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Plasma microRNA Profiling as First Line Screening Test for Lung Cancer Detection: a Prospective Study (BIOMILD)

Sponsor: Fondazione IRCCS Istituto Nazionale dei Tumori, Milano

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ABSTRACT

Three decades of research have shown that radiological screening of heavy smokers can detect resectable early lung cancers with higher frequency but benefit on mortality is still debated. The preliminary results of the first two randomized spiral-CT screening trials appear conflicting, with one study showing no benefit and the other a limited mortality reduction (-7%). In the next 3-4 years the ongoing randomised trials in Europe will provide conclusive data on the efficacy of CT- screening for lung cancer. Nonetheless, no major impact on mortality is to be expected.

A possible explanation of these finding is that not all aggressive lung tumors arise from identifiable slow-growing precursors, thus suggesting a possible paradigm shift in our understanding of the natural history of lung cancer. In this respect the identification of biologic and molecular features of indolent and aggressive disease could be useful to define clinical predictors of high risk lesions and select suitable cohorts of patients who might benefit of current treatments as well as to identify genetic signatures that might represent novel therapeutic targets.

We recently reported that microRNA (miRNA) expression expression profiles in tumors and, for the first time, also in normal lung tissue are indicative of aggressive lung cancer development and, of remarkable interest, that specific miRNA signatures can be identified in plasma samples of patients up to two years before spiral-CT detection of the disease, and able to identify the occurrence of early metastatic but spiral-CT invisible lung tumors or small spiral-CT detected lesions with aggressive potential.

In the present project we propose a large prospective study in heavy smokers volunteers based on plasma miRNA profiling to assess its efficacy as a first line screening test for lung cancer detection**.** The study will be articulated in different phases: *i*) analysis of 1000 plasma samples of disease-free smokers already collected in our biological repository in the last two years *ii*) *de-novo* enrollment of 4000 smoking volunteers, collection of their blood samples and inclusion in a program of active surveillance on the basis of their miRNA risk profile *iii*) assessment of miRNA expression profile using a custom made microfluidic card containing the 24 miRNA previously identified in the diagnostic signatures *iii*) bioinformatic analyses of miRNA ratios in the cohort in order to determine which individuals are in presence or will develop lung cancer and in particular the aggressive form of the disease *iv*) assessment of the best diagnostic and treatment algorithm for subjects with suspicious miRNA profiles *v*) functional validation of miRNAs as novel therapeutic targets using novel cellular genetically engineered models of transformation and patients' tumorgrafts models.

Overall, the results of this large prospective study will permit to establish the potential of our plasma microRNA assay as a first-line screening test for lung cancer detection in a routine clinical practice for high-risk population screening with a low cost, non toxic and non invasive procedure. Moreover, the related functional studies foreseen in the project could lead to the identification of novel, miRNA targeted, therapeutic approaches for this malignancy.

Background and Rationale

Lung cancer remains the major cause of cancer mortality in the world. In addition to primary prevention, earlier detection and more targeted treatments, tailored on the biological characteristics of the tumor and its microenvironment, could significantly reduce morbidity and mortality for this disease.
The efficacy of lung cancer screening in heavy smokers by spiral-computed tomography (CT) remains a controversial issue (1,2). The observational studies published so far have reported an up to three-fold increase in the number of lung cancer diagnoses, without major decrease in the number of advanced cancers or reduction of mortality in smokers (3, 4). The preliminary results of the first two randomized spiral-CT screening trials appear conflicting, with one study showing no benefit and the other a limited mortality reduction. In fact, the recent results of NLST trial (www.cancer.gov), showed a 7% reduction in all-cause mortality and -20% lung cancer mortality, compared to annual chest x-rays, but a real control arm is lacking and full publication of the results of this trial is still awaited. Before CT screening can be offered to millions of individuals worldwide, a number of questions have to be answered: best selection of high-risk individuals, frequency and duration of screening, efficacy in current vs. former smokers, cost and side-effects. As an example of such uncertainty, the United Kingdom will launch this year a large national randomised trial, comparing one single CT scan vs. observation in 32,000 heavy smokers (5).
In a recent study (6), for the first time, we reported the results at 10 **years** of annual CT-screening, as well as a new approach to early detection with molecular markers. Our data show that CT screening detects a high proportion of early stage tumours with excellent survival during the first two years, while as the screening progresses, the frequency of late stage tumors increases. These "early advanced" cancers have a poor prognosis similar to the clinically detected (or symptomatic) advanced cancers (7). These findings seem to indicate that not all aggressive lung tumors arise from identifiable slow-growing precursors, and indicate a bipartite biologic model of lung cancer, with one indolent and one aggressive type from the very beginning, rather than one common initial type which may turn into aggressive over time (8).
In this respect, the development of molecular markers able to identify tumors in a pre- clinical phase and to track the different aggressiveness of lung tumors, including the early metastatic cancers or even the small lesions with aggressive potential is of paramount importance.
Over the past years, large scale genetic and epigenetic profiling of lung tumors allowed to establish tumor fingerprints of diagnostic and prognostic significance. However, these signatures remained mainly restricted to tissue samples and attempts for their detection in biological fluids collected by non invasive means, such as plasma or serum, were unsatisfatory in terms of sensitivity and specificity (9,10).
MicroRNAs (miRNAs) are 19 to 25 nucleotide-long non-coding RNAs that regulate gene expression by binding complementary sequences of target mRNAs and inducing their degradation or translational repression. miRNAs expression profiles of lung tumors showed deregulation of a set of miRNAs compared to normal lung tissue and down- regulation of let-7 as well as up-regulation of mir-155 were associated with worse prognosis in Stage I adenocarcinoma (ADC) patients (11). More recently, Yu et al. identified a five- microRNA signature that was an independent predictor of cancer relapse and survival of non-small cell lung cancer (NSCLC) patients (12). In addition a diagnostic assay based on hsa-miR-205 expression provided accurate classification of lung cancer with squamous histology (13). miRNAs could constitute a new class of blood-based biomarkers useful for cancer detection and prognosis definition since, for their nature, they seem to remain rather intact and stable and are detectable quantitatively with simple assays (i.e. qRT-PCR). Initial studies in

prostate, colon and lung cancer patients showed that plasma levels of specific miRNAs had remarkable sensitivity and specificity to distinguish cancer versus healthy subjects (14-17).
In our paper (6) we investigated miRNA profiles of lung tumors, normal lung tissues and plasma samples from cases with variable prognosis identified in two independent spiral- CT screening trials with extensive follow-up where multiple plasma samples, collected up to two years before radiological detection of the disease were available. We found miRNA expression profiles associated with aggressiveness of the disease and poor survival not only in tumors but, for the first time, also in normal lung tissues of the patients, thus proving the critical influence of a, likely smoke-related, lung microenvironment permissive on tumor promotion.
Of major interest, specific miRNA signatures were identified in plasma samples collected up to two years before radiological (spiral-CT) detection of the disease, and thus able to catch the earlier biological phases of disease development. It is noteworthy that we also defined a signature that discriminates subjects according to aggressiveness of their future tumors, and in particular the occurrence of early metastatic but spiral-CT invisible lung tumors or small spiral-CT detected lesions with aggressive potential.
Anticipating clinical and hopefully pre-clinical diagnosis of one to two years, could significantly change the tumor burden and improve the efficacy of any local and systemic therapy, both cytotoxic and biological. On the other hand miRNA profiles will be helpful to reduce the extent of over-diagnosis, and consequent over-treatment of low-risk disease, that is intrinsic to spiral-CT screening.
One therapeutically relevant concept is that one miRNA can downregulate multiple target proteins by interacting with different target mRNAs. Although cancer is a multigenic disease, it is becoming clear that deregulation of a single miRNA could be cause of several of the observed abnormalities (18). Moreover because of their tissue specificity, miRNAs (or anti-miRNA) represent a promising therapeutic strategy (19). Reconstitution of miR-15a and miR-16 cluster resulted in marked regression of prostate tumour xenografts (20) and reintroduction of let-7, that regulates KRAS expression, has been shown to functionally inhibit non-small cell lung tumour development in mouse models (21).
Overall, miRNA expression profiling and related functional studies would provide greater knowledge of the regulatory events underlying the development and progression of lung cancer and lead to the identification of novel diagnostic markers and therapeutic targets for this malignancy.
In the present project we propose a large prospective study in heavy smokers volunteers based on plasma miRNA profiling, to assess its efficacy as a first line screening test for lung cancer detection**.**

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Task 1. Study population and diagnostic work-up
Milestone attainment: Months 1-36

(1.1) Study population. The study will evaluate the efficacy of plasma miRNA profiling for early lung cancer detection in two distinct populations of high risk individuals. The first one is represented by 1,000 subjects enrolled in the on-going randomized MILD trial (1), whose plasma samples have been already stored in our biological repository during the last five years. The second population is represented by 4,000 newly recruited subjects with the following characteristics:

- current heavy smokers of ≥ 30 pack/years, aged ≥ 50, or former smokers with the same smoking habits having stopped from 10 years or less;
- current or former smokers of < 30 pack/years, aged ≥ 50, with additional risk factors such as family history of lung cancer, prior diagnosis of chronic obstructive pulmonary disease (COPD) or pneumonia, professional exposure to known carcinogens (i.e. asbestos).

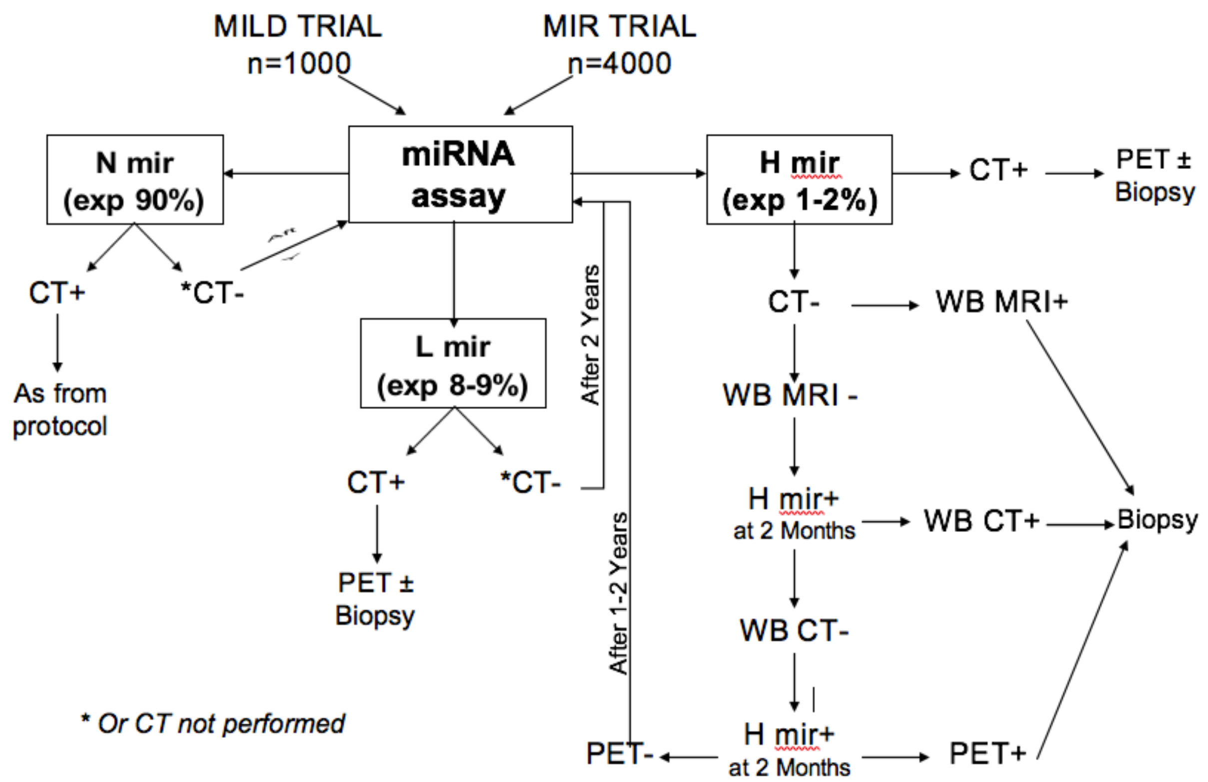
A sample size of 4000 subjects, a 70% of whom was expected to have double negative low-dose CT (LDCT) examination and miRNA test at baseline, was calculated to guarantee an 80% power with a 5% level of significance to detect:
(a) a 30% reduction in the total number of LDCTs performed during the trial;
(b) the absence of significant differences in the proportions of stage I lung cancer in double negative subjects sent to 3-year LDCT repeat as compared to other subjects;
(c) the absence of significant differences in the proportions of overall lung cancer resectability in double negative subjects sent to 3-year LDCT repeat as compared to other subjects.

(1.2) Diagnostic work-up. In the MILD population, miRNA testing (Task 3) will be performed on stored plasma samples, in addition to the screening protocol planned for the assigned arm (CT every 1 or 2 years vs. observation). In the newly recruited population, all subjects will undergo baseline LDCT, spirometry, and miRNA profiling.

All individuals with negative miRNA (**Nmir**) profile (90% expected) will repeat the plasma assay at three years (*Figure 1*).
Individuals with low-risk miRNA (**Lmir**) profile (8-9% expected) will repeat the plasma assay and LDCT at two years, without additional diagnostic examinations if not required by the screening protocol (i.e. suspicious or positive CT) *(Figure 1)*.
Individuals with high-risk miRNA (**Hmir**) profile (1-2% expected) will undergo additional diagnostic examinations consisting in positron emission tomography (PET) in case of concurrent suspicious or positive CT, or whole body MRI (WB MRI) +- needle aspiration biopsy in case of negative CT. Subjects belonging to control arm of MILD trial will undergo

chest CT first, and additional testing according to the same diagnostic algorithm. If these examinations will result negative, miRNA assay will be repeated after two and four months, and in case of persisting **Hmir** profile, whole body CT (at two months repeat) or PET (at four months repeat) will be performed *(Figure 1)*.
In case of negative results of all the entire diagnostic work-up, miRNA profile and chest CT will be repeated after one and two years.
For the analysis of miRNA efficacy at baseline examination, **Lmir** and **Hmir** profiles will be combined and classified as positive miRNA assay.

Figure 1: DIAGNOSTIC WORK-UP



Task 2. Targeted treatment
Milestone attainment: Months 1-36

(2.1) Loco-regional therapy. Lung cancer treatment protocol will be based on clinical staging and implemented by miRNA profile, in order to prevent or reduce unnecessary adverse effects for indolent disease as well as ineffective therapy for aggressive disease (2,3).
Stage I lung cancer with **Lmir** profile will receive limited resection (segmentectomy or local excision) plus nodal sampling through video-assisted thoracoscopic approach, followed by radical nodal dissection in case positive frozen section. Stage I lung cancer with **Hmir** profile and Stage II with any miRNA profile will receive standard anatomical resection (lobectomy or bilobectomy) plus radical nodal dissection. Stage III lung cancer will be offered surgical resection and/or radiotherapy after induction chemotherapy, irrespective of miRNA profile.

(2.1) Systemic therapy. Stage I and II lung cancer with **Hmir** profile will be offered systemic chemotherapy, either as induction or adjuvant, selected according to the best available histologic or molecular targeting, such as specific mutations. Stage III lung cancer will receive induction chemotherapy, irrespective of miRNA profile.

Task 3: Analysis of microRNA signatures in plasma samples.
Milestone attainment: Months 1-36

We recently reported that miRNA expression analyses in plasma samples collected 1–2 y before the onset of disease, at the time of lung cancer detection by spiral- CT and in disease-free smokers enrolled in two independent CT-screening trial, resulted in the generation of miRNA signatures with strong predictive, diagnostic, and prognostic potential (4).
In particular, 100 microRNAs were found to be consistently expressed in plasma samples of patients and controls. Since the normalization of miRNA data in plasma samples using reliable housekeeping miRNAs is still a controversial issue, the ratios between the expression values of all the 100 miRNAs were computed to bypass the problem. These "miRNA ratios" were then used to analyze differences between classes of samples resulting in the definition of ratios with clinical relevance. For the ratios showing statistically significant differences in the class comparison analyses ($p<0.05$), a cut-off value was established with the formula (mean of the ratio in one class ± S.D) to use the ratios as binomial variables in the generation of different signatures:

A signature of 16 ratios, composed by 15 miRNAs, indentifies individuals at risk to develop lung cancer in the following two years with sensitivity of 80% and specificity of 90%. Moreover, a signature of 10 ratios, composed of 9 different miRNAs, in pre-disease plasma samples is able to predict the development of tumors with worse prognosis and might even be helpful in pinpointing those early stage tumors at high risk of aggressive evolution.

A signature composed by 16 ratios, involving 13 different miRNAs, has a diagnostic value (75% sensitivity and 100% specificity). Moreover, 10 ratios, including 11 miRNAs, are able to discriminate patients with or without the aggressive form of the disease.

A total of 24 miRNAs resulted to be involved in the four different signatures.
To better define the range of values of the miRNA ratios and their power in the prediction and in the classification of the disease, we need a large scale prospective study including thousands individuals.

(3.1) Measurements of miRNA levels in plasma samples using qReal-Time PCR
Milestone attainment: Months 1-36

Whole blood is collected in EDTA vacuum tubes and plasma immediately separated by two centrifugation steps at 2500 rpm at 4°C and stored in a biological bank at -80°C. Using mirVana™ PARIS™Kit (Ambion) total RNA will be extracted from 200 µl of plasma samples and microRNA expression will be determined using the Megaplex™ Pools Protocol on custom made microfluidic cards (Applied Biosystems). These cards contain the 24 microRNA previously identified, spotted in duplicate, and on each card 8 plasma samples could be applied per time.
The retrotrascription and the preamplification steps will be performed using a custom made Master Mix (Applied Biosystems) containing the primers for the 24 microRNA of interest. For each microfluidic card the Ct of every miRNA will be determined using the program SDS 2.2.2® (Applied Biosystems) and setting a threshold of 0.2 and a manual baseline from 3 to 18 cycles.

(3.2) Bioinformatics analyses of "miRNA Ratios"
Milestone attainment: Months 18-36

Using the Ct values obtained, miRNA ratios will be calculated with the formula ($2^{\wedge}\text{-Ct}^{mir-x}/2^{\wedge}\text{-Ct}^{mir-y}$). The signatures of "miRNA Ratios" previously identified will be tested and validated on the plasma samples collected in the prospective study.
We will need to assess the distribution of values of "miRNA Ratios" in the entire cohort and thereby establish a "cut off value" or a "cut off range" for each of the signature's ratios in order to determine which individuals are in presence or will develop lung cancer and in particular the aggressive form of the disease.
We expect that the values of the "miRNA ratios" have a Normal distribution where the values that fall far from its mean should be the cases with a predisposition to develop the aggressive form of lung cancer.

Task 4: Functional validation of microRNAs as novel therapeutic targets

(4.1) Use of novel cellular genetically engineered models of transformation to gain information on microRNA changes linked to cancer development and progression
Milestone attainment: Months 18-30

In this task we will test the functional relevance of the microRNAs identified in the diagnostic and prognostic signatures in lung tissues and plasma (4). miRNAs might represent optimal therapeutic targets because *i.* they are highly tissue specific and their effects are expected to be the result of the complex modulation of multiple targets belonging to multiple pathways, *ii.* they are easily manipulated by loss-o-function and gain-of-function approaches and *iii.* as increasingly effective pharmacological means to modulate miRNA activities are being developed, identifying miRNAs that are essential for tumor maintenance or for metastasis might provide exciting new therapeutic opportunities.
To examine the contribution of relevant miRNAs composing the diagnostic and prognostic signatures identified in lung tissues and plasma we will use both established lung cancer cell lines (A549, H460) and normal immortalized bronchial epithelial cell lines (HBEC-KT). These cellular models will allow to have insight of the miRNAs role into different phases of the carcinogenetic process since cancer cell lines will permit to asses the effect of miRNAs deregulation in a context where the tumor is already established and progressed and on the other hand HBEC-KT represent a promising model to investigate the effect of selected

microRNAs in the early stages of the development and maintenance of the tumorigenic phenotype through the different phases of lung carcinogenesis. Immortal HBEC-KT cells have been additionally manipulated *in vitro* to recapitulate many of the genetic changes commonly observed in lung cancers (p53 knockdown, K-Ras over expression and EGFR mutations). Despite the presence of several molecular alterations these cells have not acquired a fully tumorigenic phenotype and do not form tumors in nude mice (5) indicating that additional changes might be needed for manifestation of the tumorigenic potential. We have received the different variants of HBEC-KT cells from Dr. J.D. Minna (UT Southwestern Medical Center, Dallas, Texas) and have already successfully transduced them with a retroviral vector carrying shRNA targeting the oncosuppressor gene FHIT. Down regulation of both FHIT and p53 recapitulates the two most common genetic alterations observed in lung preneoplastic lesions and we have therefore generated a model of initiated bronchial cells to test the contribution of specific miRNA expression to lung cancer development.

Modulation of miRNAs expression in vitro. The significance of selected miRNA will be tested in cultured A549, H460 and HBEC-KT by transiently or stably transfecting these cells with either synthetic pre-miRNA molecules (6) designed to mimic endogenous miRNA that are processed into mature products in gain-of function experiments or synthetic anti-sense RNA molecules (2'-O-methyl RNA) and/or with locked RNA oligonucleotides (7) designed to specifically bind to and inhibits specific miRNA in loss- of-function experiments. Cells transfected with scrambled oligonucleotides will be used as controls.

Functional analysis of the effects of gene manipulation in cell lines. We will investigate whether modulation of selected microRNAs result in changes in proliferative and invasive properties and also in loss of epithelial characteristics and acquisition of mesenchymal properties. This profound change in cell phenotype is believed to lead the epithelial cells to acquire characteristics of motility, invasiveness and resistance to apoptosis and could therefore play a crucial role in the acquisition of invasive and metastatic properties.

The biological effects of specific miRNA alterations will be investigated by analyzing changes in proliferation by SRB and clonogenicity assay, capacity to induce apoptosis by annexin-V/propidium FACS assay and TUNEL test, cell mobility and invasion capacity by specific assays with BD BioCoat Matrigel Invasion Chamber (BD Bioscience), Epithelial-Mesenchymal Transition (EMT) by Real Time PCR with specific Assay-on-Demand (Applied Biosystem) for the two conditions. Since our these cell lines have been already transduced with GFP reporter gene, changes in growth kinetics, mobility and invasion of these cells will be estimated with readings in a microplate reader (Tecan instruments).

Modulation of miRNA expression in vivo. To analyze the effects of microRNAs of interest on *in vivo* tumorigenicity we will inject in nude mice clones obtained by stable transfections with specific plasmids or by lentiviral vectors infections thus obtaining a near-physiological expression level of microRNAs. We will use non-tumorigenic HBEC- KT cells and their variants engineered with K-Ras and p53 mutation to assess a possible cooperation of miRNA expression with other oncogenic lesions. To assess the effects on tumor growth capability we will evaluate tumor take, size and growth rate.

(4.2) Identification of microRNA targeted therapeutic strategies using *tumorgrafts* models
Milestone attainment: Months 24-36

Modulating miRNA functions may provide novel therapeutic opportunities for cancer treatment. Since many miRNAs have been identified to impart tumor suppressive effects, restoring their expression (endogenously or exogenously) may yield therapeutic effects.

Re-expression of even a single miRNA in tumor cells could provide significant therapeutic benefit as shown in two recent reports demonstrating that viral delivery of let- 7 miRNAs suppressed tumor growth in a mouse model of lung adenocarcinoma (8,9). Importantly, let-7 directly targets *KRAS*, the initiating oncogene in this tumor model (10). Furthermore, systemic administration of miR-26a using adeno-associated virus (AAV) in an animal model of hepatocellular carcinoma (HCC) also inhibited tumor progression without toxicity (11).
Furthermore, as miRNAs are also associated with chemo- and radio-sensitization of cancer cells (12), a combination approach could also be exploited in an attempt to improve therapy outcome.
Tumorgrafts represent an extremely helpful model to test the efficacy of combined therapy *in vivo*, and will be largely employed to individualize targeted therapies.
Fragments of approximately $3mm^3$ obtained by surgical specimens are implanted subcutaneously, by trocar gauge, in one or both flanks of nude mice. Tumor lines are then propagated by serial s.c. passages of fragments from growing tumors (300-600mg) into healthy mice as we have previously described (13). So far, we successfully grew 14 tumorgrafts in nude mice starting from 31 human lung primary tumors (take rate 45%) and additional tumorgrafts will be established from the enrolled individuals who will develop lung cancer during the course of the project.

We will use tumorgrafts, serially maintained *in vivo* as tumor lines, to explore the potential of agents specifically targeting microRNAs when used alone or in combination with conventional chemotherapeutic drugs. Sequence-specific antisenses for *in vivo* inhibition of microRNAs overexpressed in the signatures or associated with resistance will be employed alone or in drug combination experiments after testing their efficiency in microRNA inhibition. Reagents from Exiqon, which are characterized by complete phosphorothioate backbone conferring stability in serum will be used. For those microRNAs dowregulated in the signatures or whose expression is associated with chemosensitivity, miRNA mimics delivered trough viral vectors or nanoparticles will be employed.
Tumorgrafts established from patient's tumors identified in the project will be treated *in vivo* with the different miRNA-tailored agents targeting the microRNA of interest (miRNA mimics/antisense oligonucleotides through viral vectors or nanoparticles) administered according to i.v. or s.c. route following optimization experiments starting when tumors are palpable ($>=50mm^3$). Optimal dose and time schedule will be defined in preliminary experiments. For combination treatments cisplatin will be then administered i.v. (10g/kg), at its optimal dose according to an every seventh day for three times schedule (5mg/kg/injection). The effects of treatments will be evaluated in the residual tumors in terms of tumor shrinkage and decreased rate or delayed tumor recurrence in serial transplantation assays.

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Feasibility

PI's expertise:
Dr. Ugo Pastorino has long standing experience in the fields which are directly relevant to the present protocol.
Peculiar features of thoracic surgical activity are the innovative techniques of minimally invasive lung resection, muscle-sparing thoracotomies and broncho-vascular reconstruction; trans- manubrial approach to apical chest tumors; management of T4 tumors with SVC and major vessel resection; volume reduction for concurrent emphysema; induction chemotherapy; and pulmonary metastasectomy.
Clinical research is focused on diagnosis, and treatment of primary and secondary lung cancer, use of PET scan for staging of thoracic malignancies, salvage surgery of lung metastases and mediastinal tumors, biology of lung carcinogenesis and chemoprevention of smoking-induced cancers. In 1991 he launched the International Registry of Lung Metastases. He has been the principal investigator of a prospective five years pilot study for early detection of lung cancer, already concluded, testing a combination of low-dose spiral CT plus PET scan and molecular biomarkers in 1035 high risk volunteers. He is the PI of the novel randomized early lung cancer detection trial named MILD (multicentric Italian lung detection) that started in 2005.

Facilities and resources:

The Unit of Tumor Genomics, Fondazione IRCCS INT Milano, collaborating In the project, is fully equipped for biological and molecular analyses, including the following dedicated to the project execution:

- Sterile room for cell cultures and transfections, equipped with 2 laminar flow hoods biohazard II e 2 CO2 incubators,
- Light and fluorescent microscopes equipped with a CCD camera and interactive systems for cell biology and molecular cytogenetic analyses (including Spectral Karyotyping);
- HyBrite Hybridysation machine for FISH; • Geneamp PCR system 9700 termocycler; • 96-well microplates reader; • General laboratory equipment (centrifuges, microfuges, apparatus for protein and nucleic acid electrophoresis, cold room at 4°C, freezers -20 and -80); • IBM PC compatible computers; TCP/IP Networks; UNIX servers
- The purchase of a real-Time PCR system with TaqMan array block module (Applied Byosytem) entirely dedicated to the experimental work described in the project is required considering that a full time use for the execution of the microRNA profiles of the 6000 plasma samples of the trial is necessary.

Institutional facilities of the Department of Experimental Oncology of INT include:

- FACS analysis and cell sorting (FACScalibur, FACS Vantage SE with upgrade for high speed sorting and FACS ARIA Cell Sorter);
- Confocal microscopy;
- Biologic irradiator;
- Immunohistochemistry equipments;
- DNA sequencing and microsatellite analysis;
- Microbiology;
- Real-time quantitative PCR Applied Biosystems HT7900 (Applera) equipped with adaptor for 384-samples microfluidic cards;

- Illumina platform with hybridization and scanning stations from Axon
- Radioactive room with standard molecular biology equipment and instrumentation for PCR, gel electrophoresis, manipulation of radioactive products
- Dedicated rooms for the manipulation of biohazard level III MOGMs, equipped in compliance with the existing Italian legislation.
- Facilities for Animal housing (nude, NOD/SCID mice of various strains) with centralized food and cage-washing services. Animals are housed in ventilated racks and subjected to 12 hour light/dark cycles. Basic husbandry is provided by certified technical staff. The experiments described in the proposal are conform to all regulations protecting animals used for research according to DL 116/90.

The protocol was approved by the Ethics Committee on April 22, 2011 (15 June 2011 n. 182DG, n. 160/2011)

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