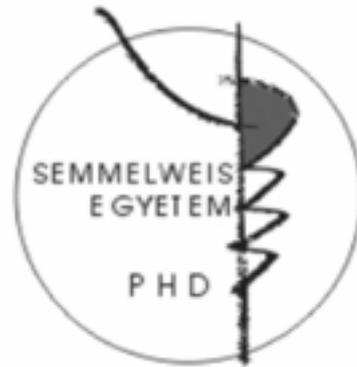


Factors in Progression of Lung Cancer

Thesis

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1. Introduction

Lung cancer is one of the leading causes of tumor mortality associated with a poor survival rate even after complete surgical removal. On average, only half of the stage I lung cancer patients and 1/3 of those with stage II cancers survive over 5-years of primary treatment. Small cell lung cancers can clearly be distinguished from non-small cell lung cancers (NSCLC) based on their histology, neuroendocrine differentiation, transient response to chemotherapy, and generally poor survival.

The NSCLC group, however, is rather heterogeneous including adenocarcinomas (ADCs), squamous cell carcinomas (SCC), large cell, and anaplastic carcinomas.

Cigarette smoking remains the main risk factor for lung cancer, accounting for about 90% of the cases in men and 70% of the cases in women. Over the past few years research groups have investigated the possible involvement of several molecular mechanisms, such as cell-cycle and apoptosis regulators, oncogenes and tumor suppressor genes, cell adhesion molecules in the pathogenesis and progression of lung cancer. Lung cancer is associated with numerous chromosomal regions, genes and pathways. Gene mutations triggered by environmental exposure responsible for cancer development. Similarly to other tumors, a cascade of morphological changes is characterized during lung carcinogenesis. It is more than obvious that the malignant phenotype and its heterogeneity within a tumor subclass or even in a given tumor is caused by the interaction of many gene changes and pathways. Focusing on single genes to use as disease markers is almost hopeless. However, it seems that the different gene changes are not equally important, since targeting single key gene lesions could cause therapeutic benefit.

Single genetic changes

The 3p LOH (80%) and pRb inactivation (90%) is probably important at the early stage of the transformation of epithelial cells with neuroendocrine character. These are followed by p53 inactivation (90%).

EGFR pathway

EGFR is occasionally amplified and/or mutated in NSCLC, and can be overexpressed with other members of the family, forming functional heterodimers.

Prognostic genetic markers NSCLC

The proliferative fraction of cancer is a common predictor of prognosis and it is relatively easy to assess histologically using Ki-67 immunohistochemistry.

The p53/p63 system is a clear characteristic of squamous cell carcinoma (SCC), and its expression is connected to poor prognosis. This histological type of lung cancer is characterized also by Bax expression which correlates with that of p53. The apoptotic index of SCC is associated with Bax level, therefore raising the possibility that Bax is a marker of good prognosis.

The heterogeneity of NSCLC cases of similar morphology, reflected by diverse clinical behavior and response to therapy, justifies molecular studies to find biomarkers associated with tumor progression, prognosis and clinically relevant disease groups based on molecular expression profiles. *TMA*s are useful for the efficient expression profiling of tumors at the protein level, where malignant phenotype and most drug therapy are manifested.

The current standard of treatment for patients with incurable metastatic NSCLC is a *doublet chemotherapy* regimen. The principal agents used are cisplatin or carboplatin. Platinum compounds are heavy metal complexes that form adducts with and cross-links between DNA molecules and thus effectively block DNA replication and transcription. Repair of these adducts and cross-links is dependent on *ERCCI* (excision repair cross complementation group 1).

Adenocarcinoma histology, non-smoking history, Asian race, and female gender were the characteristics that were associated with increased response to *EGFR TKI* (tyrosine kinase inhibitor). *EGFR* mutations are more common in patients with the same clinical characteristics as those associated with better treatment response. The latest advances in research of biological and clinical relevance of activating mutations have been reviewed recently. *EGFR* gene copy number, detected by fluorescent *in situ* hybridization (FISH), is also associated with response to gefitinib.

Small cell lung cancer (SCLC)

Microarray studies determined that the previously separated groups of neuroendocrine cancers (small- and large cell ones) are genetically highly similar if not identical. Polysialic acid (polySia) of the neural cell adhesion molecule (NCAM) is an

oncodevelopmental antigen and is found in small cell lung carcinomas as well as cell lines derived from these tumors. Polysialilation is a posttranslational modification of NCAM which modify the role of NCAM in cell to cell adhesion.

2. Aims/Objectives

1. In vitro and in vivo growth of clonal sublines of human small cell lung carcinoma (SCLC NCI-H69) is modulated by polysialic acid of the neural cell adhesion molecule.
2. Cellular site of synthesis and dynamics of cell surface re-expression of polysialic acid of the neural cell adhesion molecule in human SCLC NCI-H69 cells.
3. Investigating the expression of proteins which play a role in the progression of lung cancers (NSCLC) we focused on the prognostic value of markers involved especially in the brain metastasis formation.
4. The effect of platinum-based neoadjuvant chemotherapies on certain biomarkers, including apoptosis, cell proliferation, and DNA repair mechanisms [ERCC1 (excision repair cross-complementation group 1)], was also investigated. We studied whether the neoadjuvant therapy induces a selection of tumor cells with different biological behavior or therapeutic response.
5. The purpose of the study was to investigate whether protein biomarker overexpression detectable using immunohistochemistry is a prerequisite for the presence of increased gene copy number or activating mutations and responsiveness to the epidermal growth factor receptor (EGFR) inhibitors in patients with lung adenocarcinomas.

3. Methods

3.1. In vitro and in vivo growth of clonal sublines of human small cell lung carcinoma (SCLC NCI-H69) is modulated by polysialic acid of the neural cell adhesion molecule.

3.1.1. Tumors

Paraffin blocks from 50 surgically resected SCLC were obtained from archival files and 15 primary tumors and their metastases were used.

3.1.2. Cell culture, cell cloning and cell lines

Frozen vials of small cell lung carcinoma cell line (NCI H69) were obtained from the American Type Culture Collection (ATCC). The cells were grown in RPMI 1640 medium containing 10% FCS, 1 mM benzyl penicillin and 0.01 % streptomycin sulfate. Cells of line H69 were dissociated by passing 10 times through a 0.5mm hypodermic needle. Of the 51 clonal sublines of the NCAM-positive SCLC cell line H69 established this way, the clonal subline E2 that contained no cells expressing polySia and the subline F3 with a high proportion (~95%) of polySia positive cells were used for further studies.

3.1.3. Reagents and gold labeling

A mouse monoclonal antibody, mAb 735, that recognizes only long chain (chain length >9 units) forms of polySia was used. Protein A-purified mAb 735 was directly labeled with 8 nm gold.

3.1.4. Immunocytochemistry

Cytologic preparation and tissue sections were blocked for 5 to 10 minutes with PBS containing 2% w/v fat-free dried milk powder. Sections were then incubated with primary and secondary antibodies. Label intensification using silver acetate developer was carried out.

3.1.5. Immunoblotting

The cells were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE).

3.1.6. Cell cycle synchronization

The synchronizaton was performed in the presens of colcemide.

3.1.7. Treatment with the methylation inhibitor 5-azacytidine

3.1.8. Treatment with endosialidase N (endoN) to remove the polySia of NCAM.

3.1.9. Standard aggregation and disaggregation assay

3.1.10. Assay for assessing cell to substrate adherence

Collagen type IV, laminin, heparan sulfate and poly-L-lysine were used as substrates.

3.1.11. Assessing colony-forming efficacy & nude mice experiments

Single cells, obtained by passing cells through a 0.5mm hypodermic needle, were suspended in 0.3% agar in culture medium or 1.5% agarose over an agar base following standard protocols.

Nude mice received a single subcutaneous injection into the neck fold of 10^6 viable cells in culture media.

3.1.12. Immunoprecipitation & SDS/PAGE

3.1.13. Immunocytochemistry & immunoelectron microscopy

3.1.14. Temperature-induced (20°C) and chemical (monensin-induced) transport blocks

3.1.15. Statistical analysis

Student *t*-test and Chi^2 test

3.2. Investigating the expression of proteins which play a role in the progression of lung cancers (NSCLC) we focused on the prognostic value of markers involved especially in the brain metastasis formation.

3.2.1. Patients

Formalin-fixed paraffin-embedded archival tissue blocks of NSCLC (59 cases) and brain metastases from some of these tumors (26 cases) were used in this study

3.2.2. TMA (tissue microarray block) construction

Low-density tissue multiblocks were constructed using a manual microarray builder which allows the assembly of recipient blocks incorporating 24 pieces of 2mm diameter tissue cores in a 4x6 orientation.

3.2.3. Immunohistochemistry

Twenty-nine biomarkers potentially associated with tumor progression and brain metastatic potential were tested.

3.2.4. Scoring, hierarchical clustering, and statistics

Immunostaining scores with all patients-related data were archived in a standard MS-Excel spreadsheet format as recommended by Liu et al. Scoring data were binarized

and statistically analyzed using both the Pearson λ^2 and the Fischer exact test by declaring differences significant at $P < 0.05$. Biomarker expression and patients' outcome were correlated using the univariate analysis including the Kaplan-Meier estimates and the log-rank test. The scores were also transformed into a structure relevant for clustering analysis using the Deconvoluter software. Correlations were then visualized using Treeview heat-maps software.

3.3. The effect of platinum-based neoadjuvant chemotherapies on certain biomarkers, including apoptosis, cell proliferation, and DNA repair mechanisms

3.3.1. Patients

Seventeen patients with primary lung cancer, who were treated with cisplatin-containing chemotherapy were studied. Lung cancer tissue blocks obtained by bronchoscopic biopsies together with their corresponding biopsies after neoadjuvant chemotherapy were analyzed

3.3.2. Immunohistochemistry

3.3.3. Evaluation

The results were analysed case by case.

3.4. The value of immunohistochemical evaluation of EGFR expression in the selection of lung adenocarcinomas sensitive for EGFR-TKI therapy

3.4.1. Patients

One hundred twenty-seven primary NSCLC tissues prospectively before and a further nine samples retrospectively after EGFR TKI therapy were examined.

3.4.2. Immunohistochemistry

The expression of EGFR protein was determined by IHC using Dako EGFR PharmDx kits (DakoCytomation).

3.4.3. FISH & PCR

(all details are seen in *Ph.D. Thesis* written by F.Pintér M.D.)

3.4.4. Statistical analysis

4. Results & Discussion

4.1. Fluctuating degrees of cell surface staining intensity is very typical for polySia immunohistochemistry due to the fact that polySia represents homopolymers of a 2, 8-linked N-acetylneuraminic acid residues with a wide variation in the degree of polymerization.

The existence of H69 subclones with a consistently high or low proportion of polySia immunoreactive cells, requires that, in these clones, this property of the cells was stable under the culture conditions used. PolySia expression is hence a clonable characteristic, and cannot therefore be related to a stage in the cell cycle.

Cells from clonal sublines E2 and F3 immunoreactive for polySia were maintained in serial culture for 18 months and during this time, showed little change in the proportion of cells immunoreactive for polySia. In conclusion, the present results demonstrate that stable clonal sublines from SCLC H69 cells can be established that all express cell surface NCAM but vary with respect to the presence of polySia.

PolySia on the cell surface affected the calcium-dependent aggregation of cells. This suggests that a calcium-independent mechanism, possibly mediated by NCAM, is involved in maintaining cell-cell contacts in aggregates, and is inhibited by polySia.

In the colony forming assay no measurable differences in adhesiveness to the substrates were detected between the polySia-positive and -negative cell lines or with the polySia-positive cells treated with endo N.

In the study of synthesis and dynamics of cell surface re-expression of polysialic acid of the neural cell adhesion molecule we demonstrated that, in F3 cells, synthesis of polySia takes place intracellularly in a membrane-bound compartment, most probably represented by the Golgi apparatus. It is likely that NCAM polysialylation is a complex process involving several enzyme activities. Our data suggest that all these enzyme activities are located in the Golgi apparatus.

4.2. Immunohistochemistry combined with tissue microarrays (TMAs) is an ideal approach for rapid screening and validation of preselected protein biomarkers in many tumor samples under standard conditions. The NSCLC groups corresponding to different stages of tumor progression (primary without distant metastasis, primary with

brain metastasis, and brain metastasis of the latter) were correlated in pairs for immunostaining profiles to find markers distinctly expressed in any of the groups and/or associated with patients' survival.

When comparing biomarker profiles of LC and LCMet groups, collagen XVII, CD44v6, and caspase 9 were significantly overexpressed, whereas β -catenin and cellular apoptosis susceptibility protein (CAS) expression was down-regulated in the LCMet group.

Among the 29 biomarkers, the loss and/or delocalization of cell membrane-linked β -catenin was the only significant univariate predictor of poor patients' survival.

However, nuclear translocation of β -catenin, a feature of elevated Wnt signaling was not seen in this study.

We found higher frequency of syndecan-1 expression in the BrMet group compared with any primary NSCLC group, suggesting a role for syndecan-1 in the metastatic process, but this did not have a significant prognostic impact. In groups of NSCLC we found that (1) the delocalization and loss of β -catenin is an univariate predictor of poor prognosis; (2) there are differentially expressed biomarkers associated with tumor progression including the upregulation of collagen XVII, CD44v6, caspase-9, and possibly those of cyclin D1 and cyclin D3, and the downregulation of β -catenin and CAS, however, most of them have no prognostic impact.

4.3. We have studied the effect of cisplatin-containing chemotherapy on the lung cancer tumor tissue comparing pre-chemotherapeutic bronchoscopic biopsies and postchemotherapeutic surgical resections. Our present work revealed differences between adenocarcinomas (ADC) and squamous cell carcinomas (SCC) regarding the changes in marker expression after chemotherapy. In SCC group pronounced changes were found regarding the post-treatment decrease of ERCC1 expression, however, in ADC tumors there was no change detectable.

Comparing the expression of certain apoptotic markers in different NSCLC histologic subtypes, there was no detectable association related either to SCC or ADC, however, the difference between the expression of ERCC1 in SCC and ADC tissues was remarkable

Seven of preoperative cases expressed increased level of Ki-67 after neoadjuvant treatment, while 3 of 17 cases indicated decreased expression of the same marker. The rest of tumors ($n=7$) showed no remarkable change in proliferation marker.

The results of the present study suggest that platinum-based chemotherapy may induce the selection of tumor cells with more aggressive phenotype as it was detectable in the increased expression of Ki-67, and also affects the expression of tissue markers that could have predictive value. This knowledge might be of importance when designing treatment protocols for NSCLC patients.

4.4. EGFR protein expression was successfully evaluated in 116 patients (success rate, 99%). Overexpression was found in 68 cases (59%). There were no significant differences in the rate of IHC positivity in patients of different age, gender, and smoking status. Only the semiquantitatively estimated protein expression using the IHC score showed significant association with the frequency of mutation. Although all gene amplification caused a very strong immunohistochemical membrane staining, statistically the protein expression was unrelated to gene copy number. The advantages of IHC analysis are that 1) it is the most cost-efficient and easiest method; 2) most *in vitro* diagnostic units can perform this test; and 3) this test is the most frequently positive (in our study 59%), therefore excluding the fewest patients from therapy. Most importantly, this study does not suggest that only patients with *EGFR* mutations should be exclusively selected for EGFR TKI therapy, but provides strong evidence that all patients with *EGFR* mutations with lung adenocarcinoma should be treated regardless of other biomarkers or smoking status.

5. Conclusions

5.1. *In vitro* and *in vivo* investigations of the human small cell lung cancer (SCLC-H69) cell line and its subclones demonstrated that the NCAM- α -2,8 linked polysialic acid reduces the cell to cell adhesion and increases the metastatic ability. The synthesis and intracellular transport of polysialic acid are associated with the Golgi complex: it is a *de novo* process in SCLC cells.

5.2. The results of the investigations of protein expression profile of primary and brain metastatic non-small cell lung cancer groups concluded that the β -catenin is the only marker which has correlation with the patients' survival.

5.3. The effect of platinum-based neoadjuvant chemotherapies on certain biomarkers, including apoptosis, cell proliferation, and DNA repair mechanisms, was also investigated. The low patients' number could not allow us to compile statistics, but the results suggest that platinum-based chemotherapy has no influence on low activity level apoptosis, and it induces a selection of tumor cells with increased proliferating activity. Therapy-induced change in the expression – in our cases the loss of expression - of tissue marker ERCC1 (excision repair cross-complementation group 1), which plays a role in DNA repair, may have a predictive value in therapeutic response.

5.4. The study comparing methods to detect different levels of EGFR expression provides evidence that tumor tissue groups positive to different EGFR diagnostics only partly overlap. Most importantly, lung cancers which do not overexpress EGFR protein, therefore present negative EGFR immunohistochemical reaction, can carry activating mutations and the patients can have excellent response to EGFR TKI therapy.

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