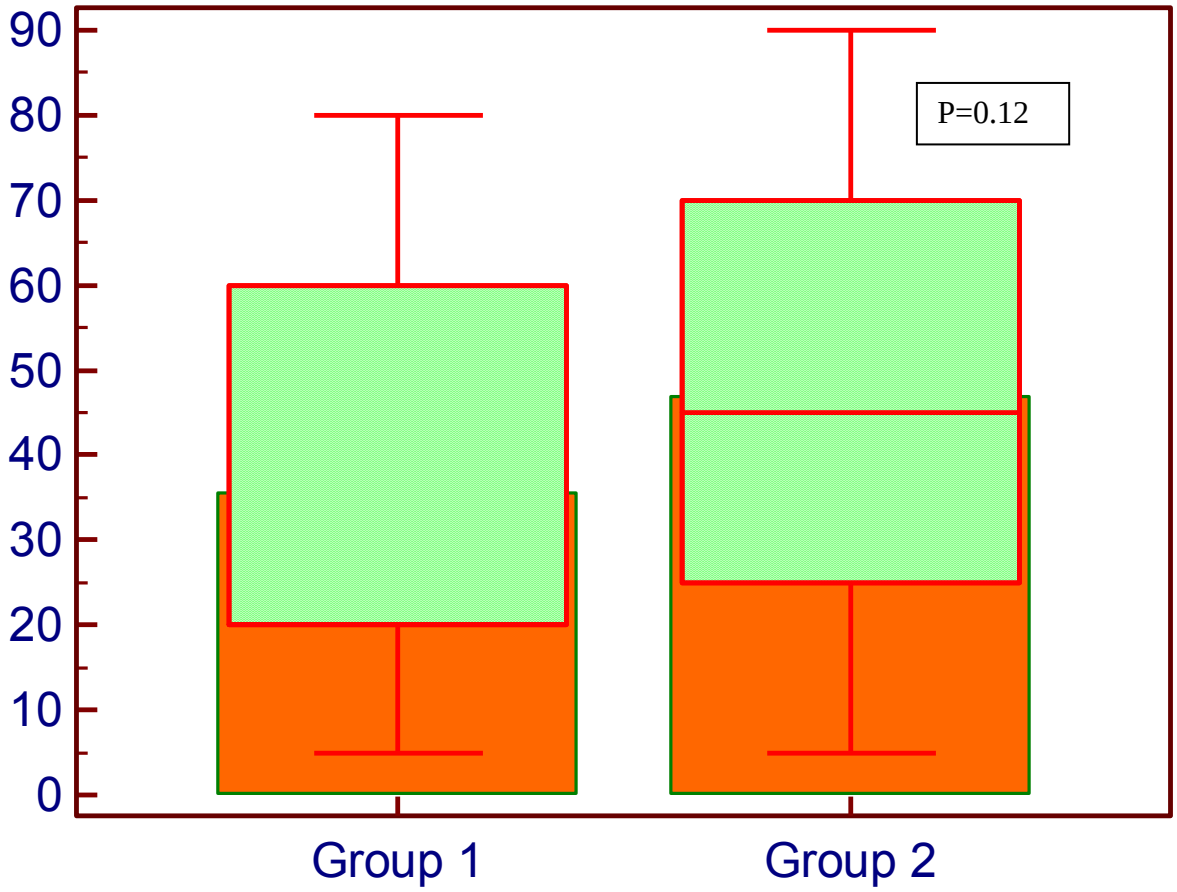
Figure 2. Comparison about HIF-1α intensity multiplied by percentage of positive cells



Group 1: Lung cancer with visceral metastases
Group 2: Lung cancer with bone and visceral metastases

Mann-Whitney U test

Figure 3.Comparison about percentage of cells expressing HIF-1α



Group 1: Lung cancer without bone metastases
Group 2: Lung cancer with bone metastases

Mann-Whitney U test

Figure 4. HIF-1α Immunostaining

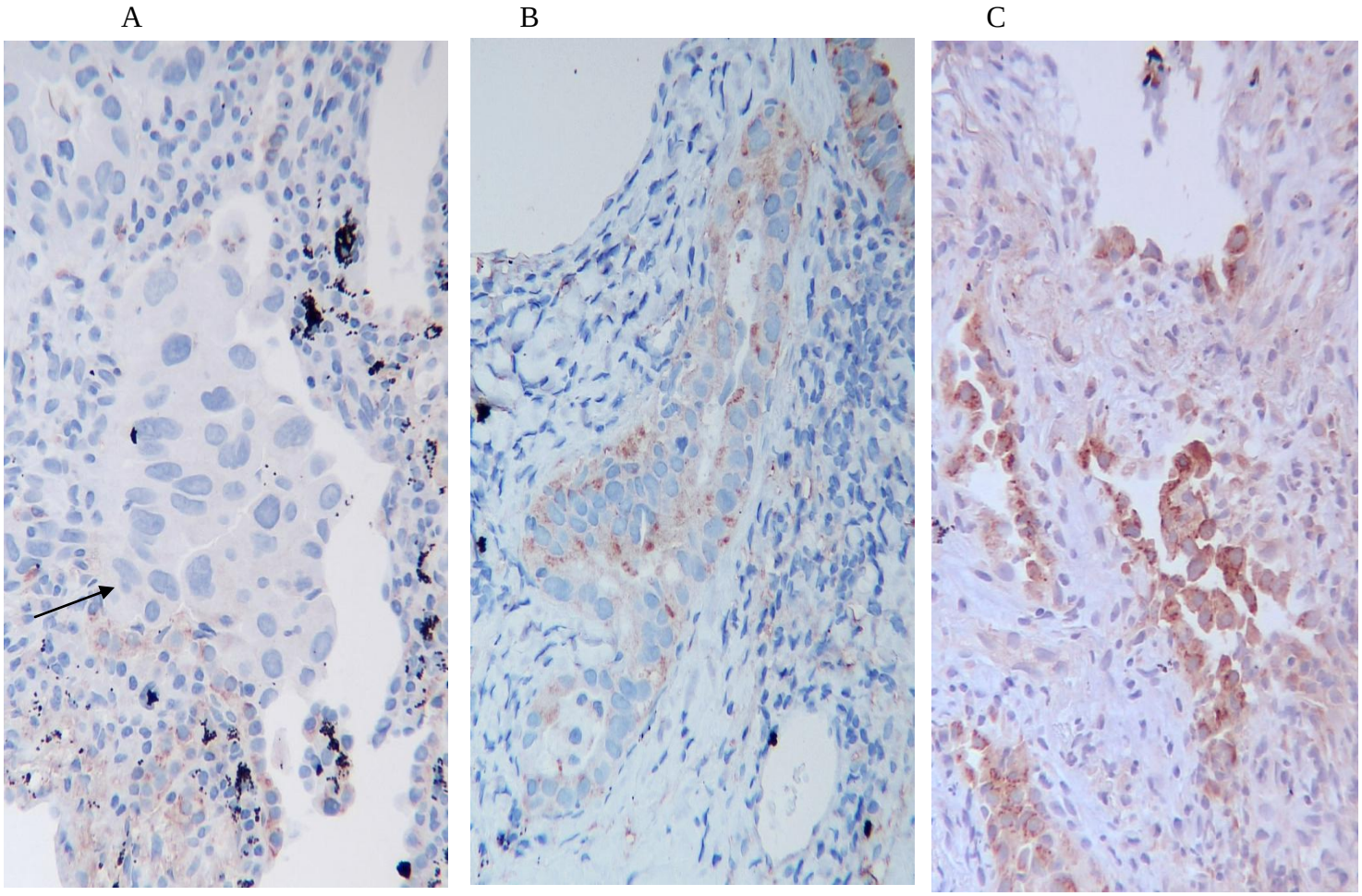


Figure A: negative for HIF-1α
B:mild positive
C:high positive
The arrow indicates neoplastic cells