

# Investigation of Early Detection Biomarkers for Lung Cancer in Plasma Using Quantitative High-Throughput DNA Methylation Analysis

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## Introduction

Lung cancer is a major oncological cause of death worldwide and has become a global public health burden, further substantiating the need for early diagnosis, risk assessment, more effective targeted therapies, and prognosis. The key to accomplishing these goals is a better understanding of genes and pathways disrupted during the initiation and progression of this disease. DNA hypermethylation is recognized as an important mechanism for tumor suppressor gene inactivation in cancer and could yield powerful biomarkers in blood for early diagnosis.

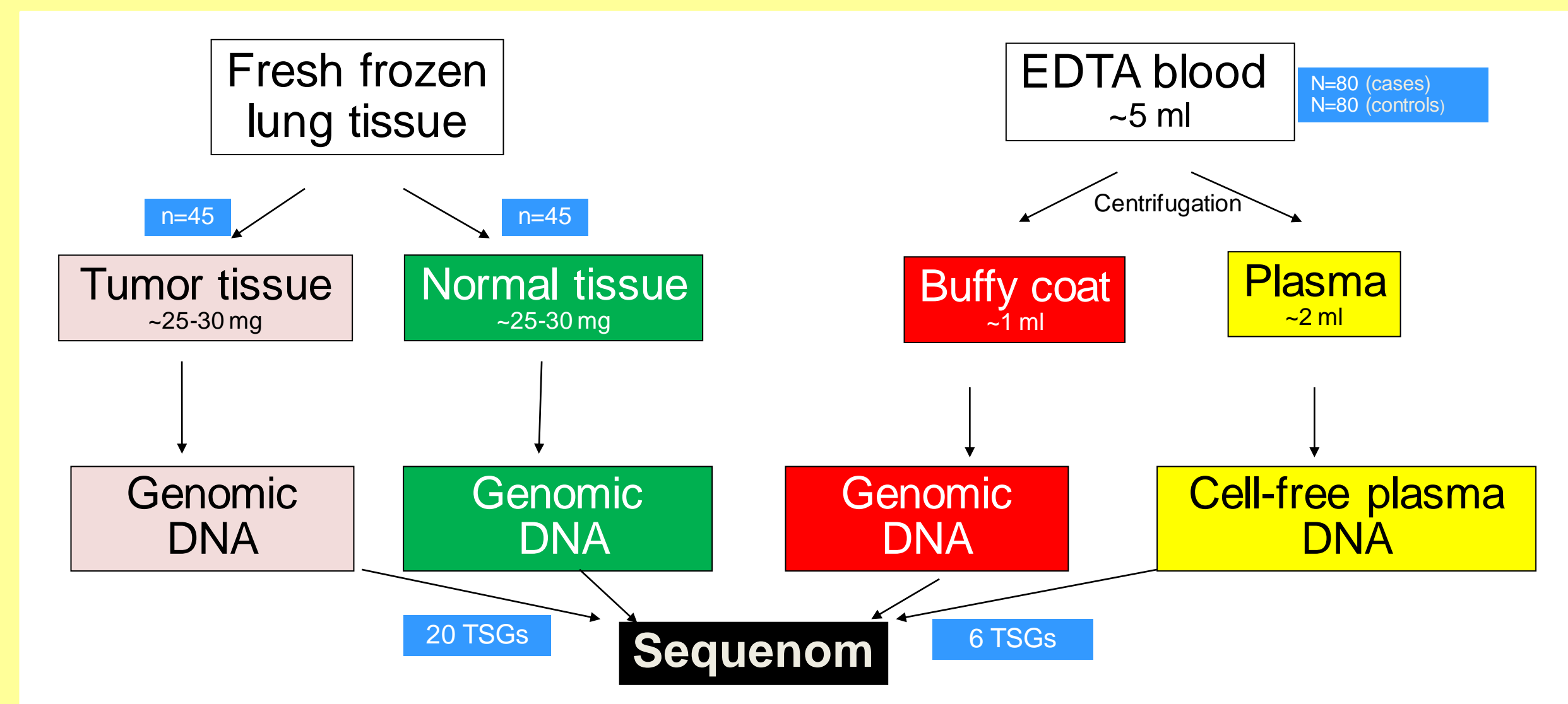


Fig. 1 Project paradigm

Here we aim to develop DNA methylation markers to facilitate early detection, and prediction of response to therapy and prognosis of patients with lung adenocarcinoma or squamous cell carcinoma. This study uses a candidate gene approach focusing on 20 genes frequently hypermethylated in lung cancer tissue vs. corresponding normal tissue. Based on initial methylation analysis in lung tumor vs. normal tissue, here we test for aberrant promoter methylation of a panel of 6 out of 20 tumor suppressor genes in cell-free plasma DNA from 80 lung cancer patients and 80 cancer-free individuals with available demographic, clinical and survival data.

## Materials and methods

Using sensitive, quantitative high-throughput DNA methylation analysis (MassARRAY, Sequenom), we examined the methylation profile of 20 previously published TSGs genes in a collection of 45 lung cancer tissue samples (T) and adjacent normal lung (N) tissue from the same patients. For 6 TSGs methylation analysis was also performed in a collection of 160 lung cancer and cancer-free individuals' plasma samples.

### Sources of genomic DNA

Genomic DNAs from fresh-frozen cancer as well as adjacent normal tissues were isolated using **DNeasy Blood and Tissue Kit** (Qiagen, Germany). Plasma DNA were extracted from cases and controls using **QiAamp Blood Midi Kit** (Qiagen).

### Quantitative Methylation Analysis

To analyze methylation status of promoter regions of the 21 selected genes, quantitative high-throughput DNA methylation analysis was performed using the MassARRAY system from Sequenom as previously described (Fig.2).

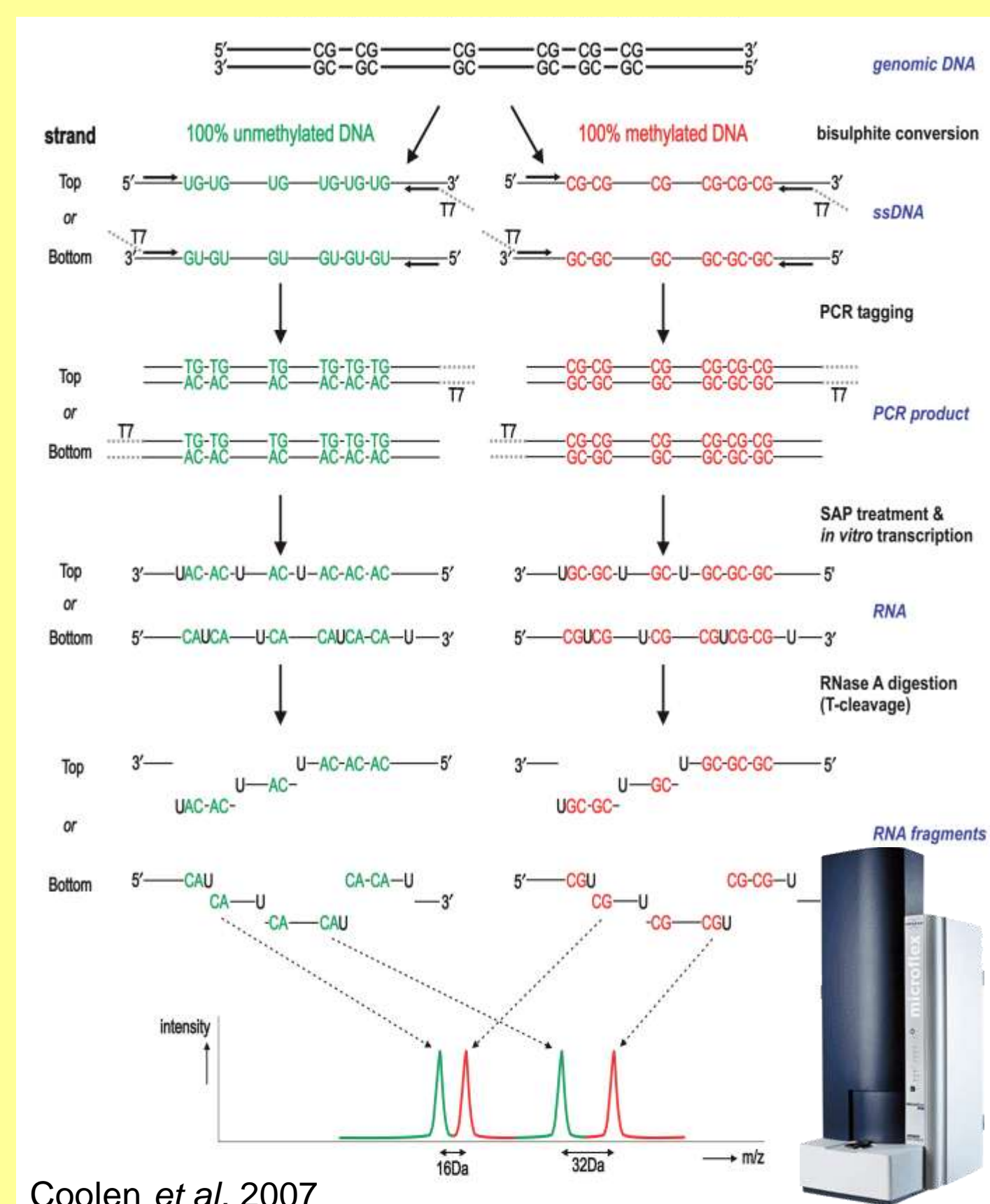


Fig. 2 DNA methylation analysis using base-specific cleavage and MALDI-TOF MS

**Step1. Bisulfite treatment** of genomic DNAs using the EZ-96 DNA methylation kit (Zymo Research).

**Step2. Amplification and introduction of T7-promoter tag** through PCR using Sequenom MassARRAY kit.

**Step3. Shrimp alkaline phosphatase (SAP) treatment** of PCR

**Step4. In vitro transcription** and RNase A cleavage base specifically (U or C) using Sequenom MassARRAY kit.

**Step5. MALDI-TOF-MS:** The G/A variations result appear in a mass shift of 16Da per CpG site (mass difference between G and A), which is detected by the matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

## Results

Initial methylation analysis in 45 T-N pairs of 25 different amplicons of 20 TSGs revealed 14 different genes to be differentially methylated in tumor tissue vs. adjacent normal lung (Fig.3). 12 TSGs showed hypermethylation in T, among those 9 – statistically significant. The overall quantitative estimate of DNA methylation levels in T vs. N was:

35% vs. 15% for <b>TCF21</b> (p<0.000)	7.8% vs. 3.8% for <b>CHFR</b> (p=0.006)
27% vs. 12.6% for <b>RASSF1A</b> (p<0.000)	10.4% vs. 2.5% for <b>p16<sub>INK4a</sub></b> (p=0.049)
18.4% vs. 9.3% for <b>OPCML</b> (p<0.000)	9.5% vs. 5.2% for <b>RUNX3</b> (p=0.67)
16.9% vs. 8.6% for <b>DLEC1</b> (p=0.03)	13.0% vs. 7.9% for <b>Pax5</b> (p=0.156)
7.7% vs. 3.1% for <b>OLIG1</b> (p=0.006)	10.3% vs. 7.5% for <b>TIMP3</b> (p=0.008)
19.2% vs. 13.8% for <b>MGMT</b> (p=0.004)	43.2% vs. 15.7% for <b>TERT</b> (p<0.000)

## Results

Interestingly, this is also the first report of highly significant hypomethylation of **14-3-3sigma** and **BLU** in lung tumor tissue. The overall quantitative estimate of DNA methylation level in T vs. N was 59.4% vs. 84.3% for **14-3-3sigma** (p<0.000) and 11.7% vs. 17.3% for **BLU** (p<0.000). For **RASSF1A** and **TCF21** genes the average methylation level in plasma DNAs was significantly higher in Ca compared with Co (p=0.0057 and p=0.034 respectively), and for **14-3-3sigma** the average methylation level in plasma DNAs is significantly lower in Ca compared with Co (p=0.0076) (Fig.4). Also, for **RASSF1A** and **p16** methylation in tumor tissue positively correlates with DNA methylation in plasma (r<sup>2</sup>=0.25, p=0.0054 and r<sup>2</sup>=0.22, p=0.0091 respectively) (Fig.5).

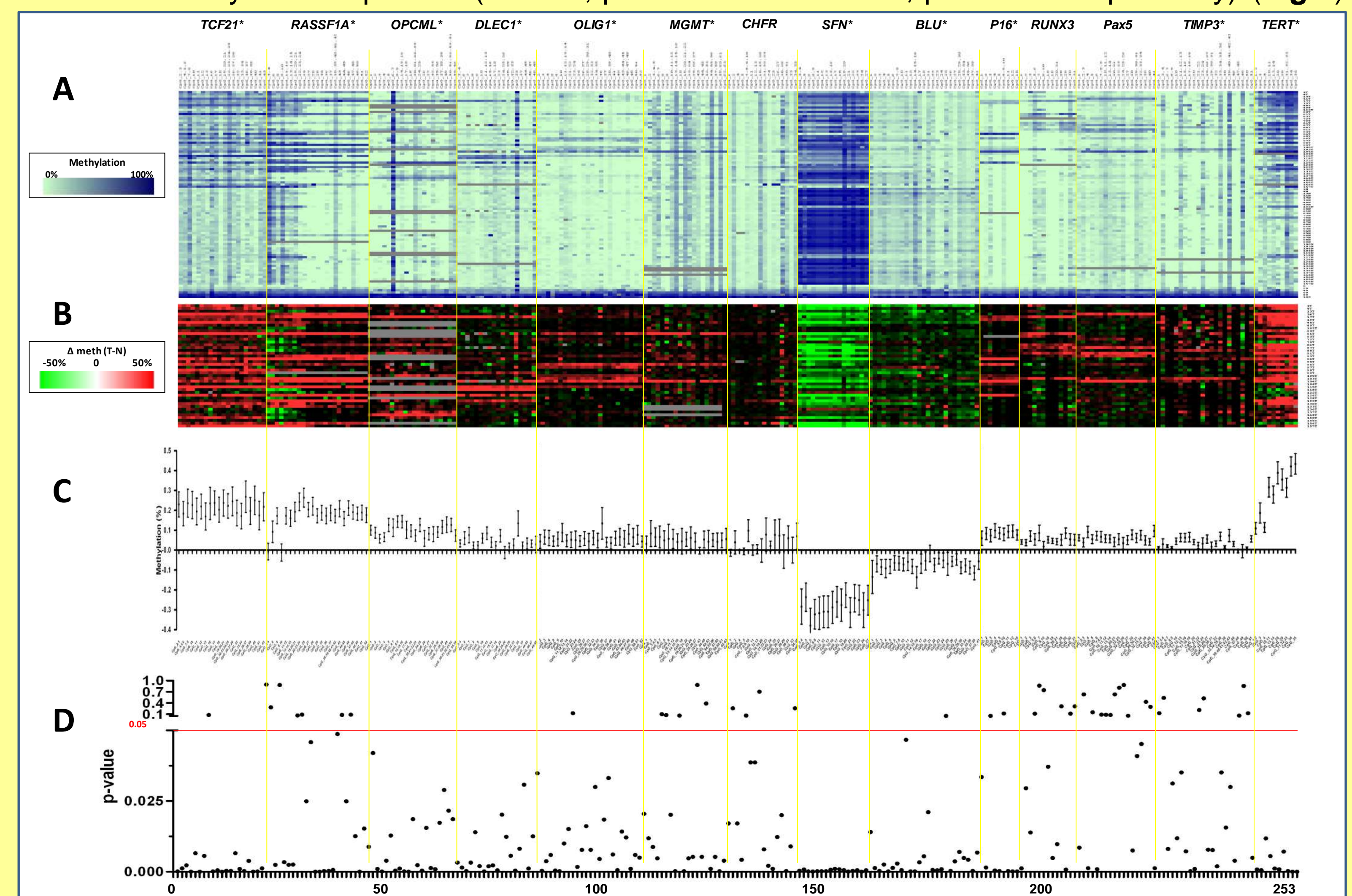


Fig.3. Methylation analysis for the 14 amplicons of 20 genes that were differentially methylated in the initial screen of T and N. The figure shows methylation across each amplicon for 45 T/N matched pairs. (A) Heatmap of methylation data of 45 T and N. Bright green indicates 0% methylation, dark blue indicates 100% methylation (B) The heatmap for 45 lung cancer patients indicates the differences in DNA methylation levels between T and N in single CpG unit resolution. Green indicates hypomethylation, red indicates hypermethylation. (C) Median and 95% confidence interval of methylation difference between T and N in single CpG resolution. (D) The significance of the differences observed between T and N in single CpG resolution (cut-off depicted is p-value 0.05).

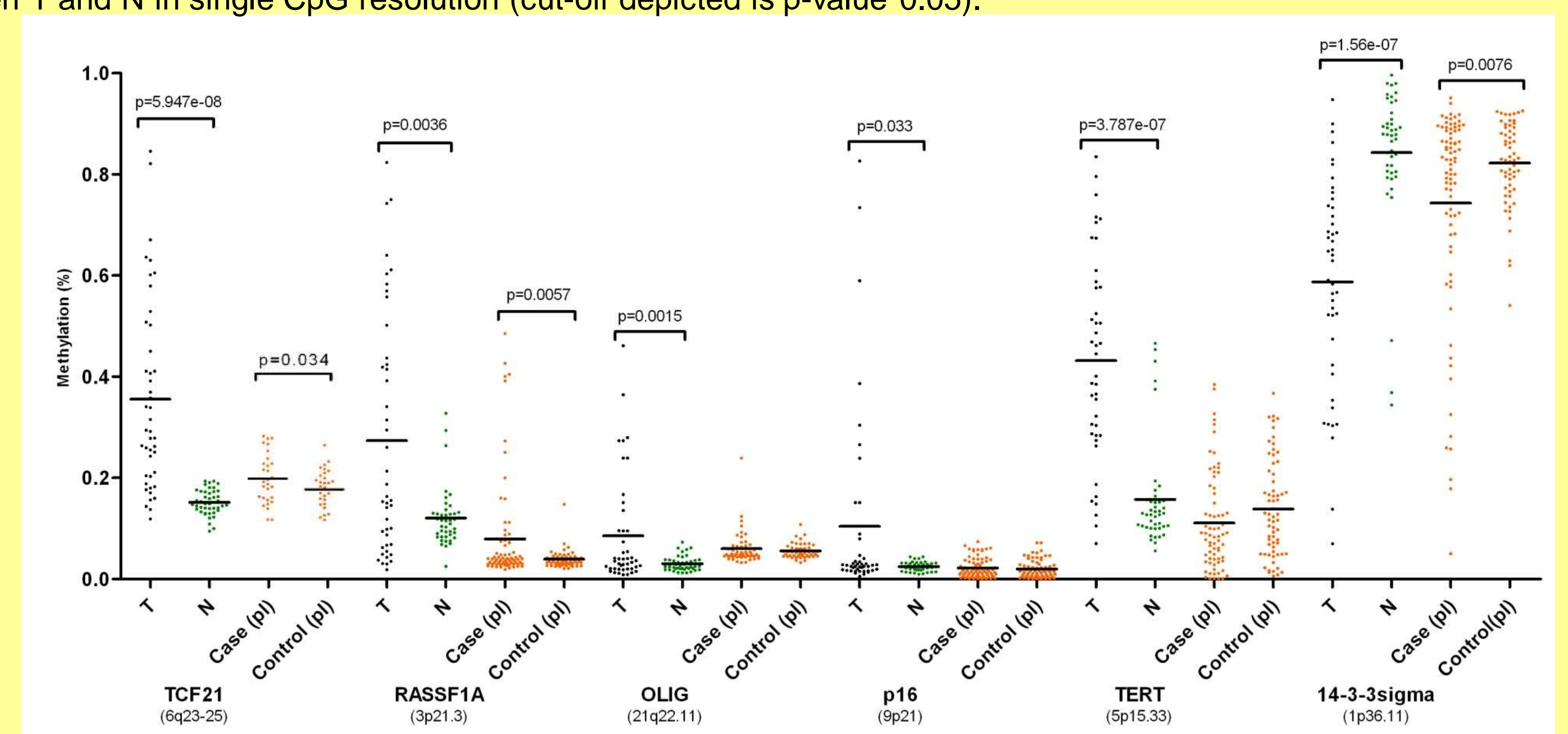


Fig.4 Methylation analysis for the amplicons that were highly significant differentially methylated in the initial screen on 45 T/N tissue matched pairs were performed in cell-free plasma DNA of lung cancer patients and cancer-free individuals. The figure shows the average methylation of each amplicon for 45 T/N matched pairs and 80 plasma samples of cases and controls.

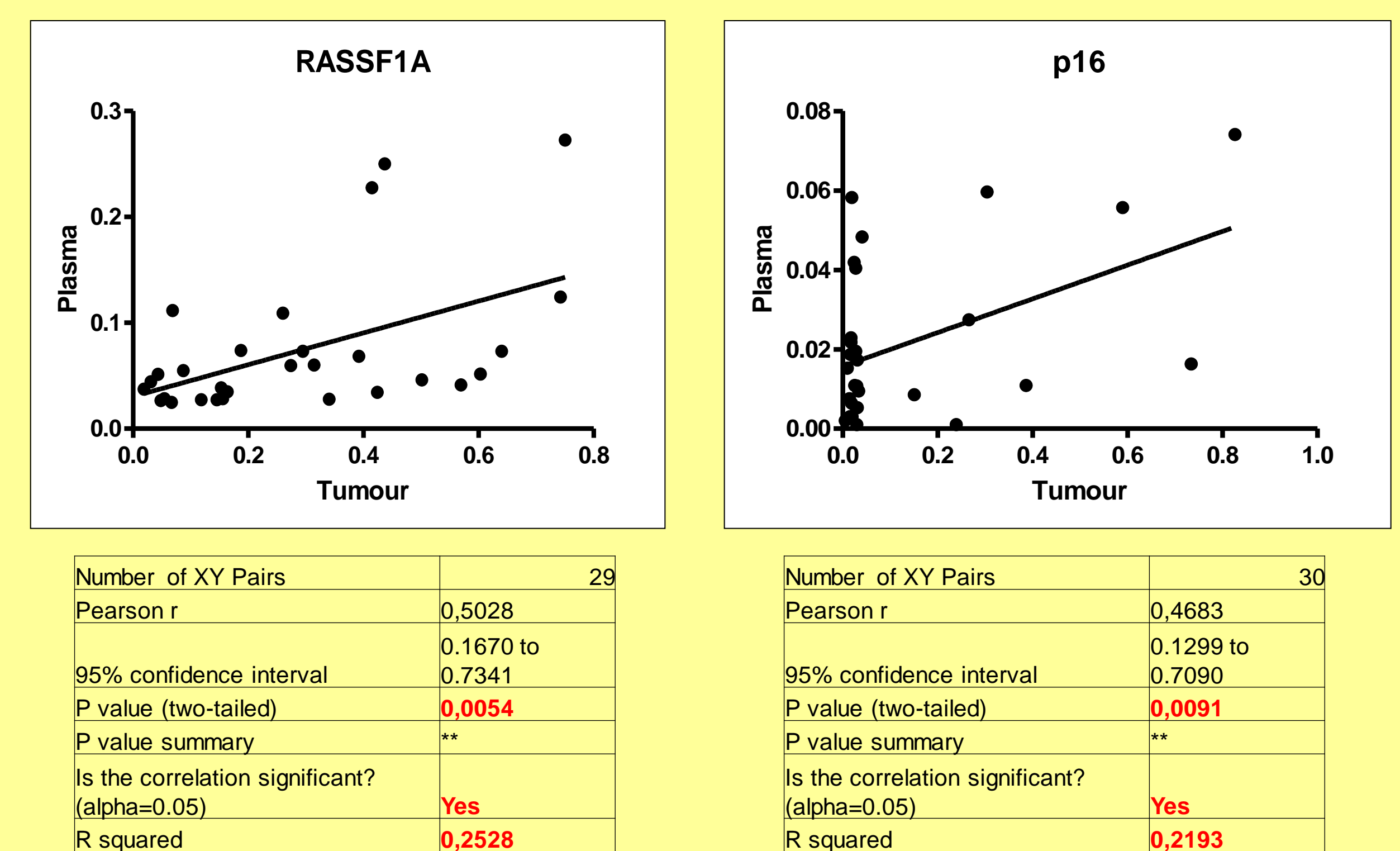


Fig.5 Correlation between methylation in lung tumor tissue and corresponding plasma samples for RASSF1A and p16<sub>INK4a</sub> tumor suppressor genes.

## Conclusion and future outlook

We have identified 3 candidate biomarkers for lung cancer in plasma that have the potential to become a viable screening approach for the early detection, treatment and prognosis. Also, functional analysis of **14-3-3sigma** will be performed to examine the expression level of this hypomethylated gene since it is not in agreement with previous reports.