Global DNA hypomethylation in prostate cancer development and progression: a systematic review.

Global DNA hypomethylation in prostate cancer development and progression: a systematic review

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Abstract

Background:

The role of global DNA methylation in prostate cancer (PCa) remains largely unknown. Our aim was to summarize evidence on the role of global DNA hypomethylation in PCa development and progression.

Methods:

We searched PubMed through December 2013 for all studies containing information on global methylation levels in PCa tissue and at least one non-tumor comparison tissue and/or studies reporting association between global methylation levels in PCa tissue and survival, disease recurrence or at least one clinicopathological prognostic factor. We summarized results using non-parametric comparisons and *P*-value summary methods.

Results:

We included 15 studies in the review: 6 studies with both diagnostic and prognostic information, 5 studies with only diagnostic information and 4 studies with only prognostic information. Quantitative meta-analysis was not possible because of the large heterogeneity in molecular techniques, types of tissues analyzed, aims and study designs. Summary statistical tests showed association of DNA hypomethylation with PCa diagnosis ($P<0.006$) and prognosis ($P<0.001$). Restriction to studies assessing 5-methylcytosine or long interspersed nucleotide element-1 revealed results in the same direction. Analyses restricted to specific clinicopathological features showed association with the presence of metastasis and tumor stage in all tests with$P<0.03$, and no association with Gleason score (all tests $P>0.1$ except for the weighted Z-test, $P=0.05$).

Conclusion:

DNA hypomethylation was associated with PCa development and progression. However, due to the heterogeneity and small sample sizes of the included studies, along with the possibility of publication bias, this association requires additional assessment.

Introduction

The introduction of PSA testing has led to a considerable increase in diagnosis of prostate cancer (PCa), which was the second most commonly diagnosed cancer in men worldwide in 2012.[1] However, due to the limited sensitivity and specificity for aggressive PCa, PSA testing may lead to over-diagnosis and possibly over-treatment of indolent tumors, and implementation of PSA screening is not recommended.[2, 3]Consequently, identification of accurate biomarkers that can distinguish aggressive prostate tumors requiring immediate treatment from indolent tumors that can be appropriately handled with active surveillance approach has become an imperative.

Both genetic and epigenetic changes have been implicated in the PCa development and progression.[4, 5] Epigenetic changes include DNA methylation, histone modifications, microRNA interference and genomic imprinting among others.[6] Although histone modifications and microRNA interferences have been suggested to have an important role in prostate tumorigenesis,[7, 8, 9, 10, 11, 12, 13, 14, 15] DNA methylation is the most investigated epigenetic alteration. Several genes have been found to be aberrantly hypermethylated in PCa in general or in more aggressive compared with less aggressive tumors, including: DNA damage repair genes (*GSTP1*, *MGMT* and *GSTM1*); hormonal receptor genes (*AR*, *ESR-α*, *ESR-β*, *RARβ* and *RARβ2*); cell-cycle genes (*CDKN2A*,*CCND2*, *RASSF1A*, *HIC1* and *SFN*); signal transduction genes (*EDNRB*,*RUNX3* and *CD44*); apoptosis genes (*DAPK*, *SLC5A8*, *SLC18A2* and*TNFRSF10C*) and tumor cell invasion and metastasis genes (*E-Cadherins*, *APC* and *TIMP-2*).[4, 7, 8, 9, 13, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27,28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38]

Gene-specific hypermethylation may coexist with a nonspecific global DNA hypomethylation. DNA hypomethylation involves the loss of DNA methylation in genomic regions where it normally occurs, and is assumed to be a genome-wide phenomenon.[36, 39, 40] Deficiency in 5-methylcytosine (5 mC) content is found in almost every type of cancer (reviewed in Ehrlich[41] and Wilson[42]), and is mainly due to hypomethylation of highly repetitive DNA sequences such as long interspersed nucleotide elements (LINEs) and short interspersed nucleotide elements.[43, 44, 45, 46, 47] Dense methylation of these repetitive sequences presumably prevents gene disruptions, translocations and chromosomal instability, thus maintaining genomic integrity.[48, 49, 50, 51, 52, 53, 54, 55] Although global DNA hypomethylation was suggested to have a role in PCa development and progression more than 25 years ago,[56] it has been studied to a much lesser extent than gene-specific hypermethylation. Recent developments and implementations of new molecular techniques have, however, increased the number of studies assessing the role of this epigenetic change in PCa development and progression.

We conducted a systematic review to summarize evidence on the role of global DNA hypomethylation in PCa development and progression. We reviewed: (i) studies in which global methylation was used to distinguish between prostate tumor tissue and benign prostate tissue (hereafter referred to as ‘diagnostic studies’), and (ii) studies in which global methylation was studied in association with clinicopathological prognostic factors and/or disease recurrence including survival (hereafter referred to as ‘prognostic studies’).

Materials and methods

Search strategy and study selection

We searched the PubMed database for original research articles published in English using the following combination of MeSH terms: ((((prostate) OR 'prostate cancer') OR 'prostate tumor')) AND ((((methylation) OR hypomethylation) OR line1) OR Alu). The last update of the PubMed search was conducted on 13 December 2013. Reference lists of all included articles as well as relevant reviews in the field of epigenetic markers in PCa[18, 57, 58] were scrutinized to identify additional articles not detected in the PubMed search.

Diagnostic studies were eligible for inclusion if they reported global methylation levels in PCa tissue and at least one non-tumor tissue: normal prostate tissue (NPT), PCa adjacent benign tissue, BPH tissue, and low- or high-grade prostatic intraepithelial neoplasia tissue. Prognostic studies were eligible for inclusion if they reported the association between global DNA methylation levels in PCa tissue and survival, disease recurrence or at least one clinicopathological prognostic factor: Gleason score or tumor grade, tumor stage, presence of metastasis and PSA level at diagnosis. If two or more studies overlapped, we included the study with the largest sample size. In the case of identical sample sizes, we included the study with the earliest date of publication.

Data extraction was initially performed by one author (RZ), followed by individual re-extraction of each paper by one of the co-authors (DZ, AP, VF, CG or LR). In the case of inconsistencies or disagreements a third co-author (LR or DZ) was involved in the decision making. For each study, we abstracted information on the molecular techniques used to assess global methylation, the types of evaluated prostate tissues and the main results, including the mean (or median and frequency if mean was not available) of DNA methylation and the reported relevant *P*-values. Whenever possible, if *P*-values and means were not available, we computed them using relevant information reported in the original article (for example, we estimated the means from the Figures or calculated *P*-values from standard errors or confidence intervals).

Data synthesis

Due to the large heterogeneity in molecular techniques used to assess DNA methylation levels, types of tissues analyzed, study aims and study designs, no quality assessment of the studies was performed. For the same reason, a quantitative meta-analysis of the findings was not possible. We limited our analyses to non-parametric comparisons of the study results.

Evidence was summarized using three statistical tests. First, we conducted a global test of association using an exact binomial distribution and a threshold of 0.05 for the study-specific *P*-values. We chose 0.05 as the threshold as it was used in many of the original articles. This test does not consider the direction of the associations, but rather focuses on a departure from the null hypothesis.[59] The other two statistical tests were used to assess the direction of the associations. By using an exact binomial test, with an expected probability of 50% under the null hypothesis of no association, we conducted a sign test to estimate the *P*-value associated with the observed proportions of inverse associations (that is, DNA methylation levels being lower in PCa tissue compared with non-tumor prostate tissue, or in more aggressive compared with less aggressive tumors).[60]This test has a very low power but permits inclusion of studies that reported only means and no *P*-values. Finally, we repeated the same test by including only the studies reporting *P*-

values. We used a *P*-value of 0.05 as a threshold for the definition of an inverse association and an expected alpha of 0.025 instead of 0.50 (vote-count test).

We performed additional combined probability tests by combining the exact one-tailed *P*-values from individual studies. Two-sided *P*-values, when reported in the original studies, were converted into the corresponding one-sided *P*-value according to the direction of the association. We assigned a *P*-value value of 0.9 for the studies reporting $P>0.05$ and the largest *P*-value for the studies reporting non-exact small *P*-values (for example, 0.001 when the reported *P*-value was $P<0.001$). Combination of the *P*-values is typically obtained through Fisher's test ($\chi^2=-2\sum_k \ln(p_k)$, where k is the number of individual studies and χ^2 follows a chi-squared distribution with 2 k degrees of freedom), and Stouffer's Z-transform test ($Z_s=\sum_k Z_k/\sqrt{k}$, where Z_s follows standard normal distribution).[60] We decided to perform Fisher's test, as it is the most commonly used combined probability test, and a variation of Stouffer's Z-transform test, the weighted Z-method, which is reportedly superior to both Fisher's and Stouffer's method.[61] The weighted Z-method was calculated using META 5.3., meta-analysis program, 1989, National Collegiate Software Clearinghouse, Raleigh, NC, USA.

We first analyzed all diagnostic and all prognostic studies indiscriminately of the type of the molecular technique used or clinicopathological characteristic assessed. For the studies using more than one technique to assess global DNA methylation levels, we used the estimate obtained using LINE-1 sequence as this is the most commonly described surrogate of global methylation levels. For the studies including more than one type of comparison non-tumor prostate tissues, we considered the group that was most similar to NPT tissue on the basis of the following *a priori* decided ranking: NPT, low-grade prostatic intraepithelial neoplasia, PCa adjacent benign tissue, BPH and high-grade prostatic intraepithelial neoplasia tissue. In most of the prognostic studies, more than one clinicopathological characteristic was evaluated. We used only one estimate for the main analyses, chosen on the basis of the following *a priori* decided ranking: (1) survival, (2) recurrence, (3) presence of metastasis, (4) Gleason score or tumor grade, (5) tumor stage and (6) PSA level at diagnosis.

We also conducted subgroup analyses. For diagnostic studies, we analyzed separately studies based on 5 mC content and those based on LINE-1. For prognostic studies, we analyzed separately results for the different clinicopathological features whenever the same feature was assessed in at least three studies.

Finally, we conducted sensitivity analysis by including the studies without reported *P*-values. Considering a conservative assumption that these studies were more likely to have a high *P*-value we assigned them with a two-tailed *P*-value of 0.9 and a one-tailed *P*-value of 0.45.

Results

Characteristics of eligible studies

The PubMed search identified 1169 articles. After excluding 1150 studies that did not meet the inclusion criteria, and 3 studies because the analysis was not performed on human samples, 16 diagnostic and/or prognostic studies were

retained[24, 45, 55, 56, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73] (Figure 1). Screening of the 16 relevant articles' reference lists yielded no additional studies. One study containing prognostic information was excluded because of overlapping samples[66] with Florl *et al.*[45] Thus, in total, we retained 15 studies, of which 6 had both diagnostic and prognostic information,[24, 56, 62, 65, 67, 69] 5 had only diagnostic information[55, 68, 70, 71, 73] and 4 had only prognostic information.[45, 63, 64, 72] The studies were highly heterogeneous, in particular in the type of tissue analyzed and in the molecular techniques used to assess global DNA methylation (Table 1). Molecular techniques included high-performance liquid chromatography, antibody immunostain densitometry, high-pressure liquid chromatography-tandem mass spectrometry, Southern blot hybridization, combined bisulfite restriction analysis (COBRA) assay, real-time PCR-based methylation-specific assay (MethyLight assay), combination of methylated DNA precipitation and restriction enzyme digestion assay (COMPARE assay) and pyrosequencing. The main characteristics of these techniques are summarized in Appendix Table 1.

Diagnostic studies

Characteristics of the 11 studies[24, 56, 55, 62, 65, 67, 68, 69, 70, 71, 73] that included samples of both PCa tissue and at least one comparison non-tumor tissue are summarized in Table 2 and Figure 2. The most commonly used methods to assess global DNA methylation were total 5 mC content[56, 62, 65, 69, 71] and LINE-1 repetitive elements estimation methods.[24, 55, 62, 67, 68, 70] Some of the studies used more than one technique.[62, 68] Most of the studies had a limited sample size; only one study included more than 100 tumor tissue samples, which were compared with a smaller number (n=30) of non-tumor tissue samples.[67] Two studies did not report P-values or sufficient information for their calculation.[70, 73]

As summarized in Table 3, seven out of nine studies reporting P-values reported a $P<0.05$ (global test: $P<0.001$). Ten of the 11 studies reported negative associations (sign test: $P=0.006$). Among the nine studies reporting P-values, seven studies with negative associations had a$P<0.05$ (vote-count test: $P<0.001$). Both combined probability tests using the exact one-tailed P-values from the nine individual studies reporting P-values returned a combined $P<0.001$. After inclusion of the two studies, which did not report a P-value we observed no change in the results with a combined $P<0.001$.

A total of six studies assessed total 5 mC content. Four out of the five studies reporting P-values reported a $P<0.05$ (global test: $P<0.001$), four out of the six studies reported negative association

(sign test: P=0.34) and, among five studies reporting P-values, three studies with negative associations had P<0.05 (vote-count test: P<0.001). Combined probability tests returned a combined P<0.004. Results remained unchanged in the sensitivity analysis (P<0.004).

When analyses were restricted to the six studies assessing LINE-1, four out of five studies reporting P-values had a P<0.05 (global test:P<0.001), all six studies reported a negative association (sign test:P=0.016) and, among the five studies reporting P-values, four studies with negative associations had a P<0.05 (vote-count test: P<0.001). Combined probability test returned similar results with a combinedP<0.001. No change in the results was observed in the sensitivity analysis (P<0.001).

Prognostic studies

Ten studies investigated the association between global DNA methylation levels in PCa tissue and survival,69 disease recurrence65and/or at least one clinicopathological prognostic factor.24, 45, 56, 62, 63,64, 65, 66, 67, 69, 72 Their characteristics are summarized in Table 4 andFigure 3.

As reported in Table 5, out of these 10 studies, 6 reported an association with a P<0.05 (global test: P<0.001). Eight out of 10 studies reported an inverse association between DNA methylation and indicators of tumor aggressiveness (sign test: P=0.054), and 5 studies with inverse association reported a P<0.05 (vote-count test: P<0.001). Combining the exact one-tailed P-values from all 10 studies returned similar results with a combined P<0.001 in both tests.

Analyses focused on specific prognostic indicators were possible for presence of regional or distant metastases, Gleason score and tumor stage. In general, results for the different indicators were consistent.

Out of six studies comparing global DNA methylation levels in metastatic (lymph node or distant metastases) vs non-metastatic tumors,45, 56, 62, 63, 64, 72 four reported an association with a P<0.05 (global test: P<0.001). All six studies reported lower methylation level in metastatic

compared with non-metastatic tumors (sign test: P=0.016), and four studies with inverse association reported a P<0.05 (vote-count test: P<0.001). Similarly, both combined probability tests returned a combined P<0.001.

Gleason score was analyzed in association with global DNA methylation in four studies,[24, 45, 67, 69] of those only one study reported an association with a P<0.05 (global test: P=0.19). Three out of the four studies reported an inverse association (sign test: P=0.31), that is, methylation levels decreased with increasing Gleason score, and one study with inverse association reported a P<0.05 (vote-count test:P=0.096). Fisher's test returned a combined P=0.10, whereas the weighted Z-test returned a combined P=0.046.

Five studies compared global DNA methylation levels across categories of tumor T stage.[24, 45, 63, 64, 67] Two out of five studies reported an association with a P<0.05 (global test: P=0.02). All five studies found an inverse association between methylation levels and tumor stage (sign test: P=0.03). Out of these five studies, two reported inverse association with a P<0.05 (vote-count test: P=0.006). Both combined probability tests returned a P=0.02.

Discussion

We included a total of 15 studies in this review. Eleven 'diagnostic studies' assessed global DNA methylation in PCa tissue compared with non-tumor prostate tissue, and 10 'prognostic studies' assessed the association between global DNA methylation in PCa tissue and survival, disease recurrence and/or clinicopathological prognostic factors. The studies were characterized by a large heterogeneity in molecular techniques used to assess global DNA methylation levels, type of tissue analyzed, patient characteristics, statistical analyses and reporting of the results. In addition, most of the studies were of limited sample size.

Global DNA methylation levels in the studies included in this review were assessed either by estimating total 5 mC levels (direct methods) or by estimating levels of surrogate markers, most commonly LINE-1 (indirect methods). Differences between these methods (described in more detail in Appendix Table 1) could account for some differences in the study results. Immunohistochemistry, as opposed to methyl-sensitive restriction enzymes and bisulfite conversion based techniques, can distinguish 5 mC from the structurally similar 5-hydroxymethylcytosine, thus providing a more accurate estimate of the total 5 mC content. On the other hand, immunohistochemistry detects methylated cytosines in both promoter and other genomic regions but makes no distinction between these two sites. A study comparing different methods used to quantify global DNA methylation[74] found levels of LINE-1 and Alu measured by pyrosequencing to be unreliable substitutes for the gold standard measurement of total 5 mC levels by high-performance liquid chromatography, although they deemed LINE-1 to be acceptable as a surrogate in many cases. Furthermore, Yegnasubramanian et al.[62]found a change in LINE-1 methylation in primary PCa without a matching change in global 5 mC content. This further supports the assumption that the use of different methods could, at least to some extent, be responsible for the different study results.

Inclusion criteria differed across studies. Moreover, global methylation levels in normal tissues depend on a number of factors (for example, age, race/ethnicity, environmental and dietary

factors)[75, 76, 77, 78] that may affect the risk of developing PCa. It is possible that confounding by these factors or selection bias may have affected results to some extent. As adjusted estimates were available in a minority of the studies, our review is based on unadjusted estimates.

In the diagnostic studies, DNA methylation levels in PCa tissue were compared with those in different types of non-tumor tissue, ranging from NPT obtained from autopsy series to non-neoplastic tissue adjacent to the tumor. This is a potent source of heterogeneity. In theory, comparison between PCa tissue and NPT is supposed to show the largest effect. However, a diagnostic biomarker can be assessed only in the prostate tissue in patients receiving a prostate biopsy. Thus, the most clinically informative comparison would be between patients with PCa and cancer-free patients who received a prostate biopsy as a part of the diagnostic process for a suspected PCa.

Global methylation levels were associated with a variety of prognostic factors. Clinically, the most informative outcome is overall survival or potentially PCa-specific mortality. However, only one study assessed the association between global DNA methylation levels and survival (finding a positive association with a *P*-value of 0.40).[69] The lack of studies assessing survival as an outcome likely reflects difficulties in conducting a study with follow-up information as opposed to studies where cases at different stages of the disease or with different clinicopathological characteristics at the time of the diagnosis are compared cross-sectionally.

As a result of the aforedescribed heterogeneity across studies, we were not able to conduct a formal meta-analysis. Instead, we performed statistical tests in which the magnitude of the effects is not considered. We did not use standardizing approaches, such as the standardized mean differences, as these approaches assume homogeneous underlying standard deviations across studies and, in our scenario, such assumptions were unrealistic.[79] Consequently, our review produced only qualitative indications of the possible existence and direction of the effects of interest. The statistical tests that we used have limitations. The global test is influenced by the strength of the association and the study size, but does not consider the direction of the association. The vote-counting and sign test have a very low power and do not take into account the magnitude of the effect observed in the studies nor the sample size of each study. The sign test is more powerful but needs an *a priori* specified direction of the association. Combined probability tests also have drawbacks. Fisher's combined probability test is asymmetrically sensitive to small compared with large *P*-values, and it may lose power when there are few large *P*-values. Weighted Z-method takes advantage of weights and is more powerful than Fisher's test when combined studies are of different sizes,[61] but still it does not take into account the magnitude of the effects.

Publication bias is also a possible potent source of bias in our review. As noted above, most of the studies were of limited sample size, based on stored material and some may have been exploratory analyses within the framework of larger studies on PCa. Selection toward publishing studies finding an association and low *P*-values is possible. As there is a strong *a priori* hypothesis that tumor tissue has lower global methylation levels, publication bias may have also affected the direction of the published associations. The role of publication bias cannot be tested in our data using the standard indirect tests, as we could not consider the magnitude of the effects and there was little variation in the sample sizes of the studies included in the review.[79]Consequently, the indications emerging from this review should be used with caution until large replication studies will become available.

Despite the abovementioned sources of heterogeneity and limitations, quite consistent results surfaced from our review. Global DNA methylation levels were consistently lower in PCa tissue compared with non-tumor prostate tissue, and methylation levels were lower in tumors with more aggressive compared with less aggressive characteristics. There were no clear inconsistencies in these findings when analyzed by method of DNA methylation assessment (direct vs indirect methods) or by clinicopathological factors.

In conclusion, DNA hypomethylation was associated with PCa development and progression. However, due to the heterogeneity and small sample sizes of the included studies, along with the possibility of publication bias, this association requires additional assessment. To increase the understanding of global hypomethylation in PCa and to assess the potential clinical diagnostic or prognostic value of this biomarker additional large and well-designed studies will be essential. Careful selection of comparison tissues is an important step, as global methylation levels vary greatly depending on the choice of the histologically benign prostate tissue. Furthermore, application of the standardized molecular techniques would not only ensure quality and reproducibility of the study results, but it would also facilitate comparison between the studies. Finally, to evaluate the possibility of clinical translation, studies should record information on established diagnostic or prognostic markers for PCa.

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Ethics declarations

Competing interests

The authors declare no conflict of interest.