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Identification of *NHLRC1* as Novel Oncogene form a Lung Cancer Epigenome-Wide Association Study

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Lung cancer is the most prevalent cancer worldwide and patients diagnosed with lung cancer have a poor survival probability. This is because most early stage lung cancers stay asymptomatic and thus are diagnosed at late stages of the disease. The vast majority of lung cancers can be attributed to tobacco smoking, so smokers are at the highest risk of developing lung cancer due to their life style. Epigenetic modifications bear a lot of hope to serve as disease biomarkers to associate disease risk to environmental exposures. The most stable epigenetic trait is DNA methylation which is therefore heavily investigated in long-term preserved samples from prospective population-based studies. This study is one of the first to investigate DNA methylation in blood from healthy smokers to assess lung cancer risk and to investigate the function of differential methylation at these sites in lung tumors.

In this thesis, a nested case-control study within EPIC Heidelberg was designed, and genome-wide Illumina 450K Bead chip array DNA methylation data was generated and analysed. The top smoking-dependent methylation alterations in blood previously reported in the literature were published to be associated with lung cancer risk in collaboration with an international multi-centre lung cancer EWAS consortium.

This thesis specifically aimed to investigate the top hits from the EPIC HD lung cancer EWAS sample set for their functional role in lung tumor tissue. Therefore, top differentially methylated CpGs were compared to publically available 450K data for lung tumor versus adjacent normal tissue from TCGA. Strikingly, the top differentially methylated CpGs from a comparison of blood samples from EPIC HD cases versus controls were also differentially methylated in a TCGA comparison of lung tumor versus adjacent normal tissue samples. Additionally, region-specific methylation specific mass spectrometry assays (MassARRAY, Agena Bioscience) were designed to analyse lung tumor versus adjacent normal validation sets. For the top hypermethylated positions upstream of *NHLRC1* and *ACTC1* these assays confirmed the statistically significant differential methylation of the entire regions tested. Additionally, the differential methylation was associated with differential gene expression in

the validation set by quantitative real-time PCR. The *NHLRC1* upstream region was confirmed to be a DNA methylation dependent regulatory region for *NHLRC1* expression.

Functional assays in lung cancer cell lines A549 and H1299 revealed *NHLRC1* to be a novel putative oncogene since its siRNA-mediated knock down lead to decreased proliferation, viability, migration, and invasion of cancer cells. Mechanistically, this was explained by the observed reduction of AKT phosphorylation at Serine 473 which is specifically set by the mTOR complex 2 constituent RICTOR. This suggests that *NHLRC1* has a previously unknown role in the activation of AKT through the mTOR pathway. Reduction of NHLRC1 accompanied by loss of AKT phosphorylation was also associated with the FOXO transcription factor regulated re-expression of tumor suppressor genes and repression of oncogenic cell cycle driving genes in lung cancer cell lines. This shows the immediate dependence of lung cancer cells on *NHLRC1*. Concordant with the reduction of AKT phosphorylation in *NHLRC1* knock down experiments, *NHLRC1* overexpression enhanced AKT phosphorylation. This effect was still dependent on upstream PI3K signalling which indicates that this is dependent on activated mTOR. Thus, *NHLRC1* may regulate an inhibitor of the mTOR complex2 through its ubiquitin E3 ligase activity.

In conclusion, to our knowledge this is the first study to show that DNA methylation alterations identified from a lung cancer EWAS can uncover novel oncogenic mechanisms of epigenetically regulated genes in tumor cells. This is evidence that DNA methylation alterations in blood of healthy individuals who develop lung cancer are not random, but occur at gene regulatory sites. Additionally, the molecular oncogenic functions of the *NHLRC1* gene have been characterized for the first time.