Ruolo dei miRNA come marcatori diagnostici e prognostici nel tumore al polmone

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Lung Cancer: epidemiology

Leading cause of cancer mortality in the world: in Europe about 400,000 new cases/year (34,000 in Italy)

75% of cases: advanced stage disease

5 years survival rate = <15%

breast > 70%
colon > 50%

NEW STRATEGIES FOR EARLY DIAGNOSIS AND RISK ASSESSMENT

Unit of Tumor Genomics, INT, Milan
Lung Cancer Screening by Spiral-CT: critical issues

Observational studies reported up to three-fold increase in the number of lung cancer diagnoses, without apparent major decrease in advanced cancers or reduction of mortality in smokers (Bach et al., JAMA 2007)

The results of the four published randomized spiral-CT screening trials appear conflicting, with three European studies showing no benefit (Infante M., AJRCCM 2009, Saghir Z., Thorax 2012, Pastorino U., Eur J Cancer Prev. 2012; ~10,000 subjects overall) whereas, the large (53,454 persons) NLST trial (NEJM 2011), showed a 7% reduction in all-cause mortality and ~20% lung cancer mortality, compared to annual chest X-rays.

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 (Bach PB, Lancet Oncology 2008)

In this respect, the development of molecular markers able to identify tumors in a preclinical 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.
Sources of blood-based biomarkers.
Novel promising biomarkers are generated by cancer cells, tumor microenvironment the host response and their dynamic interaction.

Plasma biomarkers studies at INT (Milan)

- Methylation markers plasma samples in case-control series (Bearzatto A. Clin Cancer Res. 2002)
- Circulating plasma DNA (hTERT) quantification in case-control series and in pilot INT-IEO and MILD screening trial (Sozzi G., Can Res 2001; Sozzi G., J Clin Oncol. 2003; Sozzi G., AJRCCM 2009; Roz L. Lung Cancer 2009)
- Highthroughput microRNAs expression profiles in plasma samples in independent screening trials (INT-IEO, MILD) (Boeri M. et al., PNAS 2011; Sozzi G. Cell Cycle 2011; Boeri M. et al., Cancer J 2012)
microRNA: a new class of biomarkers

small noncoding RNAs that regulate gene expression by binding complementary sequences of target mRNAs and inducing their degradation or translational repression

Evolutionary conserved

One miRNA has multiple targets

One miRNA

mRNA

mRNA

mRNA

mRNA

mRNA

mRNA

mRNA

microRNA: a new class of biomarkers

Blood-based miRNA studies are in their infancy

miRNA remain rather intact and stable in plasma/serum

Simple universally applicable assay for quantification (i.e. qRT-PCR)

In lung cancer plasma levels of miRNAs might have diagnostic (Silva J, ERJ. 2010; Shen J, Lab Invest, 2010; Foss KM, J TO 2011) and prognostic value (Hu et al., JCO, 2010).

miRNAs have been found packaged in exosomes derived from multivesicular bodies (7) or be exported in the presence of RNA-binding proteins (i.e. Ago-2)(8) or might be exported microvesicles shed during membrane blebbing (9). Once in the extracellular space, these miRNAs could be taken up by other cells, degraded by RNases, or excreted(10).
MicroRNA signatures in tissues and plasma predict development and prognosis of computed tomography detected lung cancer

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Contributed by Carlo M. Croce, January 12, 2011 (sent for review December 22, 2010)

The efficacy of computed tomography (CT) screening for early lung cancer detection in heavy smokers is currently being tested by a number of randomized trials. Critical issues remain the frequency of unnecessary treatments and impact on mortality, indicating the need for biomarkers of aggressive disease. We explored microRNA (miRNA) expression profiles of lung tumors, normal lung tissues and plasma samples from cases with variable prognosis enrolling in a completed spiral-CT screening trial with extensive follow-up. miRNA expression patterns significantly distinguished: (i) tumors from normal lung tissues; (ii) tumor histology and growth rate; (iii) clinical outcome; and (iv) year of lung cancer CT detection. Interestingly, miRNA profiles in normal lung tissues also displayed remarkable associations with clinical features, suggesting the influence of a permissive microenvironment for tumor development.

The intent of the present study was to perform an extensive miRNA profiling of primary lung tissues, paired normal lung tissues, and multiple plasma samples collected before and at the time of disease. From two independent spiral CT-screening trials, we aimed to identify biomarkers able to predict tumor development and prognosis, therefore improving lung cancer diagnosis and treatment options.

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microRNA in LUNG TISSUE SAMPLES

TUMOUR TISSUES

NORMAL TISSUES

Dendrogram for clustering experiments, using centered correlation and average linkage.

miR-128a
miR-129
miR-369
miR-193
miR-339
miR-185
miR-346
miR-340
miR-133b

miR-126
let-7c
miR-222
miR-30e
miR-16-2
miR-29b-1
miR-030d
mir-15a
let-7a-2

0= alive
1= dead
Stage (pTNN)
Detection year

Observed survival by year of screening

P=0.005

1-2
3-5
miRNA deregulated in tumor and normal samples according to CT detection years (1-2 vs 3-5)

### TUMOR TISSUE

<table>
<thead>
<tr>
<th>miRNA symbol</th>
<th>Fold-change (1-2 / 3-5)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-128a-prec</td>
<td>0.66</td>
<td></td>
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<tr>
<td>hsa-mir-129-3p</td>
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<td>hsa-mir-339-5p</td>
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<td>hsa-mir-348</td>
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<td>hsa-mir-340</td>
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<td></td>
</tr>
<tr>
<td>hsa-mir-126a</td>
<td>0.54</td>
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</table>

### NORMAL TISSUE

<table>
<thead>
<tr>
<th>miRNA symbol</th>
<th>Fold-change (1-2 / 3-5)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>hsa-mir-126</td>
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<td></td>
</tr>
<tr>
<td>hsa-mir-129*</td>
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<td>hsa-let-7c</td>
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<td>hsa-mir-222</td>
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<td>hsa-mir-1-2</td>
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<td>hsa-mir-28b-1</td>
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<tr>
<td>hsa-mir-16a</td>
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</tr>
<tr>
<td>hsa-let-7a-2-prec</td>
<td>0.68</td>
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</tbody>
</table>

Cell adhesion, invasion, metastasis

Inflammation

AKT pathways

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### microRNA in PLASMA SAMPLES

#### Training Set (INT-IEO trial)

**CONTROLS**
5 POOLS
(28 individuals)

**Validation Set (MILD trial)

**CONTROLS**
10 POOLS
(54 individuals)
100 of the 378 miRNAs are consistently expressed

TECHNICAL VALIDATION (training set) & VALIDATION SET

TaqMan® MicroRNA Assays Multiplex™ Only on miRNA of interest

Using single miRNAs no correlation with normalization on the mean expression value (gold standard)

MammU6  RNU44  RNU48
mir-16    GeNorm

NORMALIZATION ON THE MEAN OF EACH CARD/SAMPLE
(MICROFLUIDIC CARDS-TRAINING SET)
IS IT POSSIBLE TO BYPASS NORMALIZATION?

LOOKING AT ALL THE RECIPROCAL RATIOS AMONG miRNAs

100 miRNAs → \( \frac{100 \times 99}{2} \) = 4950 miRNA Ratios

miRNA RATIOS on PLASMA SAMPLES
excellent correlation with normalization on the mean values

ANALYSIS OF miRNA RATIOS in plasma

<table>
<thead>
<tr>
<th>RISK OF DISEASE</th>
<th>TaqMan® Array Human MicroRNA Cards Megaplex™</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1-2 yrs before disease)</td>
<td>Technical Validation Multiplex™</td>
</tr>
<tr>
<td></td>
<td>ANALYSIS OF miRNA RATIOS</td>
</tr>
<tr>
<td></td>
<td>Filter for Ratios with a minimal intra-pool variability</td>
</tr>
<tr>
<td></td>
<td>Validation Set Multiplex™</td>
</tr>
<tr>
<td>DIAGNOSIS</td>
<td></td>
</tr>
<tr>
<td>(at disease)</td>
<td></td>
</tr>
<tr>
<td>DIAGNOSIS OF AGGRESSIVE DISEASE (OUTCOME)</td>
<td>24 microRNAs</td>
</tr>
</tbody>
</table>

Duplicates Ctrl Pools Inter Samples

Pearson correlation

Mean normalization □ Ratios method
miRNA and miRNA ratios distribution and discrimination power in the analyses

**RISK OF LUNG CANCER 1-2 YEARS BEFORE CT**

- **Sample size**: 25
- **Sensitivity**
  - **AUC = 0.85**
  - **p < 0.0001**

**DIAGNOSIS OF LUNG CANCER**

- **Sample size**: 26
- **Sensitivity**
  - **AUC = 0.88**
  - **p < 0.0001**

**RISK OF AGGRESSIVE LUNG CANCER (RAD)**

- **Sample size**: 37
- **Survival probability (%)**
  - **p = 0.0006**

**DIAGNOSIS OF AGGRESSIVE LUNG CANCER (PAD)**

- **Sample size**: 31
- **Survival probability (%)**
  - **p = 0.0001**
CLINICAL IMPLICATIONS

- Plasma miRNA profiling as first-line screening test to for more stringent selection of higher risk individuals necessitating systematic screening by morphologic imaging techniques (spiral-CT and whole-body MRI) or metabolic imaging (PET, with FDG or other specific tracers) to pick up occult metastatic disease.

- Non-toxic prophylactic treatments such as vaccination, pharmacological smoking cessation programs to curb late-phase promotion?

- MiRNA profiles helpful to reduce the extent of over diagnosis, and over-treatment of low-risk disease.

24 microRNA signatures: extended Validation Set (MILD trial)

<table>
<thead>
<tr>
<th>controls</th>
<th>CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 disease free individuals</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RISK</th>
<th>RISK of AGGRESSIVE DISEASE</th>
<th>PRESENCE of DISEASE</th>
<th>PRESENCE of AGGRESSIVE DISEASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC = 0.89</td>
<td>AUC = 0.97</td>
<td>AUC = 0.92</td>
<td>AUC = 0.93</td>
</tr>
</tbody>
</table>
1000 controls (no disease)

130 samples (13%): haemolyzed

872 controls suitable for analyses: 594 LDCT arm; 286 observational arm

85 lung cancer patients

9 patients (11%): samples not available

7 patients (9%): haemolyzed samples

69 Lung Cancer patients: 44 at cancer diagnosis, 54 before diagnosis, 19 post-surgery

- custom-made 24 miRNA microfluidic card
  - NanoString and deep sequencing technologies (foreigner partner)
  - Functional studies: in vitro and in vivo (tumorgrafts models)

**Study Design & Aims**

miRNAs follow-up

CT-detected LC

Clinical LC

pre - vs. post-treatment miRNA test

tumor-related miRNAs

host-related miRNAs

extended validation on MILD samples
MILD TRIAL
\( n=1000 \)

MIR TRIAL
\( n=4000 \)

**bioMILD - 2011**

biomarker-based prospective study

- validate miRNA profile
- combination of CT e miRNA
- CT dose according to risk
- Biology-based therapy

Pharmacologic prevention
www.biomild.org

un semplice prelievo di sangue la diagnosi di un tumore al polmone, identificato a uno studio fino a due anni più prece di quanto sia possibile utilizzando la TAC spirale, il più avanzato degli strumenti deagnostici oggi a disposizione.

ci servono 4000 volontari

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