Diversity in epigenetic diagnostics between lung cancer samples of female and male origin

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Abstract. Lung cancer is among the deadliest tumor diseases and the only type of cancer that is increasing, both in incidence as well as in mortality, though only for women. The death rate of Austrian women suffering from lung cancer increased during the last decade by almost 30%, whereas male patient’s death rate fell by approximately 20% in the same time. This gender gap is caused by gender-specific differences characteristic for lung cancer, including the prevalence and type of mutations as well as response to targeted therapies. Epigenetic tumor markers, specifically DNA methylation, are a novel class of tumor-markers that could improve personalized tumor diagnostics. We developed pyrosequencing-based assays for the detection of DNA promoter methylation in six genes. After successful method validation, we analyzed 14 cell lines (5 of female, 9 of male origin respectively) to get a first insight into gender differences in DNA promoter methylation in vitro for the selected genes. We show the applicability of our assays to a broad range of input material and a possible gender dependency in two analyzed genes.

Keywords: Epigenetics; Non-small cell lung cancer, Pyrosequencing.

1 Introduction

In Austria lung cancer is the second most common cancer of the male and third most common cancer of the female population. 80% of cases are non-small cell lung carcinomas (NSCLC), which can be further specified in adenocarcinomas, squamous cell carcinomas and large cell carcinomas. The death rate of Austrian women suffering from lung cancer increased during the last decade by almost 30%, whereas male patient’s death rate fell by approximately 20% in the same time range [1]. Differences in genetic predisposition, hormonal influences and responsiveness to modern tyrosine-kinase-inhibitor therapies help to explain this antidromic trend [2]. Smoking is the major reason for occurrence of lung cancer. At comparable smoking behavior in women and men the risk for a woman to develop lung cancer is three times higher, as
the higher oestrogen levels accelerate cellular damage mechanisms [2]. Furthermore, in women there is a higher occurrence rate of mutations in the Epithelial Growth Factor Receptor (EGFR) and Kirsten Rat Sarcoma (KRAS) oncogenes as well as in the tumor-suppressor gene p53. In the last ten years Tyrosine-kinase-inhibitors (Erlotinib, Gefitinib), which act by inactivating EGFR at the cell membrane and thereby block proliferation signaling in tumor cells, were established as the first instruments of personalized tumor therapies in NSCLC. s. Response to these therapies is greatly influenced by the mutation status of the EGFR gene, with a significant higher efficiency in mutation-bearing patients [3,4]. The next generation tumor-therapeutics focus in addition on epigenetic alterations of tumor and stroma cells. These epigenetic changes are mainly DNA methylation pattern alterations, for which gender-specific differences are described. Therefore, a gender-dependent responsiveness to latest epigenetic therapies is presumed and a high potential as diagnostic marker for DNA methylation patterns is expected. The aim of this study is to establish and validate novel and cost-effective assays for the analysis of DNA methylation at the promoter region of selected epigenetic marker genes (i.e. p16, RASSF1, TERT, WT1, hMLH1, E-cadherin). In a second step 14 different cell lines are tested for their methylation patterns to describe their applicability as in vitro models for consecutive epigenetic studies.

1.1 Epigenetics

Epigenetics is the study of heritable changes in genetic material (DNA) along with an activity status of each gene (active versus inactive genes). These changes do not involve alterations to the underlying DNA sequence, so that only phenotype, but not genotype is affected. Epigenetic change is a regular and natural occurrence but can also be influenced by several factors including age, lifestyle, environmental factors and disease state. Epigenetic modifications can manifest as commonly as the way cells terminally differentiate to end up as skin cells, liver cells, brain cells, etc. Or, epigenetic change can have more damaging effects that can result in diseases like cancer [5]. At least three systems including DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene regulation are currently considered to initiate and sustain epigenetic changes [6]. The epigenetic mark investigated in this research project is DNA methylation. During this process methyl groups are added to the DNA molecule. DNA methylation happens primarily at cytosines followed by guanines, termed CpG sites. In humans approximately 60-80% of CpG sites are methylated. Regions that fulfill the two requirements of a G/C content greater than 50% over a range of 200 bp are called CpG islands. Those islands are commonly unmethylated in the human genome and for 60-70% of all genes CpG islands overlap the promoter region [7,8]. The methylation of DNA affects gene expression in two possible ways. On the one hand the additional methyl group is a physical obstacle for binding of transcription factors, on the other hand DNA methylation increases chromatin complexity, with tight interactions between histones and DNA forming inaccessible heterochromatin. DNA methylation is fully reversible and is a dynamic state that can be altered within days of a triggering event [9,10].
1.2 Selected Genes

The genes selected for analysis in this study are p16, RASSF1, TERT, WT1, hMLH1 and CDH.

p16 or CDKN2A (cyclin dependent kinase inhibitor 2A) is a gene encoding for the protein p16, which is a cyclin-dependent kinase inhibitor binding to the cyclin-dependent kinase (CDK) 4 and CDK6 [11]. p16, therefore, prevents the association of CDK4 and CDK6 with cyclin D during the G1 to S transition, inhibiting cell cycle progression [11,12]. In lung cancer the tumor suppressor p16 is often inactivated by mutation or hyper-methylation, which is associated with tumor progression [11].

RASSF1 (Ras association domain family member 1) acts as a tumor suppressor that undergoes epigenetic inactivation in cancers by methylation of the CpG islands in its promoter regions [13]. Several studies support the notion that RASSF1 plays a role in a large range of cellular processes, for example: microtubule stability, maintenance of genomic stability, modulation of cell cycle and apoptosis and cell mobility and invasion [14-19]. Furthermore, RASSF1A has been shown to be inactivated by promoter hypermethylation in the majority of lung cancers [20]. This correlation lead to further studies identifying the hypermethylation of RASSF1A as a useful diagnostic marker for lung cancer [21].

TERT (telomerase reverse transcriptase) encodes the catalytic subunit of telomerase, which maintains genomic integrity by telomere elongation [22]. Ectopic expression of TERT in many cell types results in telomere stabilization, and immortalization, which is a mechanism commonly exploited by tumor cells [23]. The upregulation in cancers is mainly caused by genetic amplification of the gene locus as well as epigenetic deregulation [24,25].

WT1 (Wilm’s tumor 1) is expressed during development, in cells undergoing epithelial-to-mesenchymal transition (EMT) and MET but it also plays a role in pathological states such as cancer, Alzheimer’s disease and liver cirrhosis [26]. While WT1 functions as a tumor suppressive gene in Wilm’s tumor, in other cancers – leukemia and breast cancer - it is described to act as an oncogene [26,27]. A recent study showed that in NSCLC WT1 binds to the e-cadherin promoter and thereby increases the invasiveness of the tumor into the tissue [27].

MLH1 (mutL homolog 1) is an important DNA mismatch repair and tumor-suppressor gene. Its promoter methylation was not only reported in association with cancer, but a gender dependency is described in colorectal cancer [28]. This makes it an interesting target gene also for our lung cancer study.

CDH1 (cadherin 1) encodes for the cell-adhesion molecule E-cadherin. The loss of e-cadherin via mutation or hypermethylation is associated with tumor progression and EMT [29]. In lung cancer, amongst other entities, promoter hypermethylation of e-cadherin has been observed [30].

1.3 Aim

The here presented study is part of the FFG funded FemTech project (#849800): “DNA Methylation in Non-Small Cell Lung Cancer and their Gender-specific Effect on Epigenetic Therapies”, which seeks to elucidate if and how the differences in DNA
methylation between male and female patients influences the carcinogenesis as well as the therapy response in lung cancer. In addition, we aim to develop diagnostic tools suitable and validated for routine application and to support the therapy selection in personalized medicine (Figure 1).

Fig. 1. Schematic representation of the high-level project aim, which is to describe the diversity between men and women suffering from lung cancer, with emphasis on epigenetic regulated gene expression by DNA promoter methylation of six selected candidate gene

2 Methods

For the development and validation of diagnostic DNA methylation assays the cell lines, listed in table 1 are used.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Gender</th>
<th>Genetic information</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR90*</td>
<td>Female</td>
<td>wt</td>
</tr>
<tr>
<td>H1975</td>
<td>Female</td>
<td>EGFR mut</td>
</tr>
<tr>
<td>HCC827</td>
<td>Female</td>
<td>EGFR mut</td>
</tr>
<tr>
<td>H2347</td>
<td>Female</td>
<td>KRAS mut</td>
</tr>
<tr>
<td>H1993</td>
<td>Female</td>
<td>n.a.</td>
</tr>
<tr>
<td>H441</td>
<td>Male</td>
<td>wt</td>
</tr>
<tr>
<td>MRC5*</td>
<td>Male</td>
<td>wt</td>
</tr>
<tr>
<td>HCC4006</td>
<td>Male</td>
<td>EGFR mut</td>
</tr>
<tr>
<td>H1437</td>
<td>Male</td>
<td>wt</td>
</tr>
<tr>
<td>HCC2935</td>
<td>Male</td>
<td>EGFR mut</td>
</tr>
</tbody>
</table>
Besides cell lines, DNA was also extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples, generously donated from the Pathology-Lab Zams.

DNA extraction was performed using Qiagen DNeasy Blood & Tissue Kit (#69506) or QIAamp DNA FFPE Tissue Kit (#56404), respectively. Afterwards, DNA was bisulfite converted using Promega MethylEdge Bisulfite Conversion System (#1301). The converted DNA is eventually stored at -20°C for a maximum of 4 weeks. Preferentially the converted DNA is immediately amplified in a PCR reaction, for which the Qiagen HotStarTaq Plus DNA Polymerase (#203603) is employed. Consequently, sequencing can be performed in a Qiagen Pyromark Q24. Pyrosequencing uses sequencing by synthesis for accurate and quantitative analysis of DNA sequences. A sequencing primer is hybridized to a single-stranded, PCR-amplified DNA template. Nucleotides are added sequentially and if the nucleotide is complementary to the base in the template strand it will be incorporated into the DNA strand by DNA polymerase. Each incorporation event is accompanied by release of pyrophosphate (PPi) in an equimolar quantity to the amount of nucleotide incorporated. The enzyme ATP sulfurylase quantitatively converts PPi to ATP in the presence of the substrate adenosine 5’ phosphosulfate. This drives the conversion of luciferin to oxyluciferin by luciferase, generating visible light in amounts proportional to the amount of ATP. Therefore, each light signal is proportional to the number of nucleotides incorporated. The light signal is convert into a pyrogram that is analyzed to determine the specific sequence of the sample under assessment.

The method for determination of promoter methylation was validated according to the Clinical and Laboratory Standards Institute (CLSI) guideline EP17-A: Protocol for determination of limits of detection and limits of quantification – approved guidelines, workflow, quality assurance & documentation [31].

### 3 Results

We developed assays with specific primer sets for PCR and sequencing for the introduced genes and optimized assay parameters such as PCR temperature profiles, mastermix composition and range of starting material concentration. For every assay a Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantification (LoQ) was determined in course of the validation procedure.

We characterized the extensive cell line cohort for all genes and specifically looked at differences between cell lines of female versus male origin. For RASSF1 we can show that female lung cancer cell lines have on average higher promoter methylation than cell lines of male origin, i.e. 40.4% vs 22.7% respectively. The same pattern was identified for WT1, with 69.4% average methylation in female cells vs 55.0% in male
cell lines. Here also the benign reference cell line IMR90 (female, 65.9%) shows increased promoter methylation compared to the male benign reference cell line (MRC5, 4.9%). In the four other genes, we discovered no gender-specific differences.

4 Conclusion

We demonstrate the development of bisulfite-sequencing assays applicable for the routine diagnostic analysis of promoter methylation levels of epigenetic tumor marker genes. After successful validation we analyzed methylation patterns of 14 different cell lines to identify their applicability as model systems for gender-sensitive in vitro model systems for epigenetic studies. For the six selected genes no clear gender-dependency could be identified, except for WT1 where female cell lines including a benign reference cell line showed increased levels of promoter methylation compared to the male cell lines. A second promising gene, RASSF1 shows a gender difference in the average promoter methylation, which is disease-related and not only sex-dependent. This emphasizes the importance of patient gender for lung cancer diagnostics, meaning that the patient gender should be a variable to define, which biomarkers to analyze for best diagnostic output. With the genetic and epigenetic characterization of 14 lung cancer cell lines we generated a well-defined toolkit to apply answering future research questions.
References

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