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Methylation Analyses at Lung Cancer Susceptibility Loci in Tumor and Germline Tissues

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Lung cancer is the deadliest cancer worldwide and the main risk factor for lung cancer is smoking. The fact that only 11% of the smokers develop lung cancer indicates that there are inter-individual genetic differences which predispose to lung cancer. A limited number of major lung cancer susceptibility loci have reproducibly been detected in case-control studies, however, genetic risk factors identified so far only account for a fraction of lung cancers, there is thus a considerable component of missing heritability. Additionally, the functional variants and the underlying mechanisms have yet to be identified for those genetic risk loci that have been identified. Aberrant DNA methylation is a hallmark of cancer. Thus, a hypothesis that genes harboring SNPs significant for an elevated risk for lung cancer risk might be epigenetically deregulated in the affected tissue was developed.

Quantitative DNA methylation analysis using MassARRAY (Sequenom) of all CpG islands in the major lung cancer risk loci 15q25.1, 5p15.33, and 6p21.33 was conducted in tumor/normal tissue pairs. Further, 45 loci, significant for early-onset lung cancer susceptibility, were selected based on the presence of proximal CpG islands and analyzed. Methylation screening of promoter CpG islands was performed in 34 sample pairs, and an independent sample set of 48 samples was used to validate any aberrant methylation observed. The genes showing significant differential methylation patterns were tested for changed expression patterns using realtime-PCR. Genes that were epigenetically deregulated in tissue were tested for reactivation of expression *in vitro* after treating lung cancer cell lines H1299 and A549 with 5-Aza-2´-deoxycytidine. Genotypes for tagging SNPs in the epigenetically affected regions were determined using the Typer assay platform of Sequenom, again in test set and an independent replication set. To explore the interdependencies between genetic variants and methylation patterns of the target genes deriving from overall lung cancer, a non-parametric ANOVA test (Kruskal-Wallis) between amplicon-wise methylation and SNP genotypes was applied for two datasets. For *CHRNB4* a knockdown using siRNAs (Dharmacon) was conducted in H1299 and A549 cells.

The *CHRNB4* promoter CpG island is hypomethylated and strongly upregulated in lung tumor tissue. Another candidate on 15q25.1 apparently playing a role in lung tumorigenesis is *CHRNA3*, which was previously published to be hypermethylated and downregulated, a result that was confirmed in this study. On 5p15.33 the *TERT* promoter was most significantly hypermethylated, this was associated with upregulation confirming previously published observations. These results were in concordance with results obtained in cell lines. For four SNPs known to be associated with lung cancer risk and smoking behaviour, a strongly significant association between SNP genotypes and methylation patterns in the promoter of *CHRNB4* was observed both in the screening and validation set of lung tumors. Analyzing four samples a segregation of methylation on the two alleles of SNP situated in the promoter could be detected. Functional knockdown of *CHRNB4* lead to a reduced proliferation and colony formation underscoring the oncogenic potential of the gene.

The analysis in the early onset candidate genes identified four novel genes for epigenetic deregulation namely, *AGTR1*, *ESRRG*, *GAS2* and *KANK3*. Evidence supporting causal epigenetic dysregulation of the candidate genes includes repeatability of DNA methylation results in two independent sample sets comparing tumor and adjacent normal tissues and expression analyses at least in part in concordance with methylation data. Other results e.g. partially inconclusive results for *ESRRG* after decitabine indicate the involvement of other regulatory mechanisms e.g. chromatin modifications. Results for the correlation between genotypes and CpG-wise DNA methylation levels in lung tumors were found to be significant in a screening dataset but could not be validated in a replication analysis, which might be due to heterogeneity of populations or due to the type of analysis. After crude analysis the *GAS2* gene seems to be the most promising gene for genotype-specific DNA methylation. Overall, the candidates from the early onset analyses might still have a big impact and future investigations might involve those genes for functional analyses.

As a conclusion and outlook the present results underscore the importance of analysing multiple regulatory layers in the genome. The main challenge of the future will be the functional follow up of targets identified in genome-wide studies. Further, new and innovative statistical models have to be invented to cope with the vast amount of data that is obtained owing to the rapid progress of new technologies. Future studies taking into account several parameters relevant for gene regulation will, in the end, lead to a more complete picture of gene regulation during carcinogenesis.