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Spontaneous osteoclastogenesis is a predictive factor for bone metastases from non-small cell lung cancer

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KEYWORDS
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Interleukin-7;
Osteolysis;
Bone;
Metastasis

Summary Lung cancer is a widespread disease and its incidence is growing. Since therapies have increased the life expectancy of lung cancer patients, the development of bone osteolytic metastases is becoming a common cause of morbidity. Osteolysis is caused by an increased osteoclast activity and may be reduced by inhibiting their formation and activity.

We studied 60 male patients affected by NSCLC, divided in early and advanced stage disease. Patients' blood and urinary samples were collected at tumor diagnosis and at follow-up. PBMCs were cultured to investigate the spontaneous osteoclastogenesis. IL-7 was dosed in serum and its quantitative gene expression was evaluated on tumor and healthy tissues by RQ-PCR.

Both at diagnosis and follow-up, osteolytic bone patients showed high spontaneous osteoclastogenesis level compared to non-bone metastatic and healthy controls. The presence of spontaneous osteoclastogenesis correlated with urinary crosslinks increase. Serum IL-7 levels were higher in bone metastatic patients than in patients without bone lesions and healthy controls. The serum IL-7 increase correlated with the osteoclastogenesis and, at least in part, depended on an increased IL-7 production by tumor cells. At follow-up, patients with increased osteoclastogenesis and serum IL-7 levels, were subjected to standard clinical analysis, which showed early secondary bone lesions.

The *in vitro* assay for spontaneous osteoclastogenesis and serum IL-7 dosage could be useful for diagnostic purposes and it might be able to monitor cancer patients with a high risk to develop osteolytic metastases at follow-up, especially after a curative treatment.

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1. Introduction

Lung cancer is the most common form of tumor in the world. It is a widespread disease and its incidence is growing. Yet, in recent years, the mortality rate due to lung cancer has decreased slightly, showing evidence of the preliminary results obtained from early diagnosis, aggressive surgery and new therapeutic regimens. Since therapies have increased the life expectancy of lung cancer patients, the development of metastases is a common event. Lung cancer has a strong ability to metastatize and bone is among the most frequent metastatic site [1]. Bone metastases from lung cancer are mainly osteolytic and make prognosis worse, causing morbidity and also having substantial financial implications for the health-care providers [2].

Lung cancer is divided into two sub-groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) that includes different histotypes such as adenocarcinoma, squamous carcinoma, large cell carcinoma and carcinoid. Adenocarcinoma causes bony localizations more often than squamous carcinoma [3]. At diagnosis, the incidence of bone metastasis in adenocarcinoma is about 40—47% [4], while at autopsy, the prevalence is 32—40%. After surgical resection of lung tumors at early stages (lung localized disease), 21% of patients without clinically detectable metastases before surgery, develop further bone lesions [5].

In standard clinical practice, osteolytic lesions are often diagnosed only when the local damage is advanced or irreversible because imaging or biological methods to foresee the development of osteolytic lesions are not available at this time [6]. The importance of an early diagnosis of bone metastases has been demonstrated, since it improves patients' quality of life and reduces treatment costs, although it does not increase their life expectancy [7].

We previously described that patients with osteolytic bone metastases show spontaneous osteoclastogenesis, since their peripheral blood mononuclear cell (PBMC) differentiate into osteoclast (OC) *in vitro* without adding exogenous factors. We described how the spontaneous osteoclastogenesis could depend on the increase of circulating OCP number [8]. In these patients, affected by different solid tumors, we detected also an abnormal serum level of interleukin-7 (IL-7). IL-7 has been previously shown to be involved in osteoclastogenesis [9]. Our first results suggested that these biological parameters could be useful to detect early bone lesions, so we studied on a group of NSCLC patients both osteolysis mechanisms and new sensitive diagnostic methods to disclose sub-clinical localizations of bone metastases. An alteration of *in vitro* osteoclastogenesis and IL-7 serum levels, whether associated with local symptoms or not, could help decide to further study patients with other examinations, such as bone scanning or PET, in order to disclose bone lesions. Subsequently, these patients could receive an early treatment with bone-seeking radiopharmaceuticals, radiotherapy or anti-resorbing agents in order to avoid the dismal consequences of bone metastases.

Table 1 Patients' characteristics

Cases	Bone metastases	Histotype
25	8	Adenocarcinoma
13	2	Squamous cell carcinoma
7	0	Large cell carcinoma
2	0	Atypical carcinoid
13	6	Not defined

2. Materials and methods

2.1. Patients

Peripheral blood (PB) samples were obtained from 60 male patients affected by newly diagnosed NSCLC and 60 healthy controls, matched for age and sex. The patients were aged from 45 to 84 years (median 67 ± 9.27 S.D.). The histotypes studied are reported in Table 1. On 13 cases the histotype was not defined since the cytologic patients' samples were barely sufficient to show the presence of malignant cells but not to define the histotype. We divided patients in two groups: (1) patients with early stage disease eligible for surgical lung tumor resection and (2) patients subjected to radio/chemotherapy with advanced disease. Patients belonging to the second group were consecutive cancer patients at the clinic and thus they underwent the same chemotherapeutic regimen according to the schedules adopted by the oncologists. In order to investigate factors able to foresee the potential development of osteolytic bone metastases, we collected patients' samples at 8 months after surgical treatment (for patients belonging to group 1). Since patients belonging to group 2 showed worse prognosis and reduced survival, we obtained only 6 months follow-ups. The follow-ups were updated for a median of 450 days for group 1 and 200 days for group 2.

Only male patients were selected in order to avoid bias due to female hormonal status, which is directly correlated with resorptive bone pathologies, such as post-menopausal osteoporosis. Moreover, NSCLC is not susceptible to hormonal therapies, which create biases on bone biology as happens in other tumor types such as prostate and breast cancers. SCLC patients were excluded from the study as these tumors produce neuro-endocrine agents able to interact with the bone metabolism, such as vitamin D or PTHrP [10]. The patients' performance status was accurately evaluated in order to pinpoint and eventually exclude patients with intestinal malabsorption diseases or other kind of deficient nutritional status, which might cause a secondary osteoporosis. Moreover, patients affected by other diseases causing disruption of bones such as Paget's disease, rheumatoid arthritis, osteomyelytis and patients treated with bisphosphonates, calcium, vitamin D, phosphorus, or hormonal therapies were excluded from the study. In order to investigate the patients' bone metabolism status, they were subjected to analysis of standard clinical markers of bone metabolism, such as serum PTH, alkaline phosphatase, calcium, phosphate and U-deoxypyridinoline (urinary crosslinks) [11]. In particular, the last parameter has been chosen in clinical practice to monitor bone metastatic disease and anti-resorbing treatments such as bisphosphonates [12,13]. The presence of bone metastases was confirmed using ^{99}Tc bone scanning and further imaging studies according to the standard clinical practice.

Healthy controls showed a normal bone metabolism (evaluated by bone densitometry) and were not under medications that could increase osteoclastogenesis. Informed consent from patients and healthy controls was obtained to comply with institutional policies. Data derived from patients were compared to data obtained by age-matched healthy controls.

2.2. Cell cultures

For all patients and healthy controls, PBMCs were isolated after centrifugation over a density gradient, Lymphoprep (Nycomed Pharma, Norway) and cultured in α-MEM, supplemented with 10% FBS, penicillin 100 U/ml and streptomycin 100 μg/m (Cambrex, Bio Science, Walkersville, MD). Cultures were stopped after 15 days, mature OCs were identified as multinucleated cells containing three or more nuclei and positive for the expression of TRAP (Tartrate-Resistant Acid Phosphatase, Sigma—Aldrich, St. Louis, MO) and $\alpha_V\beta_3$ (vitronectin receptor), kindly provided by Prof. G. Tarone.

2.3. Assay of bone resorption activity

PBMC were plated at concentration of 4×10^5 cells/well on the OsteoAssay plate (Cambrex Bio Science), which provides a thin layer of adherent human bone particles. The presence of OC was evaluated by TRAP staining. Cell culture supernatants were collected at days 5 and 10 of culture and the products of *in vitro* bone degradation were measured by CrossLaps for Culture ELISA (Nordic Bioscience Diagnostics A/S, Herlev, DK).

2.4. ELISA (enzyme-linked immunosorbent assay)

The amount of serum IL-7 and of C-terminal telopeptides degradation products (crosslaps) from type I collagen were determined by commercially available ELISA kit according to manufacturer's instructions. IL-7 sensitivity was 0—16 pmol/l (R&D system, Abingdon, UK) while crosslaps sensitivity was 0—84 nM (Nordic Bioscience Diagnostics A/S).

2.5. Generation of patient IL-7 and β-Actin plasmid standard

PCR primers for IL-7 and β-Actin were designed using Primer Express v1.0 software and synthesized by Applied Biosystems (Warrington, UK). The sequences were: IL-7 sense 5′-TGAAGGTAAAGATGGCAAACAA-3′; IL-7 antisense 5′-CAATTTCTTTCATGCTGTCCAA-3′;β-Actin sense 5′-CCCTGAAGTACCCCATCGA-3′; β-Actin antisense 5′-AAGGTGTGGTGCCAGATTTTC-3′. Purified fresh PCR products of 80 bp for IL-7 and 78 bp for β-Actin were directly cloned using TA Cloning Kit Dual Promoter (Invitrogen, Carlsbad, CA). Briefly, TOP10F competent *E. Coli* were transformed with 20% of the ligation reaction and plated on LB agar containing 50 μg/ml ampicillin; blue/white selection was performed with 40 μl of 20 mg/ml X-gal and 7 μl of 0.8 M IPTG. Minipreps of 10 pCRII clones containing the IL-7 and β-Actin sequences were performed (Wizard Plus Miniprep, DNA Purification System, Promega, Madison, WI). Positive clones were sequenced to confirm their identity. 10 μg of the selected plasmid for both the genes were digested with 8 U of HindIII restriction enzyme overnight at 37 °C. Linearized plasmids were finally purified with NucleoSpin clean up extraction kit (Macherey-Nagel, Düren, D), resuspended with 1× TE and OD_{260} was determined. Copy number was calculated from the plasmid concentration, mean molecular weight of the nucleotides (660 g/mol) and plasmid plus insert length.

2.6. Real-time quantitative analysis of IL-7 gene expression

Total RNA was extracted by Trizol system (Invitrogen) from cancer and healthy tissues, derived by surgical resection done to remove the lung primary tumor. The first-strand cDNA synthesis was performed as previously described [8]. Quantitative analysis of IL-7 was performed with real-time quantitative PCR (RQ-PCR) using β-Actin as housekeeping control. RQ-PCR was carried out using the iCycler iQ™ system (Bio Rad, Hercules, CA, USA). TaqMan probes were designed using Primer Express v1.0 software and synthesized by Applied Biosystems. TaqMan probe specific for IL-7 (5′-TGAGAGTGTTCTAATGGTCAGCATCGATCAAT-3′) and for β-Actin (5′-CGTCACCAACTGGGACGACATGG-3′) were both labelled at the 5′ end with 6-carboxy fluorescein (FAM) and the 3′ end with 6-carboxy-tetrametil rhodamine (TAMRA). Reactions for IL-7 and β-Actin quantification were performed in a 25 μl final volume with 2 μl of sample cDNA, 1× iQ Supermix (Bio Rad), 0.4 μM of each primer and 0.4 μM of the IL-7 probe. PCR primers were the same used for IL-7 and β-Actin cloning. The amplification conditions for quantization were: 95 °C for 15 min, 50 cycles at 95 °C for 15 s, 58 °C for IL-7 and 60 °C for β-Actin for 1 min.

2.7. Flow cytometry

PBMC samples from patients and controls were analyzed for the expression of OC precursor (OCP) markers by flow cytometry. Aliquots of 1×10^6 cells were incubated with anti-human monoclonal antibodies for CD11b, CD14, CD51/CD61 and their related isotype controls. OCP were considered triple positive cells. Samples were analyzed in a FACsCalibur instrument and elaborated by CellQuest software.

2.8. Statistical analyses

Statistical analyses were performed with the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL, USA). Continuous variables were expressed as mean and standard deviation. To explore the effect of crosslinks and IL-7 on OC, we fitted a linear regression model. Univariate analyses were performed by one-way analysis of variance. Bonferroni correction was used for multiple comparisons. Crude survival probabilities were estimated with Kaplan—Meier method and differences between early and advanced stage disease curves were assessed by the 2-tailed log-rank test. The time axis we

considered for survival analysis was months since cancer diagnosis. The results were considered statistically significant for $p < 0.05$.

3. Results

3.1. Evaluation of spontaneous osteoclastogenesis

We examined the presence of spontaneous osteoclastogenesis in patients with or without osteolytic metastases at diagnosis. As showed in Fig. 1A the number of OCs was significantly higher in osteolytic patients than in patients without bone metastases and in healthy controls, $p < 0.03$. Patients without bone lesions had a higher but not statistically significant OC number compared to healthy controls.

We investigated whether the spontaneous osteoclastogenesis assay was as effective in diagnosing bone metastatic phenotype as urinary crosslinks and other clinical markers of bone metabolism, such as serum PTH, alkaline phosphatase, calcium and phosphate. We compared the OC number obtained from patients' cell cultures with each clinical marker. By a linear regression model we demonstrated that the increase of urinary crosslinks was significantly correlated with an increase of OC number, $p < 0.04$ (Fig. 1B). PTH, ALP, calcium and phosphate did not seem to be associated with OC number (data not shown). In all newly diagnosed NSCLC patients, we analyzed the circulating OCP number, observing an its increased (10—15% triple positive cells) in 3 patients with bone metastases, while the other patients had low level of OCP (<2% triple positive cells). There were not statistically significant differences between groups (data not shown) and we did not find significant correlation between the number of OCs and OCPs.

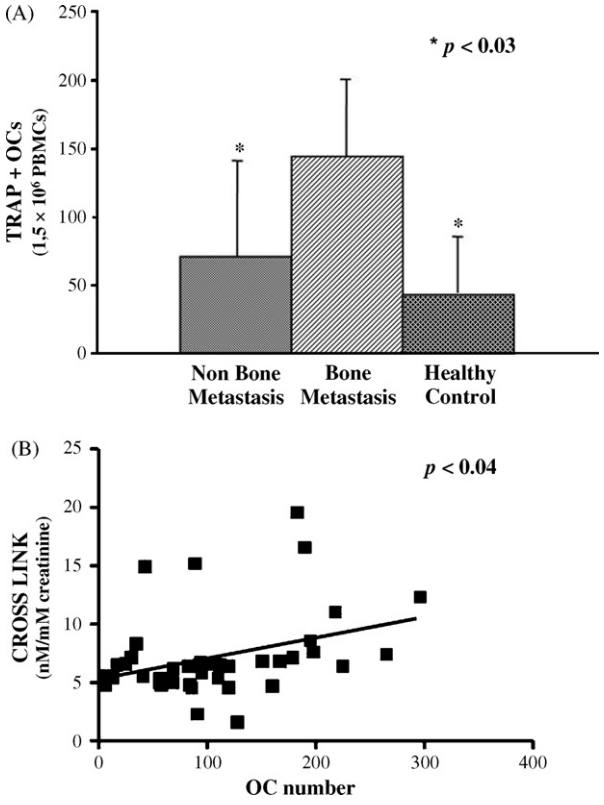

Fig. 1 Analysis of spontaneous osteoclastogenesis. At the end of culture, in both patients and healthy controls cultures, TRAP positive multinucleated cells were identified as OCs and counted. The OC number in osteolytic patients was significantly higher than in non-bone metastatic patients and in healthy controls, $p < 0.03$ (A). The increase of OC number correlates with urinary crosslinks corrected for creatinine value. In detail, a 1-unit increase in crosslinks correspondes to a significant increase, 6.75, in the mean value of OC, $p < 0.04$ (B).

3.2. Bone resorption activity in NSCLC patients

To evaluate the bone resorption activity of OC we measured the degradation products of C-terminal telopeptides of type I collagen (crosslaps) in cell culture supernatants. We analyzed the cell supernatants at days 5 and 10, measuring statistically significant differences between patients and healthy controls at day 10. The bone resorption activity was higher in patients than in healthy controls, $p < 0.02$ (Fig. 2).

3.3. Serum IL-7 expression

Having demonstrated that IL-7 is involved in regulation of osteoclastogenesis in patients with solid tumor, we investigated its potential use as serologic biomarker in monitoring bone metastases evolution [9]. Serum IL-7 levels were significantly higher in bone metastatic patients than in patients without bone lesions and healthy controls $p < 0.001$ (Fig. 3A). The mean IL-7 value for osteolytic patients was (mean ± S.D.) 15.9 ± 6.1 pg/ml; 11.5 ± 4.9 pg/ml for non-bone metastatic patients and 5.02 ± 1.83 pg/ml for healthy controls. We divided serum IL-7 levels according to three different NSCLC histotypes, but we did not find any significant variations among the three groups. The values were 11.92 ± 5.6 pg/ml for adenocarcinoma, 12.98 ± 4.9 pg/ml for squamous carcinoma and 13.22 ± 4.15 pg/ml for large cell carcinoma. By exploring a potential IL-7 effect on osteoclastogenesis, we demonstrated that the increase of serum IL-7 correlates with the osteoclastogenesis increase, $p < 0.04$ (Fig. 3B).

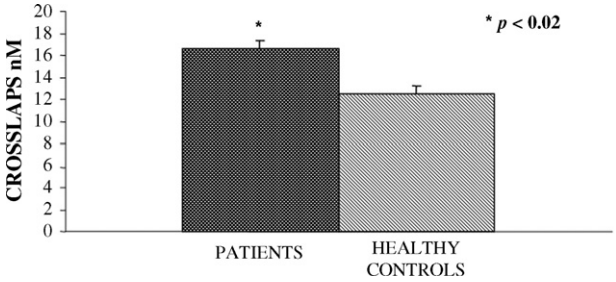

Fig. 2 Bone resorption activity. The histogram shows crosslaps levels dosed in cell culture supernatants at day 10 of culture. The bone resorption activity, evaluated as crosslaps release, resulted higher in patients than in healthy controls, $p < 0.02$.

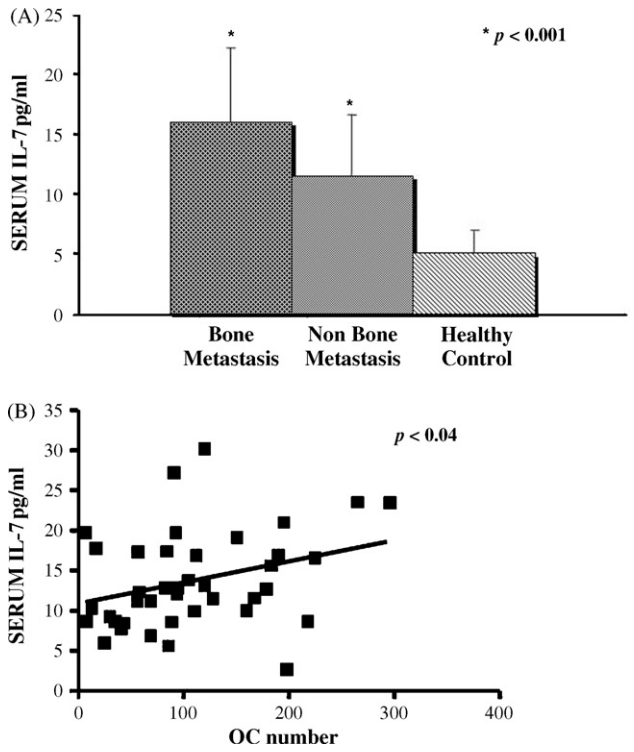

Fig. 3 Serum IL-7 dosage. IL-7 levels in cancer patients with/without bone metastases and in healthy controls were analyzed by ELISA. Samples were assayed in duplicate and data were expressed as mean values. Bone metastatic patients had significantly higher serum levels of IL-7 compared to patients without bone metastasis and healthy controls, $p < 0.001$ (A). A 1-unit increase in IL-7 is equivalent to a significant increase, 3.85, in OC number, $p < 0.04$ (B).

3.4. IL-7 gene expression in NSCLC tissues

In order to establish whether tumor cells were responsible for the increase of IL-7 production in NSCLC patients, we examined the IL-7 expression in NSCLC and in healthy lung tissues. The quantitative analysis of IL-7 gene expression demonstrated that tumor tissues expressed significantly more IL-7 than healthy tissue, $p < 0.04$ (Fig. 4).

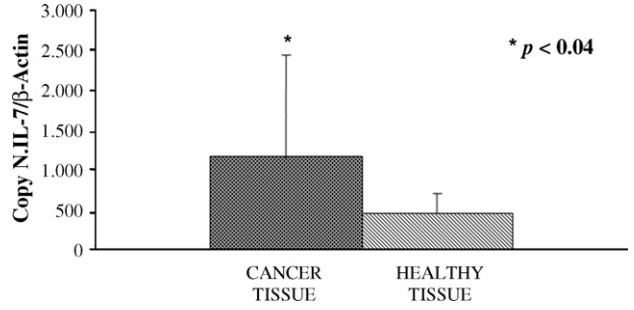

Fig. 4 Quantitative analysis of IL-7 gene expression. Twenty NSCLC and healthy tissues were analyzed by real-time PCR in order to quantify IL-7 gene expression. The IL-7 quantization was expressed as IL-7 on β-Actin (the control gene) plasmid copy number. The histogram showed a higher IL-7 expression in cancer than in healthy tissues, $p < 0.04$.

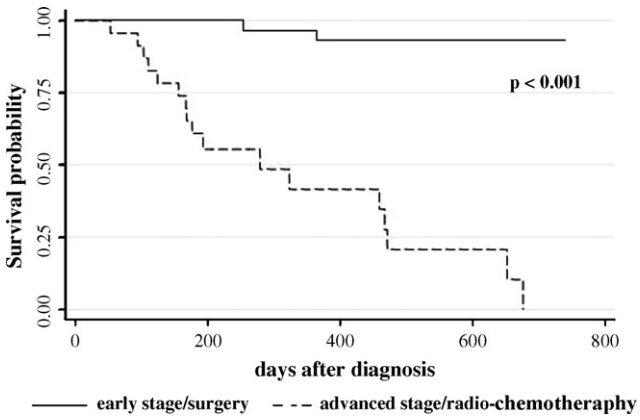

Fig. 5 Survival analysis. The Kaplan—Meier survival analysis showed that median follow-up was about 450 days for early stage cancers treated with surgery and 200 days for advanced cancers treated with radio/chemotherapy, $p < 0.001$. Survival at 200 days was 100% (95% CI: 88—100%) and 55% (95% CI: 33—73%), respectively.

3.5. Analysis of patients' follow-up

Patients affected by NSCLC have a high mortality rate, therefore we analyzed the survival rate in the two groups: (1) early stage/surgery and (2) advanced stage/chemotherapy. Median follow-up was about 450 days for early stage cancers, treated with surgery and 200 days for advanced cancers, treated with radio/chemotherapy, $p < 0.001$. Survival at 200 days was 100% (95% CI: 88—100%) and 55% (95% CI: 33—73%), for groups 1 and 2, respectively, as shown in Fig. 5.

From group 1, we collected 18/30 patients' follow-up; 2/30 patients died, 3/30 had disease progression with a low performance status and 7/30 withdrew the study. 9/18 patients showed an increase of OC number, IL-7 serum level and urinary crosslinks, confirming the direct correlation (observed at diagnosis yet) among the increase of OC number, urinary crosslinks, (Fig. 6A) and IL-7 serum levels, $p < 0.001$ (Fig. 6B). The increase in circulating OCP number was detectable in these 9/18 patients and it was variable, ranging 30—60% triple positive cells (data not shown). Since at follow-up the biological parameters were worse than at diagnosis, the 9 patients were subjected to bone scanning in order to investigate the presence of early bone lesions. In 5 cases, bone scanning evidenced hot spots in different skeletal area and further investigations demonstrated local involvement of the bone. The remaining 4 cases showed a negative bone scan for bone metastases.

As described above the survival rate of patients with advanced disease was poor, therefore at 6 months follow-up, we collected 3/30 patients, 12/30 died and 15/30 were under chemotherapy regimen or had a low performance status. The 3 patients showed a first stabilization of the disease, followed by a progressive worsening. Their disease progression was monitored by the above-examined biological parameters. A patient with a stage IV (T4N2M1) of NSCLC represents a significant example of this group: he was subjected to chest computed tomography-positron emission tomography (CT-PET) revealing ipsilateral mediastinal

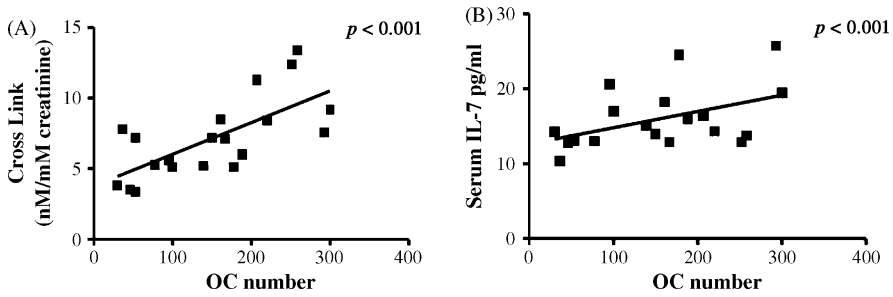

Fig. 6 At follow-up OC number correlates with urinary crosslinks and serum IL-7. At follow-up, the analysis of urinary crosslinks and serum IL-7 confirmed a correlation with the osteoclastogenesis level. A 1-unit increase in crosslinks and in serum IL-7 levels resulted in an increase of 22.3 and 10.92 OC number, respectively, $p < 0.001$.

lesions, while excluding any secondary bone lesions. The serum evaluation of bone metabolic markers did not reveal alterations of bone metabolism. The level of osteoclastogenesis was comparable to healthy controls (Fig. 7A). We did not observe any significant increase in circulating OCPs (Fig. 7B and C).

The patient was subjected to chemotherapy with Cisplatin/Gemcitabin and stereotassic radiotherapy. Three months after the end of the treatments, (about 12 months from the diagnosis), we collected patient's blood and urinary samples. We did not find any significant changes in our biological assays and in clinical examinations confirming a stable disease. During a further follow-up visit (about two years after diagnosis), we detected spontaneous osteoclastogenesis *in vitro* (Fig. 7D) and a significant increase in circulating OCPs (Fig. 7E and F). Those *in vitro* results suggested that a bone involvement could be active even if the patient was asymptomatic. The patient was promptly subjected to a re-staging of the disease. CT showed lung disease progression and secondary adrenal gland lesion. A spine MRI scan revealed destructive vertebral lesions in C4, D8 and iliac bone. The remaining patients of this group present a similar clinical history.

4. Discussion

Lung cancer frequently metastatizes to bone causing morbidity due to various skeletal complications including bone pain, pathological fractures, spinal-cord compression and hypercalcaemia [14]. These events can seriously affect the quality of life and have financial implications for health-

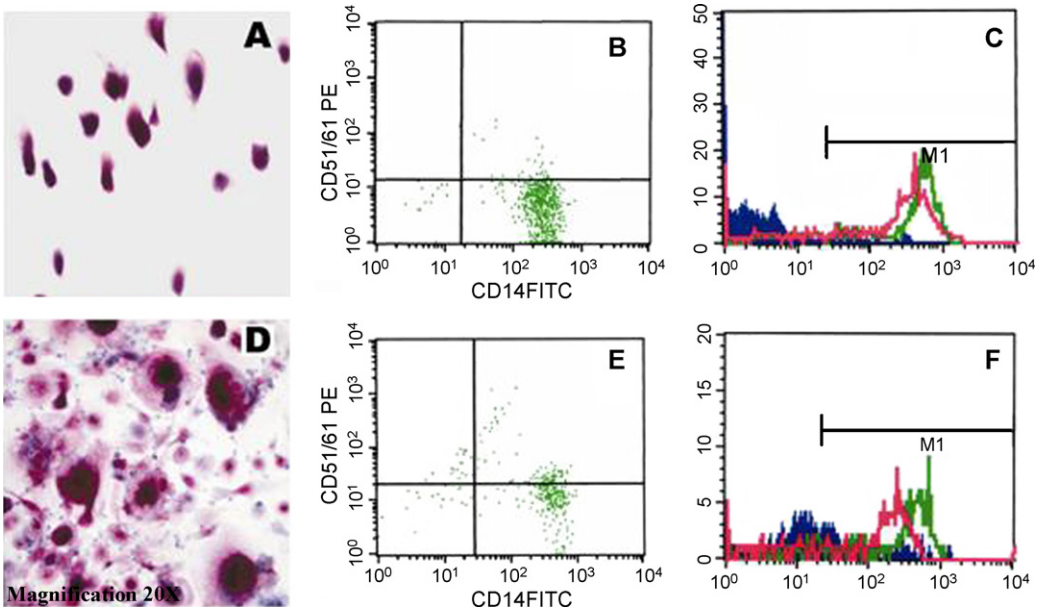

Fig. 7 Spontaneous osteoclastogenesis and circulating OCPs correlate with disease progression. PBMCs were cultured in absence of exogenous factors stimulating osteoclastogenesis. At the end of the culture, OCs were identified as multinucleated (≤3 nuclei) and TRAP positive cells. By flow cytometry, OCPs were identified as CD14, CD11b and CD51/61 positive cells. The data analysis was performed on the monocytic population and visualized as dot plots and histograms (blue is CD51/61, green is CD14 and red is CD11b). The marker (M1) was set according to the antibody isotypic controls. At diagnosis, PBMCs did not show any ability to differentiate spontaneously into mature OCs (A) and OCPs were not detected in peripheral blood (B and C). The patient's relapse was documented precociously by the development of spontaneous osteoclastogenesis (D) and the 20% of circulating OCPs (E and F). Magnification 20×.

care system [2]. Bone metastatic patients tend to live longer than patients with visceral or soft-tissue metastases. Novel therapeutic approaches, such as the introduction of bisphosphonate therapy, have recently improved treatment for bone metastases. At present, an early diagnosis of bone metastasis and the assessment of treatment for bone metastases are hindered by a lack of effective, rapid methods to measure disease response [6]. Therefore, there is a real need to develop a system for an early diagnosis of secondary bone lesions and for the monitoring of the anti-resorbing therapeutic regimens. In this study, we proposed a new diagnostic method to disclose sub-clinical localizations of bone metastases. This method is based on the evaluation of spontaneous osteoclastogenesis *in vitro* and on the dosage of serum IL-7 levels, which are correlated. The added value of the two tests proposed above, is the capability to show early metastatic bone involvement compared to the routine methods.

We previously demonstrated that spontaneous osteoclastogenesis is present in patients affected by different solid tumors with osteolytic metastases [8]. Now, we investigated the mechanism of OC differentiation in NSCLC patients, since this type of cancer often metastatizes to bone [3,15]. The osteoclastogenesis increased in newly diagnosed NSCLC patients with osteolytic metastases, while in patients without bone lesions the OC differentiation was comparable to healthy controls. These data confirmed our published results obtained from a heterogeneous group of tumors and allowed us to extend the study of the spontaneous osteoclastogenesis to a wider number of patients. We suggest to establish a cut-off value of 70 OC in the *in vitro* assay (see Section 2) as the maximum number for a normal or non-bone metastatic individual. Patients with a number of OC over 70 were considered pathological and were investigated for the possible known causes of an increase in osteoclastogenesis, namely a metabolic, inflammatory or neoplastic bone disease. In clinical practice, bone metastases are monitored by imaging techniques and by the dosage of urinary crosslinks, but its abnormal levels are often detectable when local damage to bone is advanced [6]. We explored the potential effect of crosslinks on osteoclastogenesis, demonstrating that the increase of urinary crosslinks correlated to the increase of OC number. This result documents by itself the reliability of proposed test compared to what is commonly used to monitor bone metastases [11].

The analysis of serum IL-7 revealed that patients affected by bone metastatic cancer had an IL-7 value higher than those suffering from non-bone metastatic forms, and these latter showed an intermediate IL-7 serum value between bone metastatic patients and healthy controls. Since it is known that T, B and some tumor cells produce IL-7, we investigated IL-7 production by NSCLC tissues, identifying a higher IL-7 expression in cancer than in normal tissues. This result demonstrates that NSCLC cell can be at least in part responsible for the serum IL-7 increase. The high IL-7 serum levels might be interpreted as a signal of disease progression towards the metastatic phenotype. A further effort to this hypothesis results from the finding that an increase of IL-7 directly correlate with an increase of OCs.

To determine whether the spontaneous OCs formation was dependent on increased number of circulating OCPs, we analyzed the expression of surface markers of OCPs. At diagnosis we did not find significant correlation between the number of OCs and OCPs either in patients or in healthy controls, describing an increased OCP number in 3 patients with recently diagnosed bone lesions (data not shown). At follow-up, we detected a considerable increase in OCP number of 9/18 patients subjected to surgery and in 3 patients treated with chemotherapy. This result correlated with the presence of newly diagnosed bone metastases. We believe that the increase of circulating OCPs is a temporary phenomenon, which precedes the OCP homing in the bone microenvironment and the subsequent OC differentiation and activation. Likely for this reason, patients with advanced and/or stable bone lesions did not show an increased OCP number. Therefore, since the OCP number seems to be extremely variable during patient disease and might be present only in the early stages of the bone nesting of tumor cells, we need to analyze further cases in order to define its possible use for early diagnosis of bone metastases.

The representative case of NSCLC patient (at stage IV) reported in this study demonstrated how the OC differentiation is strictly dependent on the activation by tumor cells in their metastatic phenotype. In fact we have shown how a patient free of bone metastatic localizations was unable to commit his PBMC to the OC lineage *in vitro*. A progression of the disease towards bone metastases was found to be concomitant to the ability of PBMC to differentiate into OC. The phenomenon was detected prior to the clinical appearance of symptoms related to the bone disease. We think that spontaneous osteoclastogenesis and serum IL-7 might be useful to monitor patients during their therapeutic regimens, evaluating the use of anti-resorbing agents in combination with chemotherapy when these bone markers increase from normal values. This study represents a first step to validate the use of these tests in clinical practice, in fact further clinical studies on a wider array of tumor patients are required. Nonetheless, the described data represent an effort to deepen our knowledge on the mechanisms of bone metastasis and to obtain better tools for early diagnosis of this dismal complication of cancer.

5. Conflict of interest

We do not have conflict of interest to declare.

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