Dense RASSF1A methylation in uveal melanoma correlates with expression and clinical class

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ABSTRACT
Epigenetic inactivation of tumor suppressor genes by promotor methylation is an important mechanism contributing to tumor progression. An example of a tumor suppressor gene which is frequently epigenetically inactivated in many types of cancer is RASSF1A. We analyzed promotor methylation of RASSF1A in uveal melanoma, the most common ocular malignancy. In this report we present an advanced approach to study quantity and density of methylation by combining methylation-specific digestion with digital droplet PCR. High dense methylation appeared to be associated with loss of expression and is possibly related to metastatic progression despite lack of an established prognostic marker (monosomy 3).

Keywords
Epigenetics, methylation, ddPCR, RASSF1A, uveal melanoma

INTRODUCTION
Understanding of mechanisms leading to tumor development and progression are important to effectively combat cancer. In healthy cells, the action of tumor suppressor proteins is essential to prevent uncontrolled cell proliferation. In cancer cells, inactivation of tumor suppressor genes remove this restraint.[1] Epigenetic inactivation by gene hypermethylation is one of the mechanisms leading to tumor suppressor gene inactivation.[2] By adding methyl groups to promotor DNA, the availability of DNA sequences for expression is modified. Epigenetic gene inactivation leads to loss of expression and thereby to absence of the tumor suppressor protein, driving tumor progression.

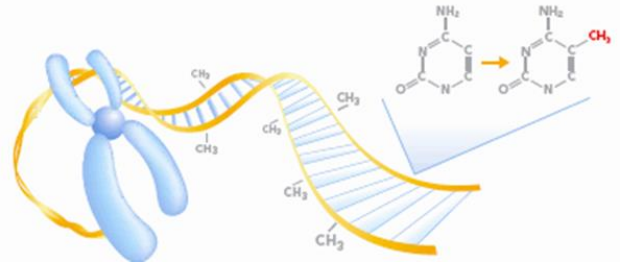

Figure 1. Schematic illustration of DNA methylation.

Whereas analyzing inactivating mutations is nowadays straightforward and powerful as a result of technical development, the study of epigenetics is more complicated. In this paper, we present a new and unique approach to determine methylation status of tumor suppressor genes in tumors. More specifically, we studied methylation of the tumor suppressor gene *Ras association domain family 1A* (RASSF1A) in uveal melanoma (UM).

Uveal melanoma is the most common ocular neoplasm. The tumor derives from uveal melanocytes of the iris, ciliary body or choroid plexus of the eye and has a strong tendency to metastasize to the liver. About 50% of the patients develop metastases, resulting in a high morbidity and mortality.[3] Based on gene expression, uveal melanoma can be divided into two classes (fig. 2).[4] Class I tumors usually do not metastasize and therefore have a favorable prognosis, while patients with class II tumors frequently develop metastatic disease. Class II tumors are characterized by the loss of one chromosome 3 (monosomy 3). Monosomy 3 is strongly associated with a poor prognosis[5], which suggests the involvement of tumor suppressor gene inactivation. A candidate tumor suppressor gene located on chromosome 3 is RASSF1A. This gene has been reported to be epigenetically inactivated in a wide variety of cancers.[6]

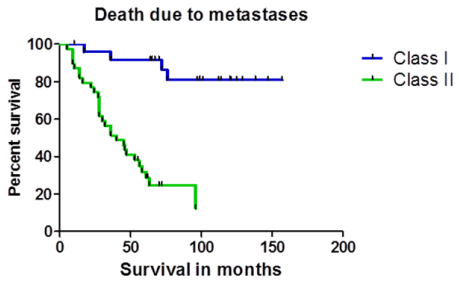

Figure 2. Survival analysis of uveal melanoma patients. Classes are defined based on gene expression.

Using a combination of methylation specific restriction enzymes and digital droplet PCR, we were able to quantify RASSF1A promotor methylation in uveal melanoma and correlate this with RASSF1A expression.

We hypothesized to find RASSF1A methylation mainly in class II tumors. In these tumors, inactivation of one RASSF1A copy in combination with loss of the other chromosome 3 copy might have contributed to the development of metastases.

MATERIALS AND METHODS

Methylation analysis
In order to analyze methylation status, we combined methylation specific digestion and digital droplet PCR

(ddPCR) to analyze a RASSF1A promotor CpG island fragment. We obtained tumor material from 64 enucleated eyes from uveal melanoma patients. Further analyses were performed using isolated DNA derived from these uveal melanoma.

Two different assays were compared: ddPCR combined with Mspa1I digestion versus ddPCR combined with BstUI digestion. Mspa1I recognizes one sequence in the RASSF1A promotor fragment. All fragments which are unmethylated at this cytosine were digested by Mspa1I, whereas methylated fragments were resistant to digestion. Thereby, only fragments with methylation at the specific cytosine were available for amplification. Consequently, this assay determines minimal methylation. The recognition sequence of BstUI, in contrast, is present at 4 locations in the RASSF1A promotor fragment. Only when all four cytosines were methylated, digestion by BstUI is prevented. Consequently, only fragments with four methylated cytosines were available for amplification, providing an indication of dense methylation (fig. 3).

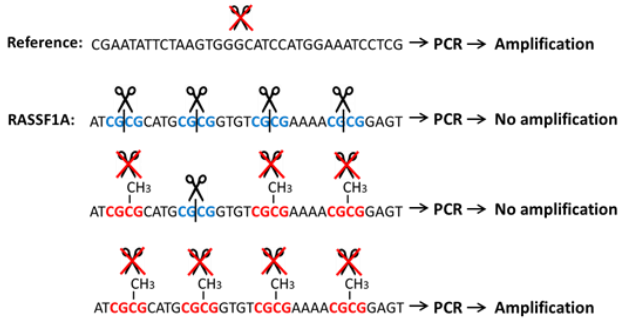

Figure 3. Schematic overview of DNA digestion by BstUI.

After digestion with either Mspa1I or BstUI, ddPCR was performed. Each PCR reaction was fractionated into 20.000 droplets. In each droplet, PCR amplification and characterization of the DNA fragment of interest and a stable reference gene took place. Subsequently, droplets were read and counted in micro flow, providing an absolute quantification.

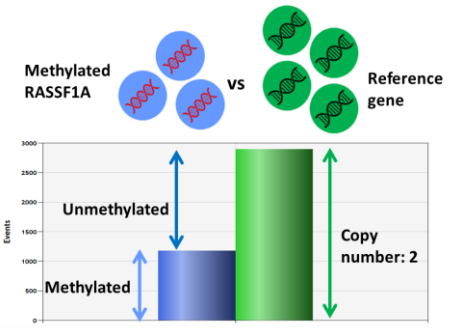

Figure 4. Two color ddPCR output. In order to determine methylated fraction, the amount of droplets positive for methylated RASSF1A is compared with the amount of droplets positive for the reference gene.

Bisulfite conversion and sequencing

In order to validate methylation with another method, UM DNA samples were bisulfite converted and sequenced by Sanger sequencing. Upon bisulfite conversion, unmethylated cytosines were converted to thymidines, while methylated cytosines were protected against bisulfite conversion.

Expression analysis

RNA was isolated from the same uveal melanoma material as described above. RNA was converted into cDNA in order to analyze RASSF1A expression. Real Time quantitative PCR (RT-qPCR) was used to determine the quantity of RASSF1A mRNA for each UM, providing an expression value.

RESULTS

Heterogeneity of RASSF1A methylation

RASSF1A methylation status of each individual uveal melanoma was determined based on ddPCR combined with methylation specific digestion. The ddPCR assay combined with Mspa1I digestion, based on the methylation of one cytosine, revealed all uveal melanoma contained a certain amount of minimal methylation. When uveal melanoma were analyzed with BstUI digestion followed by ddPCR, based on the methylation of four cytosines, a different pattern was observed: only a few uveal melanoma presented with dense RASSF1A methylation (fig. 5A).

Methylation status could be confirmed by sequencing of bisulfite converted DNA. In tumors with a low densely methylated fraction, all cytosines were converted into thymidines (fig. 5B), whereas in tumors with a high densely methylated fraction, cytosines of CpG sites were protected against conversion (fig. 5C)

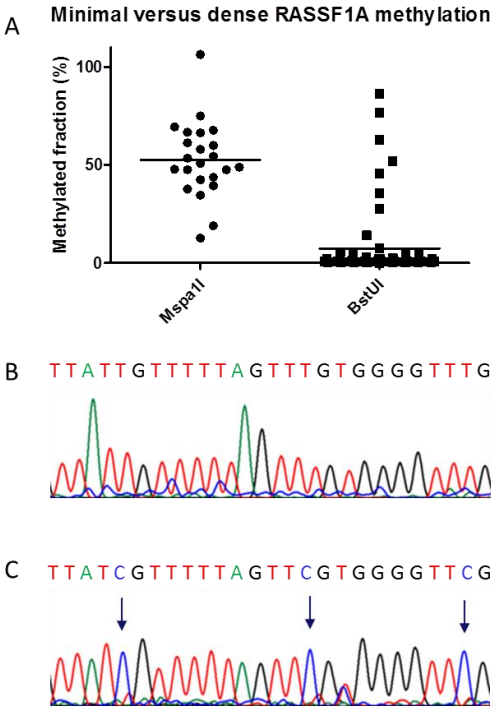

Figure 5. (A) Methylation status based on two different assays. (B) Sequence fragment after bisulfite conversion of a UM with a low dense methylation status (BstUI). All cytosines are replaced by thymidines. (C) Sequence

fragment after bisulfite conversion of a UM with a high dense methylation status (BstUI). Methylated cytosines are preserved after conversion.

RASSF1A expression is correlated with dense methylation status

In order to evaluate whether methylation status as determined by our approach is associated with loss of RASSF1A expression, we performed expression analysis. RASSF1A expression appeared not to be correlated with minimal methylation status, determined by Mspa1I digestion combined with ddPCR; although minimal methylation was found in all UM, many tumors still expressed RASSF1A. In contrast, RASSF1A expression was well correlated with dense methylation status, determined by BstUI digestion combined with ddPCR. Most UM with a dense RASSF1A methylation status lower than 5% presented with RASSF1A transcripts, whereas most tumors with a dense RASSF1A methylation status above 5% lost RASSF1A expression or showed a greatly reduced expression (fig. 6).

This threshold of 5% established by expression analysis was used to define dense methylation status; dense methylation status below 5% was defined as low, whereas dense methylation above 5% was defined as high.

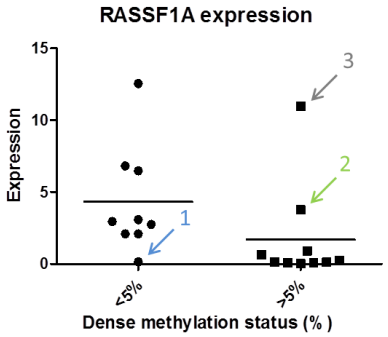

Figure 6. RASSF1A expression in UM with a low versus high dense methylation status. Methylation status above 5% is correlated with loss of expression. Three outliers were observed (arrows).

Explanation of outliers

Three UM cases did not correspond with the overall observed pattern where high dense methylation status (>5%) was correlated with loss of expression. For two outliers, we were able to find a possible underlying mechanism. Outlier 1 (fig. 6, blue arrow) presented with a low methylation status (2%) in combination with loss of RASSF1A expression. Aberrant fluorescent signals in ddPCR data observed in this UM lead to the discovery of a single nucleotide polymorphism (SNP) in the RASSF1A promotor region.

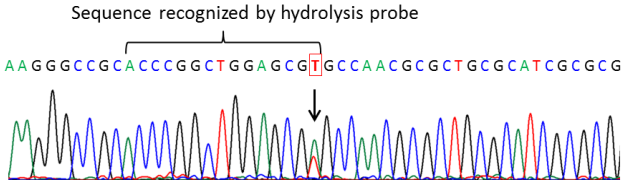

Figure 7. SNP in the RASSF1A promotor fragment recognized by the probe used in ddPCR. At position c.84 a thymidine is replaced by an adenosine (c.84T>A).

Outlier 2 presented with RASSF1A expression, despite of a high dense methylation status (63%). The melting temperature of cDNA derived from outlier 2 (~80 °C) deviated from the melting temperature of cDNA derived from other UM (~85 °C), indicating absence of RASSF1A cDNA outlier 2.

Clinical significance of RASSF1A methylation in UM

In order to study clinical significance of RASSF1A methylation in uveal melanoma, minimal as well as dense methylation status were compared between class I and II uveal melanoma. Minimal methylation was randomly distributed between class I and II tumors, suggesting absence of any clinical significance (fig. 8).

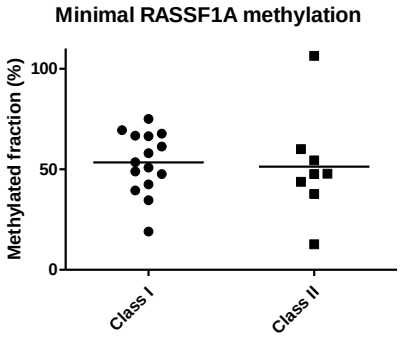

Figure 8. Random distribution of minimal RASSF1A methylation in class I and II.

On the contrary, a high dense RASSF1A methylation status (>5%) appeared to be predominantly present in class I tumors (fig. 9).

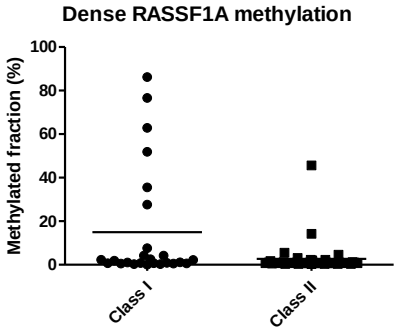

Figure 9. Dense RASSF1A methylation is more frequent in class I UM compared to class II.

CONCLUSION

In this study, RASSF1A methylation was analyzed using a new and unique approach. Digital droplet PCR in combination with methylation specific digestion gave an absolute quantification of methylation and information about methylation density.

By analyzing minimal as well as dense methylation status in 64 uveal melanoma, different methylation frequencies were observed. The ddPCR assay combined with Mspa1I digestion revealed a high frequency of minimal

methylation in all uveal melanoma, while the ddPCR assay combined with BstUI digestion demonstrated a low frequency of dense methylation.

By correlating RASSF1A expression with generated methylation status, the most informative approach to study methylation was determined. Minimal methylation status did not correlate with RASSF1A expression, whereas dense methylation status was well correlated with expression. This means minimal methylation, present in every uveal melanoma to a greater or lesser extent, does not provide any information about epigenetic inactivation of RASSF1A, while dense methylation does.

No perfect correlation between dense methylation status and RASSF1A expression was observed due to the presence of three outliers. However, for two of those a possible explanation was found. Further experiments could give more insight into the underlying mechanisms.

In one of the outliers, a SNP was observed in the RASSF1A promotor region, which might have caused the loss of expression in this tumor. This finding, together with the low occurrence of the SNP in the general population (< 1%) and the fact that the same SNP has also been described in a breast cancer patient[7], support the functionality and clinical relevance of this SNP.

As predicted by the functional uninformative character of minimal methylation, no clinical significance was observed. Minimal methylation status was randomly distributed between class I and class II tumors. In contrast, dense methylation predominantly occurred in class I tumors, representing mainly uveal melanoma with disomy 3. This contradicted our hypothesis, which was to find RASSF1A methylation mainly in class II tumors. Apparently, RASSF1A methylation is not involved in late tumor progression after the tumor has lost one chromosome 3 copy and does not contribute to the development of metastases in monosomy 3 tumors.

The impact of dense RASSF1A methylation for class I patients is still unclear. Interestingly, of the four class I patients who developed metastatic disease, despite their good prognosis and disomy 3 status, three patients had a high dense RASSF1A methylation status (>5%). This suggests RASSF1A inactivation could be involved in tumor progression in these specific class I cases. So far, no explanation has been found for the metastatic disease in these patients, which are the only four exceptions developing metastases in class I. However, nothing can be firmly concluded due to the small number of cases.

In conclusion, digital droplet PCR in combination with BstUI digestion, analyzing dense methylation, is a good approach to study the inactivation of the RASSF1A gene, in contrast to ddPCR combined with Mspa1I. Thereby we present the first quantitative epigenetic marker that correlates and possibly explains RASSF1A expression in tumors. Moreover, we provide a possible explanation for metastatic progression in so far unexplained cases, namely class I metastatic UM patients.

ROLE OF THE STUDENT

This research was performed during a Student Research Project of the BSc Biomedical Sciences at the department of Ophthalmology (LUMC). The topic was proposed by the supervisor (P.A. van der Velden). The experiments, data analysis and writing was performed by the student (D. van Steenderen) and discussed this with the supervisor.

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