243. Lung cancer: molecular pathology and functional genomics

P1949
Inhibition of hypoxia-induced phenotype alterations through epithelial-mesenchymal transition (EMT) in lung cancer by gene modulation of phosphorylation sites in tumor suppressor PTEN
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Recent studies suggested that hypoxia modulate epithelial-mesenchymal transition (EMT) process. It was also demonstrated that the biological activities of PTEN, by which cell proliferation and migration are involved, could be regulated by phosphorylation sites in its c-terminal tail. In this study, we analyzed whether gene modulation of PTEN phosphorylation sites could inhibit hypoxia-induced phenotype alterations through EMT in lung cancer cells. GFP and GFP-PTENmut were transduced into lung cancer H358 with the doxycycline (Dox) inducible gene expression system. Dox-induced expression of GFP and GFP-PTENmut in H358 were confirmed by western blot analysis. Both the Dox-untreated cells with GFP or GFP-PTEN under hypoxic condition showed the decreasing expression of E-cadherin and the de novo expression of fibronectin through EMT and significantly increased the migration ability. Only the Dox-treated cells with GFP-PTENmut kept the expression E-cadherin and inhibited the de novo expression of fibronectin against hypoxia condition. Furthermore, these cells showed the significant repression of cell migration even under hypoxia condition, supported by the finding of suppressed expression of Akt and FAK phosphorylation. In this study, gene transduction and Dox induction did not affect cell proliferation. These findings suggest that the gene modulation of phosphorylation sites in PTEN might give a new therapeutic strategy to regulate metastasis in lung cancer.

P1950
Loss of orphan G protein coupled receptor GPRC5A is associated with metastatic disease and poor survival in patients with adenocarcinomas of the lung
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Introduction: Orphan G protein coupled receptor (GPCR) 5A from the group C of GPCRs (GPRC5A) has recently been suggested to be a tumor suppressor of lung cancers. First results from knockout mice and human RNA analyses implied an association with adenocarcinomas and poor survival. The aim of the present study was to identify prevalence and clinical significance of GPRC5A protein expression in non-small cell lung cancers (NSCLC).

Materials and methods: GPRC5A protein expression was analyzed by immunohistochemistry using a tissue microarray containing samples from more than 2,000 lung cancer patients with clinical follow-up data (Mean: 35.2±34.2 months).

Results: GPRC5A expression was absent in 298 (17.4%) of approximately 1,700 analyzable NSCLC (n=792 squamous cell carcinomas, n=471 adenocarcinomas, n=309 large cell carcinomas and n=96 other NSCLC). 41% of all analyzed NSCLC showed moderate to strong cytoplasmic and membranous GPRC5A staining. Staining intensities were inversely correlated with high tumor cell proliferation (p<0.0001) and grading (p<0.05). In adenocarcinomas, absence of GPRC5A was seen in just 20 cases (4.2%) but was strongly associated with metastatic disease and staging (both p<0.0001). Cumulative survival was significantly lower in patients with adenocarcinomas lacking GPRC5A (p<0.01).

Conclusions: Loss of GPRC5A is linked to high tumor cell proliferation in NSCLC. In adenocarcinomas of the lung, it is not very frequent but is strongly associated with metastatic disease and poor prognosis. Therefore, GPRC5A expression seems to be an important prognostic factor.
**P1951**  
RARE2 methylation level in blood for lung cancer assessment  
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**Background:** Cell-free DNA bearing the same epigenetic changes as the tumor tissues was shown to be detectable in plasma circulating DNA (cirDNA) of cancer patients indicating their usefulness as complementary diagnostic and prognostic markers for lung cancer.  
**Methods:** Blood samples of 30 healthy men and 55 patients with confirmed non-small-cell lung cancer (NSCLC) before and after surgery were enrolled in this study. Methylation level of RARb2 gene in the cirDNA from blood plasma and cirDNA eluted from blood cell surface with PBS/EDTA and trypsin solutions was assessed by quantitative methylation-specific PCR.  
**Results:** It was found that RARb2 gene methylation level was significantly increased in plasma cirDNA and cell-surface-bound cirDNA from NSCLC patients compared with healthy subjects (7620 copies/ml and 1083 copies/ml in the cell-surface-bound fractions, 3589 copies/ml and 1068 copies/ml in the blood plasma, Mann-Whitney U test, P < 0.05). The increase of RARb2 methylation level was associated with stage and unfavorable outcome of the disease. 93% of NSCLC patients demonstrated the significant decrease of RARb2 methylation level in cirDNA at the time point of 10-15 days after surgery.  
**Conclusions:** Epigenetic alterations of RARb2 gene in the total cirDNA were found to be associated with lung cancer progression. Correlation of RARb2 methylation level with tumor stage, outcome of disease and surgical treatment efficacy demonstrate the possibility of cirDNA methylation marker analysis as a valuable tool for lung cancer prognosis and treatment monitoring improvement.

**P1952**  
Quantitative methylation profiles of multiple genes in patients with non-small cell lung cancer and its association with clinicopathological correlations  
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**Introduction:** Aim of this study was to examine quantitative methylation patterns using pyrosequencing in multiple genes in tumor, matching normal lung tissue and blood of patients with non-small cell lung cancer. We also analyzed possible associations between clinicopathologic features of patients and DNA methylation.  
**Material and methods:** Primary tumor samples (n=65), corresponding non-malignant lung tissues (n=65) and blood samples (n=51) were obtained from NSCLC patients, treated with curative resectional surgery. Hypermethylation status was quantified at multiple CpG sites within each promoter in multiple genes – SOX1, RASSF1A, HOXA9, CDH3, MGMT, ESR1 and DAPK by pyrosequencing.  
**Results:** For most of the genes there was a significant difference between tumor methylation, normal tissue and blood samples. Methylation in tumors was significantly higher than in normal lung for SOX1, DAPK, RASSF1A, HOXA9 and CDH3. Tumor hypermethylation was more frequently for adenocarcinoma at CDH3 and ESR1. A higher proportion of SCC tumors were hypermethylated at HOXA9 and SOX1 compared to other types of NSCLC. Patients with stage four more likely had hypermethylation at MGMT and patients with hypermethylation at HOXA9 more likely had stage two and three tumors. Gender was associated with hypermethylation at CDH3 with females being more likely to have hypermethylated tumors.  
**Conclusion:** Our results show that elevated methylation levels observed in genes SOX1, RASSF1A, HOXA9, CDH3, MGMT, ESR1 and DAPK were cancer-specific and associated with some clinicopathologic features of patients.

**P1953**  
The risk of distant metastases and prognosis prediction in early stage squamous cell lung cancer ( SqCLC) by means of 3 microRNAs expression assessment  
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**Background:** About 50% of NSCLC patients (pts) develop distant metastases following surgery. Currently, apart from stage there are no factors to select high risk pts for adjuvant chemotherapy. We previously showed prognostic value of selected microRNAs (miRks) in frozen tissues of SqCLC and the feasibility of their expression assessment in formalin fixed paraffin embedded (FFPE) samples (Skrzypski et al. JCO 28:521s-2010). In this study we validated the prognostic value of the 3-miR signature assessed in the FFPE samples in an independent patient population.  
**Methods:** FFPE tumor tissue was obtained from 89 stage I-II SqCLC pts. Of these, 40 pts developed distant metastases and 49 had no relapse after a median follow-up of 5.4 years (range, 3.4-8.2 years). Expression of miR-10b, miR-532-3p and miR-192* was analyzed by RT-PCR assays and normalized vs. U6 expression and calibrated by ΔΔCt method. After z-score transformation, the risk score was calculated based on the expression of these 3 miRs.  
**Results:** The median metastasis-free survival (MFS) was not reached in the low risk group according to 3-miR expression signature, whereas it was 26 months in the high risk group. The 3-miR (10b, 532-3p and 192*) expression signature was significantly related to the MFS (log-rank; p<0.013). With the median of the risk score as a cut-off, the sensitivity for distant relapse prediction was 66% at the specificity of 64%. After exclusion of stage IA pts, the corresponding values were 73% and 69%.  
**Conclusions:** 3-miR expression profile has been successfully validated as a predictor of dissemination in operable early stage squamous cell lung cancer.
P1956 Very early detection of small clones harboursing EGFR mutations in NSCLC by 2nd generation sequencing
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Activating mutations of the EGFR gene are present in NSCLC and respond to EGFR TKI therapy, but 50% of these patients show disease progression during the course of TKI treatment, possibly due to resistant tumor cell clones primarily present as small clones within the carcinoma. We tested 80 adenocarcinomas of the lung for known and unknown EGFR mutations comparing different methods for mutation analysis, and tried to correlate the new adenocarcinoma classification with EGFR mutation status. The study included 40 samples with proven mutation status and 40 samples with apparently acinar or papillary adenocarcinomas with undetermined EGFR status. EGFR-mutation analysis was previously done by either Sanger- or pyrosequencing, LightCycler assay, BioFilmChip microarray, IHC with mutant specific antibodies, or by DxS kit. DNA was isolated and EGFR mutations in exon 18 to 21 were tested with 454 sequencing system. Cut-off was set according to Wang et al. (Genome Res. 2007). 1829 mutations were found in 79 patient samples. 77 different previously evaluated mutations could be confirmed. Additional 158 different new mutations were detected in tumor cells. When compared to other methods our results show a better specificity and better sensitivity. Cases analyzed by LightCycler assay and pyrosequencing showed 100% correspondence to 454 sequencing, whereas all other methods showed aberrations. Mutations present in small tumor cell clones were not detected by any of the other methods. Furthermore, T790M resistance mutation combined with an activating mutation could be found in 2 untreated patient samples. The results underline the need for higher sensitive methods applicable in routine diagnostics.

P1957 Evaluation of highly sensitive PNA-LNA PCR clamp method for EGFR L858R mutation detection in lung adenocarcinoma patients
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Objective: Direct sequencing is widely accepted method for EGFR mutation identification, but has limited sensitivity. It often requires additional procedures, like microdissection, to enrich the sample in cancer cells, when their content in tissue specimen is less than 50%. PNA-LNA PCR clamping represents allele-specific approach to gene analysis and demonstrates poten accuracy and ability to detect mutant alleles even if present in low fraction of cells.

Method: 79 DNA samples isolated from fresh-frozen and FFPE tissues, which mutation status was formerly confirmed by sequencing, were analyzed by PNA-LNA PCR clamping for EGFR point mutation L858R in exon 21.

Results: L858R mutation was detected in 87/91 (100%) samples by direct sequencing, whereas in 12/79 (15%) samples by PNA-LNA PCR clamping. All mutant-positive samples by sequencing were correctly determined by PNA-LNA PCR clamp. The remaining 4 L858R mutant-positive samples were recognized as wild type by sequencing. Two of them contained only 5% and 20% of cancer cells, respectively. Surprisingly, in the other two samples PNA-LNA PCR clamping method detected only levels of EGFR mutant allele, despite the cancer cell contents were high (100% and 80%).

Conclusions: PNA-LNA PCR clamp technique enables sensitive and reliable detection of EGFR mutant allele in specimens with cancer cell content insufficient for direct sequencing or genetically heterogeneous. Regarding its extremely high sensitivity, PNA-LNA PCR clamping should be validated thoroughly prior implementation into EGFR diagnostic routine to prevent overdiagnosis.

P1958 Evaluation of four different molecular methods for EGFR mutation analysis in exon 18, 19 and 21
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Aims: EGFR mutation status detection is essential for comprehensive NSCLC treatment Most Non Small Cell Lung Carcinomas are analyzed by histological examination of a biopsy obtained by a small puncture. However, the quality of tissue is available. These conditions require methods, suitable for the detection of very small amounts of mutated DNA. The aim of this study was to compare the sensitivity of mutation detection of four different molecular methods.

Methods: 237 cases with NSCLC were included. All biopsies were formalin fixed and paraffin embedded. DNA was isolated after microdissection of tumor tissue from 20 μm slides, exons 18, 19 and 21 of the EGFR gene were investigated using pyrosequencing (PS) and BioFilmChip Hybridization (Chip). Additionally, a LightCycler Assay was performed for Exon 19 (LC). All mutations were independently confirmed by Sanger sequencing.

Results: We determined 197 cancer with exon 40 with mutations, 26 mutated in Exon 19. Chip: 24 samples, of which 4 mutated, could not be analyzed due to insufficient DNA amplification. Exon 19: 26 out of 26 detected correctly. PS: 39 of 40 mutations were detected. Exon19: 25 of 26 detected correctly. LC (target only Exon 19): 25 of 26 were recognized correctly by this method.

Discussion: A comparison of methods showed no significant difference in sensitivity. However, the Chipmethod failed to generate a sufficient PCR product out of the microdissected 20 μm slides in every tenth sample. Surprisingly, the LC based on a clamping-PCR, was not more sensitive than sequencing (PS, Sanger). A clear advantage in sensitivity could not be found for any of the applied methods.

P1959 Non-coding RNA as functional player and molecular marker in lung cancer
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Previous research has almost exclusively focused on protein-coding genes as functional players in cancer as well as diagnostic or prognostic molecular markers. However, it is now evident, that the human genome contains many more functionally important and clinically informative entities: the non-coding RNAs (ncRNA).

To assess the expression landscape of this new class of molecules, we have profiled the expression of 17000 non-coding RNAs in comparison to 22000 protein-coding mRNA in early stages of lung adenocarcinoma and matched non-malignant lung tissue. These studies revealed molecular signatures associated with tumorigenesis (tumor vs. normal, diagnostic value), with histological subtypes (acinar vs. solid) as well as with the outcome and metastasis development (prognostic value).

Eight novel, differentially expressed transcripts named “Lung Cancer intergenic RNA” (LuCaR1-LuCaR8) were selected and used for functional characterization, for which we have implemented several technologies. To identify the molecular RNA-protein-networks in which the ncRNAs could function, we use RNA affinity purification and mass spectrometry. To study loss-of-function phenotypes in human lung cancer cells, we have developed a novel technique to create functional knockouts of ncRNAs in human lung cancer cell lines using Zinc Finger Nucleases. This strategy allows the specific and stable silencing of the targeted ncRNA without off-target effects for functional studies. Our research uncovers the functions at the cellular and molecular level of the differentially regulated ncRNAs as well as their clinical importance as diagnostic and prognostic markers.

P1961 Polymorphisms A1026G and G369C of CDKN1A/p21 and p53 genes in lung cancer
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In this study, we analyzed the polymorphisms of genes regulating the cell cycle and p53 CDKN1A/p21 as factors of genetic predisposition to lung cancer in patients with lung cancer. We examined 65 patients with lung cancer (average age 56±3 years) and 100 healthy blood donors (average age 52±6 years). Diagnosis of lung cancer was confirmed morphologically, endoscopically and radiologically. The study of these genes was performed by PCR/RFLP analysis. 72-G/C- polymorphism analysis of p53 gene revealed a slight increase in the frequency of the variant 72G/C-genotype of the p53 gene in lung cancer patients compared with those in healthy (38 and 32% respectively, P = 0.695).

We have analyzed two polymorphisms CDKN1A/p21: 1026 A/G and 369 G/C polymorphism in the 5′-gene promoter. In analyzing the 1026-A/-G/p21 gene polymorphism was found halving the frequency of 5′-CG-genotype in lung cancer

340s
patients compared with those in healthy individuals (38 and 77%, respectively; OR = 0.72, CI 95% 0.081–3.95). The frequency of AA-genotype of p21 gene in patients and healthy was respectively 50 and 19% for the GG-genotype of p21 gene was respectively 40 and 3% (OR=6.59, CI 1.27–24.29). This enabled us to assume that the AG-genotype performs protective and AA-genotype - the role of predisposing to cancer of the lung. The frequency of A-allele in lung cancer patients was higher than in healthy individuals (70 and 57.5%, respectively). OR of lung cancer for carriers of the A-allele was 1.72 (CI95% 1.05–2.83). In the analysis of G369C-gene polymorphism in p21 is shown, the frequency of C-allele in lung cancer patients significantly exceeded that in healthy individuals (20.77 and 11%, respectively; OR=2.12 CI95% 1.10–4.09).

P1962 Proteinase inhibition regulates the anti-tumor activity of PAI-1 towards lung and prostate cancer cells
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Plasminogen activator inhibitor type 1 (PAI-1) plays an important role in tumor growth and metastasis formation, directly via specific urokinase complexing or indirectly due to its affinity to vitronectin. The aim of this study was to analyze the impact of the mutant forms of PAI-1: very long half-life (VLHL PAI-1) or devoid of affinity to vitronectin (Vn PAI-1) in proliferation of lung cancer (A549 and H1299) and prostate cancer (LNCaP and DU145) cells characterized by different proteinase (urokinase) production. The dose- and time-dependent inhibition of cell proliferation in the presence of VLHL PAI-1 was evident in A549 and LNCaP cultures. In H1299 cells inhibitory effect was only dose-dependent (p<0.001), while in DU145 only 100 μg/ml of VLHL PAI-1 in 72 hrs cultures suppressed prostate cancer cells proliferative activity (p<0.001). The inhibitory effect of PAI-1 on the proliferation of A549 and H1299 was observed while in prostate cancer lines (DU145, LNCaP) only the inhibitory effect of the highest Vn PAI-1 concentration (10μg/ml) was evident (p<0.05). In DU145 but not time dependent, wPAI-1 didn’t affect A549 and LNCaP proliferation while in highest concentration it had the stimulating effect on H1299 and DU145 (24-48 hrs cultures).

PAI-1 is a negative regulator of cancer cells proliferation due to its anti-proteinase activity. Its biological effect on lung cancer cells is time and dosage-dependent.

P1963 Identification of p-stage I NSCLC patients with high risk of recurrence and poor prognosis by tumor marker index (TMI)
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TMI has been shown to be a prognostic factor in early stage NSCLC (Muley et al., Lung Cancer, 10: 408-415, 2008). The objective of the study was to analyse the value of TMI to predict tumour relapse in completely resected p-stage I NSCLC patients.

112 patients entered the study (71 male/41 female; 37 SCC, 59 AC, 16 other NSCLC; relapse: n=33; death: n=20; median follow-up: 39.3 months). Preoperative markers were measured with immunoassays (Roche, Mannheim, Germany). TMI is defined as the geometric mean of normalized CEA and CYFRA 21-1 values. Statistical analysis was done with SPSS 18.0 (Chicago, USA).

CEA (HR: 2.3, p=0.015) and CYFRA 21-1 (HR: 3.0, p=0.002) differed significantly between patients with low and high risk of tumour relapse. The differentiation was improved (HR: 3.6) by using TMI (cut-off 0.58). 3-year disease-free survival was 83.8% and 54.0%, respectively (p=0.001). 953 patients in the low risk group and 25/59 patients in the high risk group had tumour recurrence. The overall survival was 95.4% in low risk and 77.5% in high risk patients (p<0.001). TMI was found to be a significant prognostic factor (DFS, OS) in multivariate analyses. A small effect of adjuvant chemotherapy (n=20) could be seen in the high risk group. The relapse rate was 2/8 patients with adjuvant chemotherapy compared to 23/51 patients without adjuvant chemotherapy. In contrast, patients receiving adjuvant chemotherapy in the low risk group had a higher rate (5/12) compared to patients without adjuvant chemotherapy (3/41).

Patients with elevated TMI levels were shown to be at an increased risk of relapse and might therefore be appropriate candidates for adjuvant therapy.

P1964 Expression profiling in hypoxic non-small cell lung cancer explants reveals a four-gene hypoxia signature
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Background: Hypoxia is frequent in solid cancers like lung cancer and contributes to chemotherapy resistance. To identify patients with hypoxic tumors would be of advantage. Direct oxygen measurements are of limited use, thus, feasible hypoxia markers are needed.

Material and Methods: A novel ex-vivo model was established using fragmented non-small cell lung cancer (NSCLC) specimens cultured three days under hypoxia (1% oxygen) or normoxia. cDNA microarrays were performed on hypoxic and normoxic fragments derived from ten patients (five adenocarcinomas, five squamous cell carcinomas). Correction for multiple testing was performed using FDR5.

Results: Histomorphology and viability/apoptosis tests confirmed the viability of the fragments. HIF-1α immunostaining and expression of carbonic anhydrate IX mRNA were increased in hypoxia. Microarray analysis revealed 129 regulated genes with at least two-fold expression change in hypoxia compared to normoxia. Hypoxia-induced gene expression was histology-dependent, only four genes were significantly regulated in both subtypes.

Conclusion: Our novel ex-vivo model is suitable to study hypoxic adaptation in lung cancer. Its advantage is the use of real tumor tissue maintained under different oxygen concentrations. cDNA expression profiling revealed a four-gene hypoxia signature that might be useful in therapies aiming to restore tumor perfusion and oxygenation.
Conclusions: Synaptophysin may be the most sensitive marker for neuroendocrine differentiation. Nevertheless, there is no specific marker to clearly separate TC from AC. We confirm that the PI, as determined by Ki67 staining is higher in AC than in TC and might be an ancillary tool for the distinction between TC and AC in addition to the mitotic rate. Moreover, the combination of PI and mitotic count in terms of a grading system may predict outcome more effectively than mitotic count alone and should be subject to further research.

P1966
Genetic polymorphism of alpha 1 antitrypsin and glutathione S transferase and lung cancer risk
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Background: Polymorphisms for genes encoding alpha 1 antitrypsin (A1AT) and glutathione S-transferase (GSTM1/GSTT1) might contribute to the variability in individual susceptibility to lung cancer.

Objectives: This is a cross-sectional, randomized, case control study for the evaluation of the frequency of A1AT (MS, MZ) and GST (GSTM1/GSTT1 null) alleles among patients with lung cancer. The study included 56 cases of lung cancer diagnosed patients (histopathological examination), recruited from the Pneumology Hospital Leon Dansello Cluj and 125 healthy unrelated controls, selected among patients observed in the Internal Medicine Department.

Methods: A1AT genotyping was carried out using PCR amplification of relevant gene segment was followed by restriction enzyme digestion Taq1. Detection of A1AT gene S and Z alleles was determined through analysis of resulting restriction fragment length polymorphism (RFLP). For GSTM and GSTT genotyping we used Multiplex PCR, followed by gel electrophoresis analysis.

Results: The molecular analysis identified the MS genotype in 3 (5,4%) patients with lung cancer and 1 (0,8%) of the controls. The heterozigous MZ state was detected neither among cases nor in controls. The prevalence of GSTM null genotype in lung cancer patients was 49,4% compared to 42,8% of controls, also the prevalence of GSTT null genotype in lung cancer patients was 24,5% compared to 18,2% of controls.

Conclusions: Our findings (positive statistical significance),suggest that heritable A1AT and GST status may influence the risk of lung cancer development.