Details matter: the role of genomic location and assay standardization in DNA methylation analyses

“Only when including quality and standardization at every level of DNA methylation analyses, will we be able to achieve the robustness to independently validate DNA methylation analyses and to compare multiple methylation studies in systematic reviews. This is the only way to more efficiently develop future DNA methylation based biomarkers.”

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Close to 60,000 papers mentioning DNA methylation have been published by April 2017, and more than 5000 of them were published last year alone [1]. This amounts to about 14 papers per day demonstrating an exploding interest for performing DNA methylation analyses.

This is not surprising, considering the essential role DNA methylation plays in regulating gene expression [2], impacting essential processes such as cell differentiation. Inactivation of the DNA methyltransferases causes embryonic lethality in mice [3,4], underscoring the importance of correct DNA methylation patterns during normal development, with implications not only in health, but also disease.

Aberrant DNA methylation has been reported in, for example, cardiovascular [5], neurodegenerative [6] and metabolic [7] disease. A link between DNA methylation and cancer was demonstrated as early as 1983, when a substantial proportion of CpG sites that were methylated in normal tissues were found to be unmethylated in cancer cells [8]. This hypomethylation, frequently seen early in cancer development, is commonly followed by locus-specific hypermethylation. Large-scale analyses have shown that DNA

methylation aberrations are frequently present in cancers [9] and that DNA methylation profiling enables distinguishing cancer subtypes [10] and classifying cancers of unknown primary origin [11].

A variety of DNA methylation aberrations have been suggested as biomarkers for early detection, prognostication and monitoring of cancer, including in noninvasive clinical material such as blood, stool, urine and bile. Great hopes are also tied to DNA methylation as a direct target for epigenetic therapy. Technological advancements, including genome-wide profiling of DNA methylation aberrations in groups of diseased individuals and healthy controls, are supporting a steady increase in the number of DNA methylation based biomarkers, especially for cancer.

However, despite the enormous amount of papers that are being published on DNA methylation, hardly any of these findings enter routine clinical practice. This low success rate can be explained by the observation that the DNA methylation marker field suffers from general methodological issues that affect biomedical and biomarker research, such as insufficiently stringent methodology, low-quality reporting (including lack of adherence to reporting guidelines such as CONSORT, STARD,

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PRISMA, ARRIVE, REMARK), lack of collaborative studies and insufficient independent validation, preregistration, rigorous systematic reviews, umbrella reviews, randomized trials and lack of (de)implementation studies [12].

However, in order to be able to adequately perform some of the steps recently recommended by Ioannidis and Bossuyt [12], such as independent validation and systematic reviews, the specific details of DNA methylation analyses regarding assay design and genomic location should be standardized and adequately reported.

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One of these details is the variety of locus-specific analyses that are available for analyzing DNA methylation, as reviewed in [13]. The individual methods have various advantages and disadvantages and the choice of method will obviously affect the end results. This is exemplified by a comprehensive review of the analysis of the methylation status of the *MGMT* promoter [14]. *MGMT* recently entered clinical guidelines as a predictive biomarker of treatment with alkylating chemotherapy in elderly patients with glioblastoma [15]. In the review, details of all published *MGMT* studies are summarized, including the choice of locus-specific analysis. The range of methylated high-grade gliomas stretched from less than 20 to more than 90% [14]. Although, this illustrates how also the choice of technology can impact on the outcome that is measured, it does not imply that individual methods to DNA methylation are not comparable. Indeed, the variation among studies using different methods was not larger than among studies using the same method [14]. In addition, we have also shown that, if carefully aligned and optimized regarding genomic location and sensitivity, multiple DNA methylation assays can yield comparable results [16] and that DNA methylation markers can be validated in independent patient series using independent technologies [17,18].

Another key to the comparability of DNA methylation assays is the genomic location of the locus-specific assays [19]. The majority of locus-specific analyses cover only a limited number of CpG sites, and the subsequent methylation frequency only reflects the methylation status of the selected region for analysis. The sites selected for DNA methylation analyses should therefore be representative. Already in 1999, Deng and colleagues demonstrated that the methylation of the *MLH1* promoter was highly variable, and that only

a small and specific region of the promoter invariably correlated with the expected absence of gene expression [20]. Similar examples exist, as addressed in an overview by van Vlodrop *et al.* [19], including the *MAL* gene. Using a genome-wide approach searching for novel DNA methylation biomarkers for early detection of colorectal cancer, we were happy to identify *MAL* which, with a sensitivity in the 80s and a specificity in the 90s, was one of the most promising biomarkers for colorectal cancers at the time [21]. This accuracy was in stark contrast to a previous study of *MAL* with a sensitivity of only 6% in colorectal cancer [22]. The discrepancy between the two studies was the result of an unequal distribution of methylation within the *MAL* promoter, and ultimately the selection of more and less representative CpG sites that were included in the analyses [21].

An additional detail is the lack of standardization of commonly used methods which can also significantly contribute to diverging methylation frequencies reported for the same locus of interest, in the same samples and using the same technology [23]. By comparing more than 15,000 individual quantitative methylation-specific PCRs (qMSP; also called MethyLight [24]), the lack of proper normalization stood out as the most significant contributor to variability in the final DNA methylation results. Normalizing using robust elements such as the repetitive ALU element in contrast to single-/low-copy genes such as *ACTB* has luckily become mainstream. However, the power of the normalization should not be overestimated. Large differences in DNA input among samples in the same study are one of many factors that will impact on the final methylation values [23].

Lack of standardization of the methylation analysis, in addition to inclusion of different CpG sites for analyses might also be a contributing factor to why the BLUEPRINT consortium recently recommended absolute quantitative analyses over qMSP/MethyLight [25]. Engaging 18 laboratories across 7 countries, they performed a quantitative comparison of the performance of the most widely used methods for DNA methylation analysis. Carefully selected, designed and validated qMSP/MethyLight assays were however acknowledged for the ability to detect small amounts of methylated DNA in an excess of unmethylated DNA in a cost-effective manner [25], in spite of the generally lower correlation between these assays compared with the absolute quantitative assays. Among the locus-specific analyses for DNA methylation assessment, qMSP/MethyLight is commonly used. Overall, the method is fairly fast and simple, relatively cheap and has the potential for high-throughput by analyzing many samples in parallel.

Conclusion

Only when including quality and standardization at every level of DNA methylation analyses, will we be able to achieve the robustness to independently validate DNA methylation analyses and to compare multiple methylation studies in systematic reviews. This is the only way to more efficiently develop future DNA methylation based biomarkers.

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