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RESEARCH HIGHLIGHT

DNA methylome and the complexity of discovering prostate cancer biomarkers

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Prostate cancer (PCa) remains the most common malignancy and a leading cause of cancer-related deaths in men. Molecular discrimination at an early stage between indolent and aggressive primary tumors in pathologically confirmed PCa is required to develop personalized therapeutic interventions.

Somatic epigenetic alterations include DNA methylation, histone modification, RNA interference, disordered micro-RNA gene expression and genomic imprinting among others. Epigenetic alterations in human PCa occur at the earliest phases of malignant transformation and remain detectable throughout the invasive and metastatic progression of the disease. Epigenetic alterations have created great promises and potentials as biomarkers for PCa screening, diagnosis, molecular staging, prognostication and risk stratification. Reversibility of epigenetic changes allows therapeutic modulation of the epigenome and can also provide preventive opportunities in PCa.

DNA methylation is the addition of a methyl group at the 5′-carbon of the cytosine ring adjacent to a guanine (CpG). This process is executed by a number of conserved enzymes known as DNA methyl transferases (DNMTs). Aberrant DNA methylation and specifically hypermethylation silencing appears to be the most frequent molecular event in several malignancies including PCa. It is not surprising that many groups are paying considerable attention to identifying the unique profile of hypermethylated CpG islands 'methylotyping or methylation signature' that could be used for screening or as predictive or prognostic biomarkers for PCa. Kobayashi *et al.*[1] explored a small-scale DNA methylome by using the Illumina Human Methylation 27 platform. They quantitatively examined promoter DNA methylation levels at 26333 CpG sites encompassing 14104 genes in 95 primary PCa and 86 adjacent benign prostate tissues of flash-frozen specimens. These tissues were obtained from patients with organ-confined PCa who were subjected to radical retropubic prostatectomies. To determine the significance of observed differences for CpG site DNA methylation levels, they employed an unpaired two-class significance analysis of microarray for the available tumors and the matched adjacent benign tissues. This analysis revealed 5912 CpG sites hypermethylated and 2151 CpGs hypomethylated in tumors compared to benign adjacent tissues. They also used a rational strategy for prediction analysis of the data by pre-sorting the CpGs with standard deviation for all samples and improving the statistical power by analyzing those CpGs with a standard deviation of equal or greater than 0.04. Using these criteria, they identified PCa methylation biomarkers for 87 CpGs (82 genes) with predictive diagnostic values and 69 CpGs of prognostic values which correlated with biochemical recurrence. Since one-third of the CpGs examined showed quantitatively significant changes in methylation, authors examined a very rational hypothesis to determine the correlation between the observed changes and the expression levels of maintenance and *de novo* DNMTs (*DNMT1*, *DNMT3A*, *DNMT3A2* and *DNMT3B*) and DNMT-interacting proteins (DNMT3L and EZH2) which are believed to target methyltransferases to certain genomic locations. TaqMan qPCR gene expression assay using FAM/MGD-labeled probes from Applied Bioscience inventory revealed a significant correlation between overexpression of *DNMT3A2* (r^2=0.272, P=0.0031), *DNMT3B* (r^2=0.197, P=0.0056), *EZH2* (r^2=0.211, P=0.0037), and global hypermethylation in tumors suggesting a possible causal role. Kobayashi *et al.* extended the investigation to recapitulate the potential cause and effect relationship between increased levels of *DNMT3A2*, *DNMT3B* and *EZH2* transcripts and global hypermethylation. They transiently transfected a primary culture of human prostate epithelial cells with various DNMTs plasmids (alone or with EZH2). After robust bioinformatics, they further discovered that *DNMT3B1* and *DNMT3B2* overexpression led to elevated methylation levels in a subset of CpG sites which demonstrated PCa-specific hypermethylation.

Overall, the strengths of the study on promoter-DNA methylation profiling by Kobayashi *et al.*[1] include: (i) the comprehensive nature of the methylation analysis involving hierarchical clustering of malignant and adjacent benign prostatic tissues, differential methylation assays, two-class significance analysis of microarray, permutations and associated bioinformatics; (ii) the inclusion of a reasonable population size; (iii) searching 26333 CpG representing 14104 genes; (iv) the prediction analysis of the methylation data to identify the most predictive diagnostic methylation signature; and (v) the identification of prognostic DNA methylation changes which correlate with biochemical tumor recurrence.

Compared with other malignancies, gene-specific and genome-wide methylation analyses in PCa were conducted with a limited number of samples or limited scopes.[2–4] Previous studies have identified over 30 hypermethylated CpG loci in PCa.[5] Among these, GSTP1 promoter methylation occurs in up to 90% of prostatic tumors and in 70% of prostatic intraepithelial neoplasia lesions.[6,7] Other well-characterized PCa-hypermethylated genes include *RASSF1A*, *CDH1* and *CDKN2A*.

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In a recent study by Liu *et al.*,[4] association between *APC*, *TGFβ2*, *HOXD3* and *RASSF1A* genes, promoter hypermethylation and PCa progression was examined in 219 radical prostatectomy samples. This study discovered an elevated promoter hypermethylation of the *APC*, *HOXD3* and *TGFβ2* genes, and an association with PCa progression. Another report by Kron *et al.*[2] which searched for genome-wide methylation profiles using an agilent human CpG array and 20 frozen PCa tissue samples, led to the identification of the *HOXD3* and *BMP7* genes' hypermethylation in high-grade tumors. None of these investigations compared methylation profiling between malignant and matched adjacent benign prostate tissues. Kobayashi *et al.*[1] also confirmed the hypermethylation status of previously discovered diagnostic biomarkers such as *APC*, *RASSF1* and *GSTP1* in PCa when compared with adjacent benign tissues.

In relation to the methylation profile of PCa, several investigations were conducted to examine DNMTs and DNMT-interacting proteins in PCa cells and tissues that searched for the mechanisms underlying hyper- or hypomethylation changes. *In vitro* verification of the involvement of DNMTs as the potential mechanism that is responsible for the differential methylation data in the study by Kobayashi *et al.*[1] was demonstrated in a single prostate epithelial cell type (primary culture). In the future, these could be extended to a variety of PCa cell lines presenting different phenotypes relevant to prostate carcinogenesis, androgen sensitivity, androgen-independent, or metastatic progression under *in vitro* or *in vivo* experimental conditions. In addition to the predictive or prognostic biomarker values, therapeutic strategies for tumor cells-targeted inhibition of DNMTs could be further investigated. Since a relatively considerable number of CpGs were hypomethylated (n=2151), an understanding of demethylating processes is essential to address the biological and clinical significance of hypomethylated genes in primary PCa. Taking into consideration that the human genome presents with approximately 28 million CpGs (i.e., ~1000-fold higher than Illumina Human Methylation 27 chip), it remains to be known whether increasing the size of analyzable CpGs in high throughput arrays (e.g., new Illumina Human Methylation chip with ~450 000 CpGs) validates the findings reported by Kobayashi *et al.*[1] It is noteworthy that not all hyper- or hypomethylated CpGs are effective or efficient in transcriptional silencing or activation of the affected genes. In addition to the important downstream post-transcriptional, translational and post-translational factors, other genetic (e.g., mutation) and epigenetic processes (e.g., histone modifications, miRNA) might equally contribute or determine the fate of transcription regulation of the genes.

Overall, the missing areas for genome-based methylation analyses include the comparison: (i) between completely normal prostate tissues and independent PCa samples with risk stratification; (ii) between high-grade prostatic intraepithelial neoplasia lesions and adjacent benign prostatic hyperplasia (BPH); (iii) between African Americans and Caucasians to address PCa racial disparities; (iv) among multiple tumor foci to evaluate intratumoral DNA methylation heterogeneity; (v) between primary PCa and micrometastases or metastatic tumor foci at different locations; (vi) between indolent and clinically significant organ-confined tumors; (vii) between Gleason minor and major or between Gleason major and Gleason sum in individual tumors; and (viii) between PCa tissues and their adjacent stromal cells (e.g., endothelial, inflammatory, *etc.*) in the tumor microenvironment. Limited knowledge is available for inter-racial differences on gene-specific or genome-wide methylation or other epigenetic processes in normal individuals or patients with PCa. Kwabi-Addo *et al.*[8] examined the methylation levels of six genes (*GSTP1*, *AR*, *RARβ2*, *SPARC*, *TIMP3* and *NKX2-540*) which have been previously shown to be hypermethylated in Caucasians with PCa or cell lines. They compared matched and PCa tissues from African Americans and Caucasians who had comparable Gleason score. They observed significant differences in the methylation levels in all genes, except *GSTP1*, in the African-American samples in comparison with Caucasian samples.

A deeper understanding of the biological mechanisms underlying PCa progression and finding the most reliable predictive or prognostic biomarkers necessitates an integrative analysis which includes genome-wide screening of epigenetic events (e.g., DNA methylation, histone modifications, miRNAs), gene fusions and expression, and copy number variations combined with available methodologies to detect the relative expression levels of proteins of interests (e.g., immunohistochemical staining). Future investigations will certainly confirm the biological complexity of epigenetic processes in PCa. Like other known omic-based profiling techniques, high-throughput functional analyses of DNA methylome (robotic-functional methylome) are lacking and remain a challenging task, but are required for future personalized PCa therapy. Furthermore, at this time, it is unclear whether medical or biological therapies can meet the more complex demands as projected by an individualized therapy on the basis of the molecular signature of malignant diseases.

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