Dottoranda in Oncologia e Chirurgia Sprimentali (Internazionale, XXX Ciclo): Dott.ssa Marzia Pucci

Exosome analysis in Non Small Cell Lung Cancer: from *in vitro* models to preclinical application.

Background

Lung cancer is a major cause of cancer-related death. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and clinical outcomes are often poor. The high mortality rate seen with NSCLC is related to the difficulty of early detection and inadequate treatment strategies (1). Early detection is particularly crucial for tumors without clinical manifestations during the initial stages of their development, among which lung cancer holds a special place. International Agency for Research on Cancer (IARC) provided estimates of 1.82 million new lung cancer cases and 1.59 million lung cancer-related deaths in the world in 2012 (2). Currently more than 70% of lung cancers are loco-regionally advanced or metastatic at diagnosis. Patients with stage IIIB/IV NSCLC have a poor prognosis, with a median survival of approximately 12 months using chemotherapy**.** The era of molecular targeted therapy in lung cancer had its origin in 2004, when activating mutations in the epidermal growth factor receptor (EGFR) and their correlation with clinical response to EGFR tyrosine kinase inhibitors (TKIs) were discovered. Until today, 7 randomized trials demonstrated superior overall response rates (ORRs) and progression free survival (PFS) for EGFR-TKIs (afatinib, erlotinib, gefitinib), compared with standard chemotherapy, in patients with EGFR mutated lung adenocarcinoma. EGFR mutations in NSCLC are present in 13% to 18% of Caucasian patients, and in 60% to 80% of Asian patients. After the EGFR mutations, ALK fusion is the second most frequent oncogenic driver in NSCLC for which a targeted therapy is available based on regulatory approval (3). The patients with ALK translocation respond to ALK tyrosine kinase inhibitors, such as Crizotinib® (4). However, EGFR TKI and ALK inhibitors are not curative because resistance against these drugs arises. The elucidation of resistance mechanisms to molecular treatment provides a basis for the development of new strategies to overcome this resistance and to enhance the outcome in NSCLC patients.

The role of the tumor microenvironment in the resistence to therapy is becoming very interesting.

The tumour microenvironment plays an essential role in the development and spread of cancers. Tumour cells interact with the surrounding extracellular matrix where it is possible to find a variety of non-cancer cells including cells of the vasculature, immune system and fibroblasts. The essential role of fibroblasts in the maintenance of an environment in which tumour cells are able to maintain their aggressive phenotypic traits is becoming increasingly well documented. Cancer associated

fibroblasts (CAF) are able to secrete a vast array of extracellular matrix-modulating factors, meaning that they have potential for a functional role in every step of the carcinogenic process. Cancer-associated fibroblasts, can be key regulators of the cellular sensitivity to molecular-targeted therapy. As seen before Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) have marked therapeutic effects against non-small cell lung cancer (NSCLC) with EGFR mutations, but some patients have exhibited primary resistance to EGFR-TKIs.

As seen by Yoshida T et al. lung adenocarcinoma cell lines became more resistant to EGFR-TKI when cocultured with podoplanin-expressing CAFs, compared with control CAFs *in vitro*. The knockdown of podoplanin on CAFs cancelled the resistance to EGFR-TKIs in cancer cells. Compared with control CAFs, the cancer cells that were cocultured with podoplanin-positive CAFs continued to exhibit significantly higher p-ERK levels after treatment with gefitinib. Furthermore, postoperative recurrent patients with podoplanin-positive CAFs had a significantly lower overall response rate to EGFR-TKIs compared with those with podoplanin-negative CAFs. These data indicate the important role of the CAFs in primary resistance to EGFR-TKIs and they may be an ideal therapeutic target for use in combination therapy with EGFR-TKIs (5).

The most important risk factor in lung cancer is the cigarette smoke, there is in fact a clear dose-effect relationship between this habit and disease and this also applies to passive smoking. Recent studies clearly establish the two signaling pathways that are activated during cigarette smoke exposure in the lung airway. One focused on the activation of neutral sphingomyelinase2 (nSMase2), an enzyme that hydrolyzes sphingomyelin to ceramide. The other pathway focused on the oncogenic EGF receptor (EGFR), which becomes aberrantly activated but not degraded, leading to prolonged proliferative signaling. Goldkorn et al. demonstrated that during cigarette smoke exposure EGFR is favorably co-localized in ceramide-enriched regions of the plasma membrane, proposing that nSMase2/ceramide plays a role in the aberrant EGFR activation, leading to augmented tumorigenic signaling. Moreover, new findings indicate that cigarette smoke exposure may induce resistance to the tyrosine kinase inhibitors (TKIs), used for treatment of NSLC, merely through posttranslational molecular alterations. Ceramide is emerging in cancer research for its potential roles in proliferation and cell-to-cell communication via exosomes (6). Recently it was demonstrated that extracellular vesicles are naturally adapted for the transport and intracellular delivery of proteins and nucleic acids (7). This makes them particularly able in modulating the pathways that leads to the development of cancer drug-resistance.

For their properties, the exosomes are also particularly attractive for the delivery of pharmaceutical proteins and nucleic acids. Extracellular vesicles as carriers for biological therapeutics is a promising strategy to delivery drugs (8). Specific proteins differential expressed in extracellular

vesicles can be used as biomarkers for the early detection and diagnosis of disease for determining prognosis. In order to develop new cancer biomarkers and therapeutic strategies might be interesting to study the extracellular vesicles purified by plasma of patients with NSLC at different states and released by different lung cancer cell lines. In this proposal project, the isolation of exosomes from cell lines with oncogenic potential and correlating in vivo with NSCLC patients, could have an interesting impact for this disease.

Proposal main body

Cell may communicate and exchange information by different mechanism, using secreted signals or sophisticated vehicles, such as extracellular vesicles (EV), to relay important information to other cells, often over large distances. The dynamic and reciprocal interplays between the tumor and its microenvironment orchestrate events critical to the establishment of primary and metastatic niches and maintenance of a permissive environment at the tumor−stroma interface.

Recently the attention is focused on cell-cell communication that involves the extracellular vesicles. There are different kinds of secreted membrane vesicles that have distinct structural and biochemical properties depending on their intracellular site of origin. The two most studied vesicles population are: microvesicles and exosomes. Microvesicles originate from the plasma membrane through a mechanism morphologically similar to virus budding. These vesicles are relatively large and heterogeneous in size (100 to 1000 nm).

Exosomes are nanometer-sized vesicles that represent a distinct class of membrane vesicles (40-100 nm diameter) of endocytic origin that are released from different cell types under both normal and pathological conditions (9). Exosomes have been implicated in several cellular functions and disease states where they could constitute valuable biomarkers. Indeed most body fluids contain significant amounts of exosomes. Extracellular vesicles play a pivotal role in information transfer between cells. Cancer cells can promote neoplasia mainly by pathways that involve cell-to-cell contact and the release of soluble factors. Recently it was demonstrated the involvement of the exosomes, released by cancer cells in tumour progression and angiogenic process. Several studies support a role for exosomes in remodeling the tumor microenvironment and thereby contributing to tumor progression via enhanced angiogenesis and metastasis. Melanoma-derived exosomes have recently been shown to promote metastasis through the preparation of the metastatic niche via crosstalk between the released exosomes and bone marrow progenitor cells (10).

It was demonstrated that exosomes mediated transfer of oncogenic EGFR from human squamous cell carcinoma to tumor-associated endothelial cells activated MAPK and AKT cell signaling pathways and promoted endothelial VEGF expression (11).

Therefore, the regulatory properties attributed to tumor-derived exosomes are essential in shaping the tumor microenvironment and promoting tumor growth.

EV contain diverse tumor-associated proteins, including epidermal growth factor receptor (EGFR), K-ras, EMMPRIN, claudins and RAB-family proteins. EGFR is overexpressed in several human cancers and its overexpression correlates with poor prognosis in a large number of malignancies, including NSCLC. EV proteins may reflect pathological processes associated with the disease. Of the identified EV proteins from pleural effusion (PE) isolated from NSCLC, many proteins have been known to be critical for tumorigenesis and biomarkers for diagnosis and prognosis of NSCLC. Exosomes are enriched in small RNA species, including microRNAs (miRs). The profile of miRs in exosomes is specific, since particular repertoires of miRNAs are selectively sorted into exosomes, while other miRs are usually excluded. miRs are short single-stranded, endogenous, non-coding RNA molecules involved in binding partial complementary sequences within the 3′-UTR of the target mRNAs. Global gene expression profiles revealed numerous miRs that were deregulated in cancer compared with normal tissues. The miRs associated with oncogenesis are also termed “oncomirs”. Depending on their main targets, oncomirs are generally classified as tumor suppressive and oncogenic miRs, where tumor suppressive molecules repress protein-coding oncogenes and oncogenic miRs repress protein-coding tumor suppressors. Some miRs may play both tumor suppressive and oncogenic roles, depending on tissue and tumor contexts. miRs are highly tissue-specific and can be used to classify cancers, provide diagnostic and prognostic information, and may represent a cancer therapeutic tool (12). A number of miR profiling studies are currently in clinical trials (13) and miR-based therapeutic approaches are being developed by a number of pharmaceutical companies.

As in other forms of cancer, differential expression of miRs was observed in lung cancer cell lines and patient samples compared with non-malignant cells and normal tissues and data collected from these studies indicate that miRNAs are involved in several critical processes of lung cancer including the initiation, metastasis and drug response. Current research has found that the miRNAs can not only be secreted in tumor tissues but also in body fluids and even in some extracellular organelles, such as exosomes, all of which have the potential to serve as biomarkers for lung cancer.

Decreased expression of the let-7 miR family is frequent in many cancers and in 60% of patients with lung cancer, where it is often associated with a poor prognosis. Low expression of another tumor suppressive miR family, the miR-34 cluster, was reported to be correlated with poor survival of male smokers with squamous cell carcinoma. Expression of miR-34a in the H1299 NSCLC cell line resulted in massive apoptosis and exogenous delivery of lipid formulated miR-34 reduced tumor size in a mouse model of NSCLC, suggesting its possible therapeutic potential. miR-146a,

targeting the EGFR, is differentially expressed in various types of cancer, that indicates its tumor type-specific mode of action. Experiments with five tested NSCLC cell lines revealed the miR146a-dependent suppression of cell growth, induction of apoptosis, inhibition of cell migration, and suppression of EGFR downstream signaling. Surprisingly, miR-146a was also able to enhance the inhibition of cell proliferation induced by drugs targeting the EGFR. These effects were independent of the EGFR mutation status. miR-146a was also shown to be generally downregulated in lung cancer patients and low levels of this miRs correlated with the presence of metastasis. Recent findings revealed the important regulatory roles of miRs in a complex multistep process of invasion-metastasis. For example, overexpression of miR-126 reduced NSCLC cell adhesion, migration, and invasion, which may be partially due to Crk regulation by this miR (2).

MicroRNA are also involved in the drug responsiveness of lung cancer cells. It was reported that overexpression of miR-181b could sensitize A549/Cisplatin (CDDP) cells to CDDP-induced apoptosis by decreasing the levels of the anti-apoptotic protein BCL2 (14). Additionally, miR-181a and miR-630 were reported to be modulators of CDDP response in non-small-cell-lung cancer (NSCLC) A549 cells (15). In contrast, down-regulation of miR-17-5p expression was associated with paclitaxel resistance by up-regulation of the autophagic protein Beclin-1 (BECN1) expression in NSCLC (16). Similarly, let-7a, miR-126, and miR-145 could sensitize the responsiveness of the large-cell cancer cell line H460 and A549 cells to Gefitinib (17).

As said before, in addition to tumor tissues, miRNAs are also found in body fluids such as blood, serum, plasma, urine, and cerebrospinal fluid (CSF), as well as in sputum, saliva, and bronchoalveolar lavage (B/L) . Several studies indicate that body fluid miRNAs are stable even under extreme conditions, such as repeated freeze-thaw cycles and extreme pHs (e.g., pH≈1 or pH≈13). This feature makes body fluid miRNAs suitable biomarkers for clinical detection. Chen et al. showed that there is a distinct difference between the profile of miRNAs found in sera of healthy individuals and NSCLC patients (18). Exosomal miRNAs, are also body fluid miRNAs. Numerous studies indicate that the expression of miRNAs in exosomes is different in the normal condition and in pathological conditions such as tumor. Riccardo et and colleagues screened 742 miRNAs in circulating exosomes and selected 4 miRNAs (miR-378a,miR-379,miR-1395p, and miR-200b-5p) as screening markers for segregating lung adenocarcinoma and carcinomas patients from healthy former smokers (19). Guilherme et al. compare 12 specific miRNAs (miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR203, miR-205, miR-210, miR-212, and miR-214) in peripheral circulation exosome-derived miRNAs and tumor-derived miRNAs in lung cancer patients and healthy people. The results showed no significant difference between peripheral

circulation miRNA-derived exosomes and miRNA-derived tumors, and thus the exosome-derived miRNAs can be used as biomarkers for lung cancer (20).

The aims of the proposal are:

- to study the proteins and the microRNAs composition of exosomes derived from different lung cancer cells sensitive and resistant to conventional chemotherapeutic drugs; in order to evaluate from a functional point of view the exosomes produced by resistant cancer cells and evaluate their ability in modulating the pathways that leads to the development of cancer drug-resistance in sensitive-cells through the "transfer of resistance to therapy"**(**for example through the transfer of mutant forms of EGFR)
- to study the proteins and the microRNAs composition of exosomes derived from different lung cancer cells treated with Cigarette smoke extract (CSE); in order to evaluate the role of exosomes produced by these cancer cells in the development, progression and cancer drug-resistance.
- to study the proteins composition of exosomes released in body fluids as plasma or pleural effusion of patients with lung cancer at different stages of tumour progression;
- to study the microRNAs composition of exosomes released in body fluids as plasma or pleural effusion of patients with lung cancer at different stages of tumour progression. The identification of microRNAs selectively shuttled by the different exosomes may permit to find a novel target for lung cancer diagnosis and therapy;
- to study the role played by the lung cancer exosomes in the establishment of a paracrine crosstalk between tumor cells and the cells of the tumor microenvironment (endothelial cells and cancer associated fibroblasts, caf); with particular attention at the role of exosomes produced by CAFs and their involvement in the resistance to therapy in lung cancer.

Vesicles produced by lung cancer cells during a 24-hour culture period, will be isolated from conditioned culture medium by different centrifugations as described in Taverna et al (21). The *in vitro* study of this project will first make use of different cell lines, representing the subtypes of lung cancer. Purity of exosome fraction will be assessed by identification of specific exosomal markers such as Alix, TSG 101 and CD63 and by the activity of acetylcholinesterase, an exosome-specific marker protein (21). The morphological analysis of exosomes will be performed by scanning electron microscopy and Nanoparticles tracking analyses, to evaluate size and shape of the vesicles.

In order to identify the proteins of exosomes released by different lung cancer cells sensitive and resistant to conventional chemotherapeutic drugs and exosomes purified by plasma of patients with lung in different condition. I'll perform, a proteomic analysis of exosomes, to obtain a comprehensive proteome profile of the exosomes. I'll apply a MudPIT-based proteomics approach, by using a high mass accuracy Triple TOF 5600+ and rigorous identification criteria (22). The MudPIT (Multidimensional protein Identification Technology) is a shotgun proteomic approach that enables the detection of several thousand proteins in complex biological samples with a high level of confidence. Moreover I'll also evaluate, based on spectral counts obtained by MudPIT analyses, the proteins showing most significant difference in expression levels between exosomes released by different lung cancer cells sensitive and resistant to conventional chemotherapeutic drugs; exosomes released in plasma of patients with lung cancer at different stages of tumor progression. I'll validate this different expression by western blotting, ELISA and FACS analyses. The identification of proteins differential expressed may be useful to identify new molecular targets for biomarkers or as a new therapeutic approach in lung cancer treatment. Recently Valadi and colleagues demonstrated that exosomes also mediate transfers of genetic materials. This transfer can include transmission of specific mRs, it can contribute to the epigenetic and proteomic properties of target cells. I'll analyze mRNA and/or microRNA contain in exosomes released by different lung cancer cell lines and in plasma of patients with lung cancer at different stages of tumor progression. The identification of lung cancer exosome-specific miRs cluster may be used as marker of disease. In order to study if exosomes released by lung cancer cells can alter the phenotype of endothelial and caf (cancer associate fibroblast), I would like also study the mRNA and microRNA profile of endothelial cells and caf using microarray and microRNA chip analysis. The understanding of the manner in which endothelial cell and caf may affect lung cancer progression is important to elucidate the cross-talk between cancer cells and host. The analyses of MiRs selectively shuttled by lung cancer-exosomes can offer interesting information to identify novel biomarkers useful to diagnosis and prognosis. It was demonstrated that exosomes-derived miRs have been associated with disease progression. Rabinowits and colleagues showed the significant difference in total exosome and miRNA levels between lung cancer patients and controls, suggesting that circulating exosomal miRNAs might be useful as a screening test for lung adenocarcinoma (23). Taylor and colleagues showed a miR signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. I'll analyse if there are microRNAs selectively shuttled by exosomes derived from plasma of patients with lung cancer at different states of tumor progression. In order to find some potential biomarkers (proteins, peptides) I'll analyze the proteins and peptides composition of the exosomes released in the patient's blood. The targeted measurement of peptides derived from large

proteins (obtained by triptic cleavage of the intact protein) and biological peptides in a complex matrix can be done by a technique called selected reaction monitoring (SRM). Some potential biomarkers could be validated in other clinical samples, including pleural effusion and urine. In order to validate the correlation with exosomes composition and lung cancer in different stages of tumor progression; I could test the composition of the exosomes released in blood samples of patients Phase I of Clinical Trials in comparison with exosomes released in blood samples of patients before the treatment, to investigate the prognostic value of exosomes. I'll carry out this part of the project in collaboration with Prof Rolfo, Head of Phase I- Early Clinical Trials Unit, in UZA (Universtitar Ziekenhuis Antwerpen).

CONCLUSIONS

The study of extracellular vesicles can elucidate on resistance mechanisms to molecular treatment and provide a basis for the development of new strategies to overcome drug resistance and to enhance the outcome in NSCLC patients. EV released in body fluids as blood o pleural effusion can help to discover new biomarkers for an early diagnosis. EV can be used as carriers for biological therapeutics as promising strategy to drug delivery.

References

1. Jemal A., Siegel R., Ward E., Hao Y. Cancer statistics 2009. CA Cancer J. Clin.2009, 59,225–249.
2. Zagryazhskaya A., Zhivotovsky B. Ageing Research Reviews xxx (2014) in press
3. Sacha I. Rothschild, Oliver Gautschi Crizotinib in the Treatment of Non Small-Cell Lung Cancer. Clinical Lung Cancer September 2013
4. Camidge DR, Bang YJ, Kwak EL, et al. Activity and safety of crizotinib in patients with ALK positive non-small-cell lung cancer: updated results from a phase 1 study. Lancet Oncol. 2012 Oct;13(10):1011-9
5. Podoplanin-Positive Cancer-Associated Fibroblasts in the Tumor Microenvironment Induce Primary Resistance to EGFR-TKIs in Lung Adenocarcinoma with EGFR Mutation.Yoshida T, Ishii G, Goto K, Neri S, Hashimoto H, Yoh K, Niho S, Umemura S, Matsumoto S, Ohmatsu H, Iida S, Niimi A, Nagai K, Ohe Y, Ochiai A.Clin Cancer Res. 2014 Nov 11. [Epub ahead of print]
6. Lung cancer and lung injury: the dual role of ceramide. Goldkorn T, Chung S, Filosto S. Handb Exp Pharmacol. 2013;(216):93-113. doi: 10.1007/978-3-7091-1511-4_5. Review.
7. Astrocytes secrete exosomes enriched with proapoptotic ceramide and prostate apoptosis response 4 (PAR-4): potential mechanism of apoptosis induction in Alzheimer disease (AD). Wang G[1], Dinkins M, He Q, Zhu G, Poirier C, Campbell A, Mayer-Proschel M, Bieberich E. J Biol Chem. 2012 Jun 15;287(25):21384-95. doi: 10.1074/jbc.M112.340513. Epub 2012 Apr 24.
8. Kooijmans SA, Vader P, van Dommelen SM, van Solinge WW, Schiffelers RM. Exosome mimetics: a novel class of drug delivery systems. Int J Nanomedicine. 2012; 7:1525-41.
9. Théry C, Zitvogel L. & Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol; 2:569 2002
10. Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, García-Santos G, Ghajar C, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med. 2012

11. Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, Rak J. Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. ProcNatlAcadSci U S A. 2009;
12. Du L. and Pertsemlidis A. MicroRNA regulation of cell viability and drug sensitivity in lung cancer. Expert Opin Biol Ther. 2012
13. Nana-Sinkam SP. and Croce CM. Clinical applications for microRNAs in cancer. Clin Pharmacol Ther. 2013 11
14. miR-181b modulates multidrug resistance by targeting BCL2 in human cancer cell lines. Zhu W, Shan X, Wang T, Shu Y, Liu P.Int J Cancer (2010) 127(11):2520–9.10.1002/ijc.25260
15. miR-181a and miR-630 regulate cisplatin-induced cancer cell death. Galluzzi L, Morselli E, Vitale I, Kepp O, Senovilla L, Criollo A, et al.. Cancer Res (2010) 70(5):1793–803.10.1158/0008-5472.CAN-09-3112
16. miR-17-5p downregulation contributes to paclitaxel resistance of lung cancer cells through altering beclin1 expression. Chatterjee A, Chattopadhyay D, Chakrabarti G... PLoS One (2014) 9(4):e95716.10.1371/journal.pone.0095716
17. MicroRNAsreduce tumor growth and contribute to enhance cytotoxicity induced by gefitinib in non-small cell lung cancer. Zhong M, Ma X, Sun C, Chen L... Chem Biol Interact (2010) 84(3):431–8.10.1016/j.cbi.2010.01.025
18. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Cell Res (2008) 18:997–1006.10.1038/cr.2008.282
19. MicroRNAs derived from circulating exosomes as non-invasive biomarkers for screening and diagnose lung cancer. Cazzoli R, Buttitta F, Di Nicola M, Malatesta S, Marchetti A, Rom WN, et al. J Thorac Oncol (2013) 8(9):1156–62.10.1097/JTO.0b013e318299ac32
20. Exosomal microRNA: a diagnostic marker for lungcancer. Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH.. Clin Lung Cancer (2009) 10:42–6.10.3816/CLC.2009.n.006
21. Taverna S, Flugy A, Saieva L., Kohn E., Santoro A., Meraviglia S., G. De Leo, Alessandro R. Role of exosomes released by chronic myelogenous leukemia cells in angiogenesis"(2011) Int j of cancer.
22. Kislinger T, Cox B, Kannan A, Chung C, Hu P, Ignatchenko A, Scott MS, Gramolini AO, Morris Q, Hallett MT, Rossant J, Hughes TR, Frey B, Emili A. Global survey of organ and organelle protein expression in mouse: combined proteomic and transcriptomic profiling. Cell 125, 173, 2006;
23. Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer, 2009