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Analysis of Methylated Genes in Peritoneal Fluids of Ovarian Cancer Patients: A New Prognostic Tool,

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Epithelial ovarian cancer causes more deaths in the United States and Europe than any other cancer of the female reproductive organs. The latest cancer statistics indicated an estimated 25 400 new cases of ovarian cancer and 14 300 deaths in the United States *(1)*. The primary treatment for early-stage ovarian cancer is surgery, which in theory could be curative in low-risk patients whose disease is limited to the ovaries. The relative importance of prognostic factors such as tumor grade, histologic cell type, and other factors for defining individuals at low risk of recurrence is unknown *(2)*. Cytologic examination of peritoneal fluids forms part of the staging process for ovarian cancer and influences therapeutic interventions *(3)*. It is known that regardless of International Federation of Gynecology and Obstetrics (FIGO) stage, positive peritoneal washing cytology predicts poor prognosis for women with epithelial tumors of the genital tract, except for patients with borderline ovarian tumors *(4)*. Because of the diagnostic pitfalls entailed in the cytologic examination of peritoneal washings *(5)*, some additional methods for peritoneal fluid diagnosis have been tested, including flow cytometric DNA analysis *(6)* and a telomerase assay *(7, 8)*.

Changes in the status of DNA methylation, known as epigenetic alterations, are among the most common molecular alterations in human neoplasia *(9, 10)*, including ovarian cancer *(11–13)*. Moreover, it is now widely known that methylated DNA can be detected in various body fluids and that the methylation status of some genes can be used for risk assessment of various types of human neoplasia [summarized in Ref. *(14)*].

This proof-of-principle study aimed to clarify whether it is possible to define a high-risk group of ovarian cancer patients solely by looking at methylation changes in peritoneal fluids collected at the time of primary surgery. Because nothing is known at present about methylation changes in peritoneal fluids of ovarian cancer patients, we designed this study with a restricted number of analyzed genes. The 15 screened genes were chosen for (*a*) their demonstrated role in regulating cellular adhesion and their possible role in metastasis (*TIMP3*, *CDH1*, *CDH13*, and *APC*), or (*b*) their putative role in carcinogenesis (*PPP1R13B*, *HSPA2*, *HSD17B4*, *ESR1*, *GSTP1*, *CYP1B1*, *BRCA1*, *MYOD1*, *SOCS1*, *TITF1*, and *GSTM3*). The methylation status of these 15 genes was analyzed in 61 peritoneal fluids (58 ascites and 3 peritoneal washings) from ovarian cancer patients. These specimens were brought to the pathologist immediately after collection during primary surgery. One part of the fluid was routinely analyzed by cytologic examination (Papanicolaou staining). The rest of the fluid was centrifuged at 2000*g* for 10 min at room temperature, and the supernatant was stored at −70 °C until further analysis. Peritoneal fluids and clinical data were collected with the patients' consent.

Genomic DNA from peritoneal fluid samples was isolated with use of the High Pure Viral Nucleic Acid Kit (Roche Diagnostics) according to the manufacturer's protocol with some modifications for multiple loading of the DNA extraction columns to gain a sufficient amount of DNA *(15)*. Sodium bisulfite conversion of genomic DNA and the MethyLight assay were performed as described previously *(16–18)*. The primers and probes used in this study were published recently *(15)*. A gene was deemed positive for methylation if the percentage of fully methylated reference value was >0.

For further statistical analysis we included 57 peritoneal fluids (55 ascites probes and 2 peritoneal washings) from patients with primary ovarian cancer (4 of the original 61 cases were diagnosed with borderline ovarian tumors and were excluded). All patients were treated and followed up at the Department of Obstetrics and Gynecology, Innsbruck University Hospital, between 1990 and 2000. All patients except four [FIGO stage Ia (grades I or II) or patients showing poor physical condition] received plat-

inum-based chemotherapy after surgery. The clinicopathologic features of the 57 analyzed patients are shown in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue11/.

We addressed whether it is possible to classify patients by unsupervised hierarchical cluster analysis (average linkage, Manhattan distance), looking solely at the methylation status of the 15 analyzed genes and whether a specific methylation pattern has any prognostic value. We consequently revealed two clusters (see the online Data Supplement): Patients in cluster 1, showing fewer methylated genes, had a shorter overall survival in the univariate analysis ($P = 0.015$; Fig. 1, black line). Additionally, this clustering was a strong prognostic indicator in the multivariate Cox analysis ($P = 0.004$; Table 1), independent of age, FIGO stage, or grading. For those patients with negative peritoneal cytology, a survival benefit was seen in the univariate but not in multivariate analysis (data not shown). This survival benefit seems to be driven by the FIGO stage at the time of diagnosis as positive peritoneal cytology was associated with advanced FIGO stage ($P < 0.0001$). Two clusters were not associated with peritoneal cytology, grading, FIGO stage, or age.

Ahluwalia et al. *(19)* reported that ovarian cancer has epigenetic signatures. The methylation profile of ovarian cancer tissues makes it possible to predict the outcome of a given treatment *(13)*. Similarly, two histologic subtypes of lung cancer can be differentiated on the basis of a specific methylation pattern revealed by cluster analysis *(20)*. We now demonstrate that a group of high-risk patients can be identified solely from their methylation profiles in peritoneal fluids collected at the time of primary surgery (Table 1; see also Fig. 1 in the online Data Supplement) and that the methylation pattern is a prognostic factor independent of established prognostic factors, such as age at diagnosis, FIGO stage, and grading.

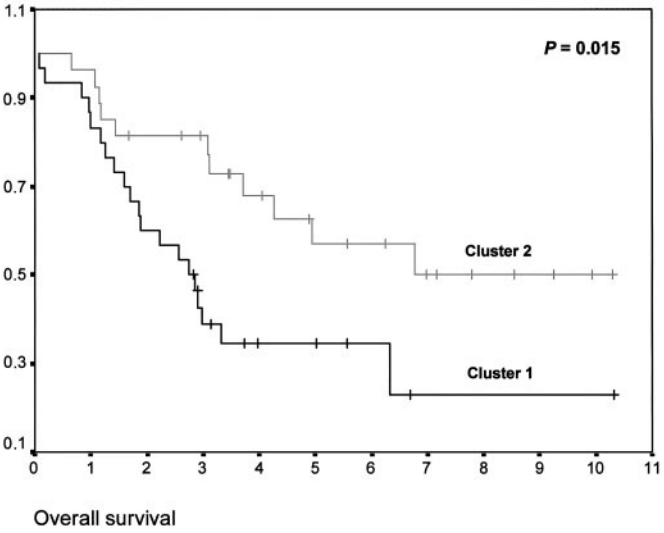

Fig. 1. Overall survival for the two revealed clusters: cluster 1 (*black line*) and cluster 2 (*gray line*).

Kaplan–Meier curves were used for univariate survival analysis. $P < 0.05$ was considered statistically significant. For statistical analysis, we used the SPSS 10.0 software package.

Table 1. Multivariate analysis (overall survival).[a]

	Relative risk (95% CI)[b]	P
Age at diagnosis	1.06 (1.02–1.11)	0.002
FIGO stage (I + II vs III + IV)	11.5 (2.7–50.2)	0.001
Grading (I + II vs III)	1 (0.5–2.1)	0.99
Cluster 1 vs cluster 2	0.3 (0.13–0.7)	0.004

[a] Cox's proportional hazards analysis was used to estimate the prognostic effects of genes adjusted for clinicopathological features.
[b] CI, confidence interval.

Epigenetic changes in neoplasia include genome-wide hypomethylation as well as regional hypermethylation *(9, 10)*. We found a cluster that shows greater regional methylation is associated with better prognosis (Table 1; see also Fig. 1 in the online Data Supplement). Recently published studies have reported improved survival associated with loss of *hMLH1* expression (regulated by methylation) in advanced ovarian cancer *(21)* and that demethylation of FANCF leads to cisplatin resistance in ovarian cancer patients *(22)*. Our results demonstrate that hypermethylation of certain genes is associated with a better prognosis. Because all but four of the patients whose peritoneal fluid had been analyzed received a platinum-based chemotherapy, we speculate that the methylation pattern in cluster 2 represents a surrogate marker for improved chemosensitivity to platinum-based agents. Moreover, DNA hypomethylation in cancers is associated with chromosomal instability *(23, 24)*, widespread derepression of ectopic gene expression *(25)*, transcriptional activation of transposable elements *(26)*, up-regulation of transcriptional noise *(27)*, and an association with a more aggressive histologic type of cancer *(28)*.

In summary, we here show for the first time that DNA methylation of various genes predicts the outcome of ovarian cancer patients independently of age at diagnosis, FIGO stage, or grading. We speculate that DNA methylation analysis of peritoneal fluids may serve as a new tool for risk assessment or proper staging in primary human ovarian cancer or even during second-look surgery. Further studies are needed to identify the role of the methylated genes in ovarian cancer and to evaluate the best group of genes for clinical testing of this new diagnostic approach.

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Procollagen Type I Amino-Terminal Propeptide: Pediatric Reference Data and Relationship with Procollagen Type I Carboxyl-Terminal Propeptide, *Patricia M. Crofton,[1,2*] Nancy Evans,[2] Mervyn R.H. Taylor,[3,4] and Celia V. Holland[5]* ([1] Department of Paediatric Biochemistry, Royal Hospital for Sick Children, Edinburgh, UK; [2] Section of Child Life and Health, Department of Reproductive and Developmental Sciences, University of Edinburgh, Edinburgh, UK; [3]The National Children's Hospital, Tallaght, Ireland; [4]Department of Paediatrics, Trinity College, Dublin, Ireland; [5] Department of Zoology, Trinity College, Dublin, Ireland; * address correspondence to this author at: Department of Paediatric Biochemistry, Royal Hospital for Sick Children, Sciennes Road, Edinburgh EH9 1LF, United Kingdom; fax 44-131-536-0410, e-mail patricia.crofton@luht.scot.nhs.uk)

Type I collagen is the predominant collagen in bone and soft tissue. The rate of synthesis of type I collagen can be assessed by measuring plasma concentrations of the C-terminal (PICP) and N-terminal (PINP) propeptides released during extracellular processing of its procollagen precursor *(1)*. However, the propeptides have different clearance routes, PICP being cleared by mannose receptors *(2)* and PINP by scavenger receptors *(3)* in liver endothelial cells. Clearance of PICP may be modulated by the hormonal milieu, whereas scavenger receptors apparently are not influenced by hormones *(4, 5)*. Within-individual biological variability is similar for PICP and PINP *(6)*, but PINP displays greater dynamic changes than PICP in response to disease and interventions *(7, 8)*. PINP has been shown to be a useful marker of bone formation in adults *(7–12)*.

During childhood growth, markers of bone turnover circulate at higher concentrations than in adults and correlate with height velocity *(13, 14)*. These markers have been used to investigate bone dynamics in childhood disorders of bone and growth *(13–15)*, but a lack of appropriate reference data has hampered use of PINP in pediatrics. Here, we report age- and sex-related reference data for plasma PINP in children from birth to 19 years of age. We also investigated the relationship between PINP and PICP to determine whether their relative clearance rates differ through childhood and adolescence.

Surplus plasma remaining after routine biochemical tests had been completed was retrieved for 43 neonates, infants, and children (23 males) younger than 5 years, who presented with various minor conditions that were considered not to have either a short- or long-term effect on growth; children with systemic disease or concurrent infections were excluded. Samples were deidentified and stored at −70 °C until analysis.

We also analyzed stored plasma from 284 children (140 males), ages 4–19 years, who had participated in an earlier population-based epidemiologic study on the seroprevalence of toxocariasis in Irish schoolchildren *(16)*. Ten samples from each gender and age group were analyzed, except for girls 4 years of age and boys 16 and 18 years of age, for whom only 4, 6, and 4 samples,