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Lung cancer epigenetics and genetics

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Lung cancer is the leading cause of cancer-related death and thus a major health problem. The efficiency of current treatment modalities for lung cancer depends strongly on the time of diagnosis, with better chances of survival if a tumor has been detected at an early stage. Thus, there is an urgent need for rapid and efficient early detection methods. Biomarkers represent a possible alternative to current, rather expensive, screening tools such as spiral computer tomography (CT), or may allow the identification of high risk groups for whom screening would be cost efficient. Although most lung cancers are the consequence of smoking, a substantial fraction of molecular-epidemiological studies point to high-prevalence, low-penetrance genetic polymorphisms as modifiers of environmental lung cancer risk. In the past the genomics field has also made significant advances in identifying genetic lesions that can now be harvested with the goal of identifying novel biomarkers for lung cancer. Furthermore, the importance of epigenetic changes that occur during lung cancer development has been reported, but has been underestimated in the past. Novel high-throughput, quantitative assays for the detection of DNA methylation or histone tail modifications are now applied, to search for alterations in the lung cancer genome and will identify novel cancer-related genes that may become attractive targets for treatment, provide new insight into the biology of lung cancers, and could also become useful biomarkers for the early detection of lung cancer in sputum, or may be used as prognostic markers. Thus, an integrative approach in lung cancer research combining epidemiological, genetic and epigenetic information becomes an important concept for the future.
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Lung cancer is the leading cause of cancer-related death in both males and females worldwide.[1,2] Only 13% of lung cancer patients survive more than 5 years. In 2007 estimates calculated 213,380 new cases of lung cancers (about 15% of all cancer cases) and 160,390 deaths (about 29% of all cancer deaths) [from: Surveillance Epidemiology and End Result (SEER) statistics http://seer.cancer.gov/]. Clinically, lung cancer can be divided into 2 groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).[1,2] Approximately 75% of lung tumors are NSCLC,[2] which includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma.[1] The incidence of adenocarcinoma appears to be increasing worldwide, possibly due to modern cigarettes that contain higher concentrations of certain carcinogens.[3] Previous studies have demonstrated that lung cancer development involves both environmental and genetic factors. Epidemiological studies indicate that cigarette smoking, as well as exposure to asbestos and radon, have a strong causal association with lung cancer.[4,5] About 80–90% of lung cancers are attributable to cigarette smoking.[6] An estimated 20% of all lung cancers are caused by a combination of environmental (*e.g.,* asbestos, radon) and/or genetic factors.[1] Although, the majority of lung cancer patients are smokers, only a minority of heavy smokers will develop this disease,[7,8] suggesting environmental or genetic determinants in disease initiation and progression. Since many carcinogenic compounds require metabolic activation to enable them to react with cellular macromolecules, inter-individual differences in carcinogen metabolism may play an essential role in the development of this environmental cancer.[9,10] Early detection of lung cancer could change disease outcome: however, current diagnostic tools are either too costly or not sensitive enough to allow early detection.[11,12] Hope comes from major technological advances in molecular oncology and promotion of integrative oncology that, for the first time, provide the unique opportunity to combine data from genomic, epigenomic and epidemiological screens. In this review, we will summarize our current knowledge in the field and discuss possible strategies for the future. Because of the availability of data the review will mainly focus on NSCLC and only occasionally highlight work and known differences in SCLC.

Lung cancer genetics/genomics

Molecular genetic studies have shown that multiple genetic loci contribute to sporadic lung cancers. The molecular abnormalities are found in both growth-promoting oncogenes and growth-suppressing tumor suppressor genes. While DNA amplification is an indication for the presence of an oncogene that was activated by increased copy number, loss of heterozygosity (LOH) is currently used as an indicator for the presence of a tumor suppressor gene locus. In lung adenocarcinomas, the oncogene *KRAS* is mutated in ~30% of cases.[13,14] *MYC*, *Cyclin D1* and *EGFR*[15] are amplified and over-expressed in 2.5–10%, 5% and 6% of NSCLC, respectively. *C-erbB2* (Her-2/neu) or *BCL2* over-expression are involved in ~25% of cases.[14] Systematic resequencing of oncogenes identified novel mutations in, for example, *BRAF,* present in about 2% of adenocarcinoma patients and restricted to tumors that did not show *KRAS* mutations.[16,17] More recently, mutations in the EGFR gene were detected, and the mutation status correlated with response to small molecule kinase inhibitors (*e.g.*, gefitinib or erlotinib).[18,19] A subset of NSCLC patients appears to express a transforming *EML4-ALK* fusion gene.[20] Novel candidate oncogenes have been identified either based on high resolution screens for copy-number changes (*e.g.*, IAP1/2) or in an approach to combine expression with genomic data.[21] Recent data suggest that oncogenic alterations of transcription factors involved in lung development may be a more common feature of lung cancer than previously realized.[22]

A number of chromosomal regions with LOH or homozygous deletions in lung cancer have been described but so far candidate tumor suppressor genes located in these regions have been identified only for a subset.[23] Tumor suppressor genes involved in lung cancer include *p53*, *p16* and *RB*. Additionally, the *FHIT* (*fragile histidine triad*) gene, located on 3p14.2, was cloned in 1996 by positional cloning.[24] Abnormal transcription of the *FHIT* gene was reported in 40% of NSCLC[25] and its function was related to proapoptotic signaling.[26] *DLC1* (deleted in lung cancer 1) was cloned through large-scale sequencing of 3p21.3 and was found to show aberrant transcripts in 3 of 30 (10%) cases or no transcription in 8 of 30 (27%) in primary NSCLC.[27] Recently, p34 was identified as another candidate tumor suppressor gene for lung cancer.[28] CYGB, previously implicated only in sporadic head and neck cancers, was recently also added to the list of candidate tumor suppressor genes involved in the pathogenesis of lung

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cancer.[29] Most likely due to tissue unavailability, much fewer studies have been conducted on SCLC than NSCLC. However, studies on cell lines indicate that SCLC and NSCLC frequently undergo different specific genetic alterations.[30]

Although previous studies investigated targeted regions in the genome, novel technology does now allow the interrogation of the whole genome.[31] As an example, in a recent study the 250K single nucleotide polymorphism (SNP) array from Affymetrix was used to evaluate copy number changes in 371 adenocarcinomas of the lung. This approach allows high resolution and the identification of even small sized copy number changes. Surprisingly, this approach identified 57 recurrent alterations, many of which had not previously been discovered, and now candidate lung cancer genes have been identified in these regions. For example, a common amplification of chromosome 14q13.3, which was found in about 12% of the samples, identified a novel proto-oncogene (*NK2 homeobox 1* or *NKX2-1*) located in the commonly amplified region.

Lung cancer molecular epidemiology/susceptibility

The familial relative risk ratio (RR) of relatives of lung cancer patients has been estimated from several registry-based studies and calculated to be RR$\sim$2.00, very similar to what has been reported for breast (RR = 2.02) or prostate cancer (RR = 1.89).[32–34] The elevated familial risk can not only be explained by shared environmental factors, as a meta-analysis reported a 1.5-fold elevated risk of lung cancer among never smoking probands with affected first degree relatives.[35] Linkage analysis of 52 high risk pedigrees localized a lung cancer susceptibility locus at chromosome 6q23-25.[36] This region contains many potential genes of interest, including *SASH1, LATS1, IGF2R, PARK2* and *TCF21*[36,37]; however, the exact inheritance mechanisms of lung cancer remain to be elucidated. *P34*, though a recently identified candidate tumor suppressor gene at this locus, does not appear to be the candidate familial lung cancer susceptibility gene.[28]

While the exposure to tobacco carcinogens is known to be the major risk factor for lung cancer, only a small proportion of smokers develop the disease.[7,8] Aside from recent family studies, association studies have thus been carried out in order to identify the genes involved in modifying lung cancer risk. These studies have focused on genes that would influence lung cancer risk as a result of gene-environment interaction, and genotyping analyses on lung cancer were first carried out on xenobiotic metabolising enzymes with known genetic polymorphisms involved in the metabolism of environmental- or tobacco carcinogens, and on DNA repair enzymes, involved in DNA repair resulting from endogenous and exogenous mutagens.[38] The list of investigated genes includes: *N*-acetyltransferases (*NAT1, NAT2*), Cytochrome P450 dependant monooxygenases *CYP1A1*, *CYP1A2*, *CYP1B1*,[9] Glutathione-S-transferases *GSTM1*, *GSTT1*, *GSTP1*, myeloperoxidase, transporter genes such as *MDR1*, DNA repair genes *XRCC1* and *XPD* and the human 8-hydroxyguanine-specific DNA glycosylase *hOGG1*, and, more recently, cell cycle regulation genes (reviewed in Ref. [10]). Genes with potential impact on smoking behavior, such as *CYP2A6* have also been studied.[39] These candidate gene approaches frequently employed the following criteria for selection of genetic polymorphisms to investigate (*i*) biological plausibility of risk modification by this enzyme; (*ii*) known (*e.g.*, by activity measurements) or suspected (*e.g.*, amino acid substitution in active site) phenotypic relevance of the genetic polymorphism; as well as c) medium to high frequency of polymorphism in Caucasian population (to ensure public health relevance of results).

Results from such case-control studies have been inconsistent, likely due to heterogeneity of study populations, failure to consider effect modifiers such as environmental exposures (gene-environment interaction), lack of statistical power causing false negatives and multiple testing creating false-positive results, as well as publication bias. Increasingly, meta-analyses or pooled analyses are being employed to determine the association of certain SNPs with cancer risk.

Glutathione-S-transferase M1 (GSTM1) conjugates known carcinogens such as epoxides of polycyclic aromatic hydrocarbons. The frequent homozygous variant 'null' genotype has in large meta-analyses been associated with a small but significant risk of lung cancer.[40] A recent study also provided evidence of a GSTM1 and radon interaction in risk of lung cancer.[41] The glutathione *S*-transferase theta 1 gene (*GSTT1*) is a particularly attractive candidate for lung cancer susceptibility because of its involvement in the metabolism of polycyclic aromatic hydrocarbons found in tobacco smoke and of other chemicals, pesticides, and industrial solvents. In a meta- and pooled analysis no significant interaction was observed between *GSTT1* (null- or non-null genotype) and smoking on lung cancer risk, whereas *GSTT1* genotype appeared to modulate the occupational-related lung cancer risk.[42] Furthermore, the NAD(P)H:quinone oxidoreductase 1 (NQO1) Pro187Ser variant allele was associated with a 22–30% decrease in lung cancer risk among Asians, whereas for *myeloperoxidase* (*MPO*) the G-463A polymorphism was associated with a 30% decreased risk of lung cancer in Caucasians.[43] The latter results were confirmed by a pooled analysis of 10 studies.[44] A meta-analysis of *epoxide hydrolase 1* (*EPHX1*) polymorphisms and lung cancer risk associated low activity variant alleles with decreased risk of lung cancer.[45]

A meta-analysis of repair gene polymorphisms found an increased risk of lung cancer for carriers of the OGG1 326 Cys/Cys genotype,[46] which is consistent with experimental evidence that this isoform exhibits decreased base excision repair activity.[47,48] Another study found statistically significant associations between *XPD*/*ERCC2* SNPs in codons 312 and 751 and lung cancer.[49]

For other SNPs more recently investigated there are no meta- or pooled analyses available as yet. Polymorphisms resulting in lower dopamine bioavailability have been associated with increased risk of NSCLC.[50] Common genetic variation in TP53 could modulate lung cancer pathways, as suggested by the association with lung cancer in African Americans and somatic TP53 mutation frequency in lung tumors.[51] Genes involved in metabolism of nicotine and other genes with potential effects on addiction are also very interesting targets for association studies.[52] CYP2A6 metabolizes a variety of procarcinogens but also catalyzes nicotine-C-oxidation, leading to cotinine formation. A number of polymorphisms have been identified for *CYP2A6*, including deletion polymorphisms (*CYP2A6*4*); however, the low allele frequencies as well as technical genotyping difficulties for *CYP2A6*[53] have thus far prevented case-control studies with the appropriate power.

The effect of a single common sequence variant might not be detectable in population association studies; the combination of multiple polymorphisms in the same gene and in genes belonging to the same biological pathway might nevertheless be important in carcinogenesis.[54] Evidence for gene–gene interactions in lung carcinogenesis comes from a pooled analysis investigating *CYP1A1*, *GSTM1* and *GSTT1*. A cumulative effect of the combination of the *a priori* 'at-risk' alleles for these genes (p for trend 0.004) was observed. The risk of lung cancer was increased with the combination of *CYP1A1*2B* or *CYP1A1*4* alleles and the double deletion of both *GSTM1* and *GSTT1* up to an odds ratio (OR) of 8.25 (95% confidence interval 2.29–29.77) for the combination including *CYP1A1*4*.[55] A strategy taking into account the pattern of linkage disequilibrium across a gene, using tag-SNPs to cover common sequence variation may shed further light on lung cancer susceptibility affecting polymorphisms: however, this approach is not effective for rare markers, and can therefore only supplement the strategy of specifically targeting rarer SNPs that are likely to alter gene function.[56]

In conclusion, there is substantial evidence that genetic polymorphisms in certain metabolic and DNA-repair related genes modulate lung cancer risk, albeit with low-penetrance. However, while many of the candidate gene studies have been labor intensive, novel SNP array-based technology now allows whole genome association (WGA) studies evaluating over 500,000 SNPs. This approach has been successful in breast[57–59] or prostate cancer[60–63] but not yet with this SNP density for lung cancer. Dense

SNP maps have been used to screen mouse models for lung cancer.[64] For human lung cancer one genome-wide SNP analysis using DNA pools has been published involving 100,000 SNPs. It identified a polymorphism relevant to splicing of the *KLF6* gene as a possible modifier of lung cancer risk; however, without a positive replication.[65] Other genome-wide SNP analyses in lung cancer have used arrays with 1,500 SNPs in cell lines[66] and on human tumor samples[67] to conduct first genome-wide LOH/copy number analyses and arrays with 10,000 SNPs to investigate drug resistance.[68]

Lung cancer epigenetics

DNA methylation in the promoter sequence of genes has been shown to be part of the silencing mechanism of tumor suppressor genes in human lung cancers. This epigenetic modification acts in cooperation with histone tail modifications and has the ability to alter the chromatin condensation status.[69] While an open form of chromatin allows active transcription a closed and condensed form does not. It is now recognized that not only genetic mechanisms, such as gross chromosomal alterations or single nucleotide mutations, but also aberrant DNA methylation provides one or both of the two hits postulated in Knudson's two hit hypothesis for the inactivation of tumor suppressor genes. A number of aberrantly methylated genes have been identified in lung cancer. Furthermore, methylation has been described as an early event in lung tumorigenesis. Most of the genes were identified based on the candidate gene approach. Protein complexes containing methyl CpG-binding proteins and histone deacetylases as major components are able to bind to methylated promoters and induce the deacetylation of histones, which mediates the formation of transcription-repressing chromatin.[70–73] In *in vitro* experiments, re-expression could be achieved by adding 5-aza-2′-deoxycytidine, a DNA methyltransferase inhibitor, and/or trichostatin A (TSA), a specific inhibitor of histone deacetylases.[74] There is now ample evidence from the literature that describes the involvement of promoter hypermethylation in lung cancer. A well-studied example in lung cancer is the aberrant promoter methylation of the tumor suppressor gene, *p16*, which correlates with gene silencing and is an early event in tumorigenesis.[75–77] Additional examples include *H-cadherin*,[78] *death-associated protein (DAP) kinase* 1 (*DAPK1*),[79] *14-3-3 sigma*[80] and the candidate tumor suppressor gene *RASSF1A*.[81] Since most of the reports describe methylation in single cancer genes, no measurement for the overall contribution of promoter methylation to lung carcinogenesis exists. As an initial step to address this question, Zochbauer-Muller *et al*. showed that numerous genes, including *retinoic acid receptor β-2* (*RARβ*), *tissue inhibitor of metalloproteinase 3* (*TIMP3*), *p16*, *O^6-methylguanine-DNA-methyltransferase* (*MGMT*), *DAPK1*, *E-cadherin* (*ECAD*), *p14ARF* and *glutathione S-transferase P1* (*GSTP1*), were methylated at various degrees in a collection of 107 primary nonsmall cell lung cancers.[75] The reports that describe DNA methylation events as diagnostic markers for certain tumor types or stages indicate the potential of DNA methylation as a molecular marker. For example, *p16* promoter methylation is proposed as a biomarker for early detection of lung cancer and monitoring of prevention trials.[82,83] Using sensitive PCR-based methylation analysis, methylation in *p16* and/or *MGMT* promoters was found in sputum of smokers, up to 3 years prior to clinical diagnosis of squamous cell lung carcinoma.[84] A variety of epigenetic biomarkers are being investigated for lung cancer detection in cytologically negative sputum[85] or plasma.[86]

Gene inactivation by promoter methylation is not only found in 'classical' tumor suppressor genes with normal functions in cell proliferation. For example, silencing of *RASSF1A*,[81] *FHIT*,[87] *RIZ1*,[88] *FUS1*[89] and *SEMA3B*[90] and *C/EBPα*[91] has been reported in lung tumors but tumor suppressor function is still under consideration. It might be possible that promoter methylation silences genes with weak or no tumor suppressing activity, including target genes with other cellular functions such as DNA repair or drug metabolism as in the case of *MLH1*, *MGMT* and *GSTP1*. However, silencing of these genes, or groups of these genes, would contribute to the overall malignant phenotype.

We and others have recently completed a genome-wide scan for aberrant promoter methylation and have identified novel targets for methylation indicating that the contribution of methylation events in lung tumorigenesis was previously underestimated. In these studies, aberrant DNA methylation was detected either by direct approaches or by indirectly utilizing the drugs that help to reactivate epigenetically silenced genes in lung cancer cell lines. Rauch *et al*. used tiling arrays in combination with a novel assay that enriches methylated CpG island sequences for lung cancer cell line DNAs to screen for novel target genes that become epigenetically silenced[92,93] Surprisingly, half of all CpG islands associated with homeobox genes were found methylated in the lung cancer cell line A549. Furthermore, this study identified *HOXA7* and *HOXA9* as frequent targets in primary stage 1 lung cancers. Shames *et al*. used a genome-wide screen for aberrantly-methylated genes utilizing the ability of 5-aza-2′-deoxycytidine to reactivate gene expression of silenced genes in lung cancer cell lines. Expression profiles of cancer cell lines were established before and after reactivation treatment. This screen identified 132 novel genes that are targets for aberrant DNA methylation in lung cancer.[94] Similarly Zhong *et al*. identified novel epigenetically silenced genes using pharmacological inhibition of DNA methylation and histone tail modifications.[95] Brena *et al*. utilized a direct method to scan lung cancer genomes for promoter methylation. Restriction Landmark Genomic Scanning (RLGS), an assay that evaluates the DNA methylation status of thousands of *Not*I or *Asc*I restriction sites, preferentially located in CpG island sequences was used in primary NSCLC tumor samples and matching normal controls.[96] An average of 3,442 promoter sequences was evaluated in 40 lung cancer samples and a total of 395 RLGS sequences were identified that were methylated in at least one of the tumor samples. Most importantly, this study determined that about 4.8% of all CpG island promoters in a lung cancer genome are targeted for aberrant DNA methylation. Considering 29,000 CpG islands in the human genome, this number would indicate that 1,400 CpG islands in the lung cancer genome could be aberrantly methylated. Protein expression of one of the target genes, *OLIG1*, identified in this study correlated significantly with survival in lung cancer patients. These genome-wide searches for aberrant DNA methylation in lung cancer now provide a wealth of information and have also identified novel candidate cancer genes that await further investigation.

There is now good evidence that aberrant DNA methylation could serve as a marker for the early detection of lung cancer in sputum.[97] Palmisano *et al*. demonstrated that promoter methylation of *p16* or *MGMT* predicted the development of squamous cell carcinoma even 3 years before clinical diagnosis[84] and there are now efforts underway to develop standards that will foster the clinical application of DNA methylation marker not only for detection of lung cancer.[98]

Future

Lung cancer prevention remains an important goal—recently some progress has been made by the introduction of smoking bans in public places throughout Europe. However, the reduction of smoking rates in men that occurred in the late 1960s through the 1980s continues to drive down mortality rates for men but rates in women have not yet begun to decrease.[5] Efforts at chemoprevention of lung cancer have thus far not been successful, in 3 randomized, double-blind, placebo-controlled chemoprevention trials during the 1990s.[99,100] In fact beta-carotene supplementation was associated with increased risk for lung cancer among the high-risk populations in two of these studies.[100,101] However, the prognosis for lung cancer patients is considerably better if the tumor is diagnosed early. Improvement of early lung cancer detection technolo-

gies thus represents an important goal for the scientific community. The advances made in the field of lung cancer epigenetics are one promising step in the direction of biomarker screening studies. Additionally, the most cost-effective way of applying any screening methods will be to optimize the identification of high risk groups. The recent advent of affordable genome-wide SNP genotyping will make for a quantum leap in the area of lung cancer association studies. A number of genome-wide association studies are expected shortly to be forthcoming. Existing case-control studies have formed a worldwide consortium to allow the study of subgroups, lower frequency SNPs,[56] and for fine mapping of the results from the whole genome association studies. Additionally, the study of copy number variants holds some promise for future efforts towards the identification of high-risk groups for lung cancer.[31,102]

Potential future areas for research will be those of Epi-epidemiology, *i.e.*, the application of Epigenetics in Epidemiology, and epigenetic markers for individualized treatment. There is great interest in how epigenetic modifications affect both cancer susceptibility and prognosis. Recently it has been shown that heritable germline epigenetic mutations can predispose to cancer.[103,104] In the search for epigenetic markers (*i*) of lung cancer susceptibility and (*ii*) as potential predictive prognostic markers the following questions are of interest: Can DNA methylation patterns as found in plasma samples provide information on tumor specific methylation patterns (free tumor DNA)? Are gDNA methylation patterns useful to assess lung cancer susceptibility or prognosis? If so, do the markers track heritable epimutations or acquired changes in methylation pattern (*e.g.*, as a result of age or as a result of environmental influences such as smoking)?

Thus, integrative oncology becomes an important concept for the future of lung cancer-related research (see Fig. 1). This concept will necessitate improvements in study design and technology, allowing the integration of genetics, epigenetics, and epidemiology in order to identify candidate gene loci with relevance to risk assessment and clinical parameters such as histology and treatment outcome. Important future challenges in this context include the better characterization of gene-environment interactions, and epigenetic influences in carcinogenesis. The identification of high-risk groups is of great importance for preventive measures such as the setting of exposure threshold values, public health campaigns and chemopreventive approaches. Overall, integrative oncology promises the potentially huge reward of individualized treatment of lung cancer patients.

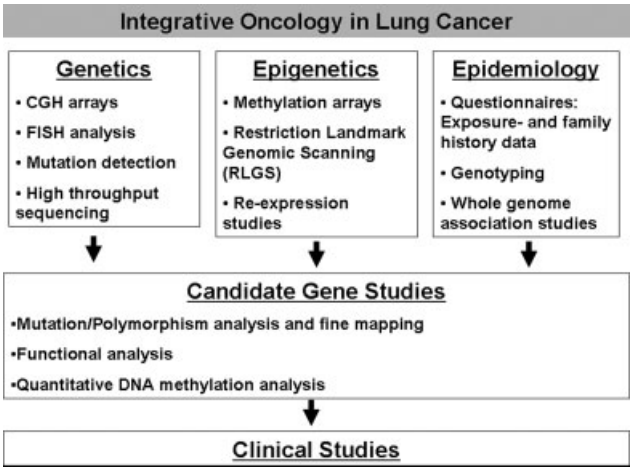

Figure 1 – The integrative oncology approach. Employing genetics, epigenetics and epidemiology in an integrative oncology approach will improve our understanding of lung carcinogenesis and make it possible to identify candidate gene loci for further studies. The best of these can then be used in clinical studies.

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Note Added in Proof

Three genome-wide association studies investigating 371K SNPs have just identified variation on the long arm of chromosome 15 (15q24/15q25.1), a region encoding subunits of acetylcholine receptors, as associated with lung cancer risk, however, it is not clear whether this link is direct or mediated through nicotine depence.[105–7]

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