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What will it take to obtain DNA methylation markers for early cervical cancer detection?

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The Papanicolaou (Pap) smear has become the most successful cancer screening method in the world, and has reduced U.S. cervical cancer deaths by 74% since its widespread adoption in the 1950s [1]. The subsequent identification of the human papilloma virus (HPV) as the primary causal agent of cervical cancer has resulted in even greater disease detection, and recently developed subtype-specific HPV vaccines likely hold great promise for disease prevention [2]. Despite these advances, the management of advanced premalignant dysplastic disease, i.e., grades 2 - 3 cervical intraepithelial neoplasia (CIN2/3), remains inadequate to predict post-treatment recurrence or the likelihood of progression to invasive cervical malignancy [3,4]. Furthermore, current vaccines do not confer 100% protection against CIN2/3 lesions or cervical cancer, resulting in questions of cost-effectiveness [5], and the use of HPV vaccines will also mandate more efficient screening approaches. Consequently, highly sensitive and specific biomarkers, in combination with HPV testing and Pap smears, could improve diagnosis of rapidly progressing disease and detect high-grade precursors likely to advance to invasive cervical carcinoma, while also reducing unnecessary follow-up of non-recurrent lesions.

Deoxycytosine methylation, within the context of a 5'-CpG-3' (cytosine-guanine) dinucleotide, represents one such promising biomarker. Methylation is the only known covalent modification of DNA itself, with 5-methylcytosine often referred to as the "fifth base" present in DNA [6]. While DNA methylation is a normal intracellular process, functioning to "silence" parasitic elements or regulate gene dosage, aberrations in DNA methylation are widely observed in all phases of carcinogenesis, including tumor initiation, progression and metastasis [7]. In particular, abnormal DNA methylation often seen in tumors is hypermethylation in regions of the genome described as CpG islands (CGIs), short stretches (500–2000 bp) of genomic DNA enriched for CpG dinucleotides present in almost half of all genes and typically unmethylated in gene promoters in normal tissues [6]. In human tumors, aberrant de novo DNA methylation occurs in 1 - 5% of the estimated 38,000 CpG islands in the human genome [8-10] and can thus serve as an alternative to mutation or deletion in the inactivation of tumor suppressors, representing one of the most prevalent molecular markers yet identified. In addition, DNA methylation is a stable and heritable mark that is readily amplifiable and easily detectable using PCR-based approaches for its sensitive and quantitative detection [11].

As DNA methylation has been firmly established as deregulated in cervical cancer, methylation-prone sequences represent potential biomarkers for this disease. Furthermore, several specific loci have been reported to be methylated multiple independent times in the

literature, suggesting general consistency in the observed methylation frequency for these loci in cervical cancer. As aberrant DNA methylation occurs very early in the transformation process [7,12], some of those previously reported methylated genes in cervical cancer but not normal cervical tissue could potentially serve as early detection biomarkers in the disease.

While a plethora of loci are reported to serve as potential DNA methylation-based early detection markers for cervical cancer, in this issue of *Gynecologic Oncology*, Wentzensen et al [13] asked the important question of which should be chosen for further evaluation, and eventually for screening of subjects. The review encompassed 51 studies and 68 genes, 15 of which have been analyzed in at least five studies. All histological stages and cytological diagnoses of cervical cancer were represented by the nearly 4400 specimens (2836 cancer, 841 normal, 709 CIN). The majority of the studies used qualitative or quantitative methylation-specific PCR (MSP) to analyze gene methylation. Most reports used fresh-frozen samples or exfoliated cells, with combinations of assays and sample types further analyzed by Wentzensen et al [13], finding that MSP of DNA from fresh frozen tissue samples the most common combination. Although normal cervical tissues were analyzed in the majority (51%) of studies, whether those samples represented corresponding normal cells from the same patient was not clear.

As might be expected given the above variables, a wide range of methylation frequencies in cervical cancer was seen for most genes, which could arise from the use of divergent methylation analysis techniques (Table 1), differences in the population in each study (e.g., ethnicity), differences in the HPV subtypes of the cervical cancer collection studied (not fully addressed), and the choice of normal control tissues (as mentioned above). By using weighted mean and frequency ranges, significant heterogeneity of the published methylation frequencies was observed (Table 2; Figure 1). Only a few studies analyzed the same genes; of these, the most frequently (55-58% of specimens) methylated genes were *CDH1* (*E-cadherin*), *DAPK1* (*death-associated protein kinase-1*), *CADM1* (*cell adhesion molecule-1*), and *TERT* (*telomerase*). An intermediate methylation frequency (29-33%) was observed for these genes during cervical cancer progression (CIN2/3 and high grade squamous intraepithelial lesions), Wentzensen et al. [13] could draw no conclusions about their potential for predicting progression to invasive cervical cancer. Similar methylation frequencies for most genes were observed in squamous cell cancer (SCC) and adenocarcinoma (AC), although differential methylation frequencies were seen for a few genes between the two cancer types (Table 3). The distinct nature of SCC and AC means an optimal cervical cancer early detection methylation panel will probably require markers for both subtypes. Due to the wide heterogeneity of the data, Wentzensen et al [13] were not able to identify a reliable DNA methylation biomarker(s) for cervical cancer early detection.

It is actually not surprising that the comprehensive analysis by Wentzensen et al [13] did not identify any consistently methylated loci for early detection of cervical cancer. The likelihood of identifying a single DNA methylation marker with 100% sensitivity and specificity is negligible [14]. If the methylation frequency is low, sensitivity will suffer as the locus yields too few cases. Even for the more frequently methylated loci, like *DAPK1* (methylated in 57% of the samples), one DNA methylation marker cannot be expected to be used for early detection of cervical cancer. Multiple loci (a panel) of frequently methylated markers in a panel would increase sensitivity, and based on their analysis, Wentzensen et al [13] suggest that *DAPK1* and *RARB* (one or both methylated in 33% of the specimens) may be considered for inclusion on such a panel.

What will it take to discover reliable DNA methylation markers for early cervical cancer detection? Microarray and sequencing approaches for genome-wide analysis, such as tiling array hybridization to immunoprecipitated methylcytosine (methyl-DNA

immunoprecipitation or MeDIP), and next generation sequencing platforms (e.g., 454, ABI SOLiD, Illumina Solexa) have not yet been utilized to examine the cervical cancer DNA methylome. These approaches could yield additional biomarkers, as well as new information about general DNA methylation patterns in cervical cancer. Furthermore, recent initiatives for global “mapping” of DNA methylation in various normal and cancer tissues [15] may yield specific methylated DNA sequences that could ultimately be useful for cervical cancer early detection. While such non-targeted approaches have the potential to rapidly identify many more biomarkers, the resulting candidate biomarker loci must still be validated in primary tumors using traditional approaches. Such genome-wide analyses must also be carried out on all histological subtypes of cervical cancer. Translation from the research laboratory to the clinic and potential commercial utility comes with its own set of challenges [16].

Bioinformatic algorithms may also be implemented for methylation biomarker discovery in cervical cancer, similar to recently performed comparative genome hybridization and transcriptional profiling analyses [17]. Recently, Ongenaert et al utilized a new methodology to sort high-throughput microarray data from both cervical cancer cell lines and primary cervical cancer samples and applied a new relaxation ranking algorithm to enrich DNA methylation markers in cervical cancer [18]. While significant enrichment of methylated genes in cervical cancer was demonstrated, no overlap was apparent between the candidates identified in that study (*CCNA1*, *TIMP2*, *TFPI2*, *PEG3*, *RUNX3*, *IGSF4*, *PTEN*, *TNFRSF10D*, *TIMP3*, *APC*) and the three markers (*DAPK1*, *CADMI* and *RARB*) showing elevated methylation in cervical cancer in the current review.

While the major conclusion by Wentzensen et al [13] may be disheartening for patients, clinicians, and laboratory researchers, particularly given the number of studies and significant investment of resources, it underscores the need for greater standardization of current approaches and suggests that large-scale, non-targeted studies may be warranted to further characterize DNA methylation biomarker discovery in cervical cancer. In the near future, the prospect of genome-wide interrogation of DNA methylation in cervical cancer is extremely exciting. The resulting information may provide new candidate early detection markers for study in clinical trials, similar to ongoing studies in other cancers (www.clinicaltrials.gov), and methylation information could be linked to pathobiology and clinical characteristics, potentially providing indicators for treatment and prognosis. If a strong DNA methylation marker panel were developed, the manner in which it would be applied would depend on its sensitivity and specificity [14]. It is unlikely that DNA methylation markers would be used alone for early detection of cervical cancer, and much work remains to be done. However, using epigenomics, applied in concert with now well-established cytology and HPV testing methods, reliable, early cervical cancer detection may become a reality.

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