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Analysis of HOXA9 methylated ctDNA in ovarian cancer using sense-antisense measurement

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ABSTRACT

DNA promoter methylation is an early event in tumorigenesis and holds promise as a valuable marker in ovarian cancer (OC). It can be measured using circulating tumor specific DNA (ctDNA) isolated from the bloodstream. Sensitivity, however, is a limiting factor of its diagnostic feasibility in OC. DNA methylation analyses are based on bisulfite conversion, resulting in two DNA strands that are no longer complementary. The current standard strategy would then target only one of the double stranded DNA strands, but the potential to increase the sensitivity by targeting both DNA strands is available. In this study, we aimed at evaluating the diagnostic potential of methylated HOXA9 ctDNA in OC by targeting both the DNA sense and antisense strand. Methylated HOXA9 was detected in the plasma of 47/79 (59.5%) patients with newly diagnosed OC using sense-antisense droplet digital PCR. Simultaneous sense-antisense measurement increased the sensitivity by 14.6% (51.9% to 25%). In conclusion, simultaneous measurement targeting both DNA strands can increase the sensitivity and the analytical approach appears valuable in the diagnostic setting of OC.

1. Introduction

Early diagnosis of ovarian cancer (OC) remains an unmet challenge in gynecological oncology. Despite progress in both surgical and nonsurgical treatment, including the use of PARP inhibitors especially in patients with homologous recombination deficiency (HRD positive) and/or BRCA1/2 mutations, early diagnosis is still the key to improve the prognosis. OC can be cured in up to 90% of the cases if diagnosed at an early stage when the disease is still limited to the ovaries [1].

So far, screening by serum cancer antigen 125 (CA125) and/or ultrasound (US) has failed [2] despite several attempts to develop algorithms and diagnostic tools to distinguish benign from malignant pelvic masses [3].

The development of a risk of OC algorithm (ROCA) [4] following large randomized controlled trials [5,6] and improvement in diagnostic US including the ‘simple rules’ by International Ovarian Tumor Analysis (IOTA) to determine the malignancy risk [7] has not managed to improve early diagnosis significantly. The fact that the disease may start in the fallopian tube [8] and tends to spread into the abdomen before it manifests itself in the ovaries, makes diagnostic US even more difficult.

Ideally, a liquid biopsy with sufficient sensitivity and specificity would be a cornerstone for earlier diagnosis, but so far, no marker has shown sufficient clinical utility. CA125 is currently used for treatment monitoring, but as its sensitivity and specificity is poor, it does not meet the criteria of an ideal marker for screening [9,10]. Also, a proportion of OC patients express little or no CA125, especially in early stage disease [11].

Malignant tumors are characterized by aberrant DNA methylation. Hypo- or hypermethylation of the promoter region of a gene holds important biological information and seems to be rather stable. It is also thought to be one of the earliest molecular changes and therefore appears promising for detection of even early-stage disease [12–16]. Since...
cancer cells undergo changes such as apoptosis, necrosis, and phagocytosis, cell-free DNA (cfDNA) is released into the bloodstream. Part of the cfDNA derived from the primary tumor or metastatic sites can be detected as circulating tumor DNA (ctDNA), which holds perspective as a marker of DNA methylation changes in the plasma.

Methylation changes of the Homeobox A9 gene (HOXA9) have to some extent been investigated for early diagnosis in OC [13,17,18] but with limited success so far. The HOXA genes coordinate the patterns of the Müllerian system during embryogenesis, with HOXA9 normally expressed in the fallopian tubes [19]. Aberrant methylation of HOXA9 has been found in OC tissue, but the knowledge of its significance to the development of ovarian malignancy is insufficient.

To bring methylated ctDNA into clinical application the optimal methodology for accurate and reproducible quantification of methylation status needs to be established. Digital PCR allows detection at very low numbers of molecules, but its sensitivity remains the limiting factor for early stage disease with a low amount of ctDNA shed by small asymptomatic tumors.

The standard method for detection of DNA methylation is based on the conversion of unmethylated cytosines to uracils after bisulfite treatment, leaving methylated cytosines unchanged. Consequently, the two DNA strands are no longer complementary and the subsequent analysis targeting one DNA strand for methylation detection thereby utilizes only half of the available DNA. Potentially, the sensitivity could be increased with assays targeting both the sense and the antisense strand of the DNA methylation marker [20,21].

For this study, we developed assays targeting the DNA sense and antisense strands of the genomic region of HOXA9 using methylation specific droplet digital PCR (ddPCR), as we aimed to evaluate the diagnostic potential of methylated HOXA9 ctDNA (meth-HOXA9) in OC applying a simultaneous sense-antisense technique.

2. Materials and methods

2.1. Material for determination of limit of blank and establishment of cutoff for a positive sample and method validation (Cohort 1 and Cohort 2)

Plasma from 64 self-reported healthy women was collected to establish the limit of blank (LOB) and cutoff for a positive sample [22] (Fig. 1, Cohort 1). To validate the sense-antisense ddPCR assays as compared to antisense assay alone, 26 patients with recurrent OC undergoing palliative chemotherapy were included (Ethics Committee; S-20160049, Cohort 2). The patients had previously undergone meth-HOXA9 antisense analysis and were selected to represent a range of meth-HOXA9 values.

2.2. Material for diagnostic potential of methylated HOXA-9 (Cohort 3)

Plasma from patients with newly diagnosed OC was obtained from two protocols. From 40 patients with localized disease (FIGO stage I-II) the plasma was collected from The Danish CancerBiobank under Bio- and GenomeBank Denmark, RBGB. Access to the data was approved by The Regional Committee on Health Research Ethics for Southern Denmark (S-20190040), the Danish Data Protection Agency (19/16314) and a signed contract between researcher and RBGB.

Plasma from 39 patients with advanced OC (FIGO stage III-IV) referred for neoadjuvant chemotherapy was prospectively collected in a translational biomarker protocol (S-20160051) between December 2018 and February 2021. All patients gave written and orally informed consent to the blood sampling. Plasma was obtained within 14 days prior to surgery (FIGO stage I-II) or just before initiation of neoadjuvant chemotherapy (FIGO stage III-IV).

Patients were histologically classified according to the World Health Organization (WHO) criteria and tumor stage was established according to the International Federation of Gynecology and Obstetrics (FIGO) criteria [23].

2.3. Isolation and quantification of DNA

Blood collection and plasma isolation was performed using the same standard operating procedure for patients and healthy controls. Within 4 h after collection in 9 mL EDTA tubes the plasma was separated by centrifugation at 2000g for 10 min and stored at minus 80 °C until use. It was then centrifuged again for 10 min at 10,000g and CPP1 was added as exogenous purification control before DNA extraction [24], ctDNA was extracted from 1.6 to 4.1 mL (mean: 3.2 mL) plasma by the QIA-symphony purification system (Qiagen, Hilden, Germany), using the QIA-symphony DSP Circulating DNA kit as specified by the manufacturer. The purified ctDNA was eluted in 60 µl M–elution buffer after which 340 µl water was added to all samples. A qPCR for the beta-2-microglobulin gene (B2M) and Glycine max cysteine-rich Polycomb-like Protein (CPP1) was then performed to quantify the amount of DNA in each sample for quality control [24]. Samples were concentrated to 20 µl on Amicon Ultra-0.5 Centrifugal Filter Units (Merck, Darmstadt, Germany).

2.4. HOXA9 methylation analysis

The purified DNA was bisulfite converted using the EZ DNA Methylation-Lightning Kit as recommended by the manufacturer (Zymo Research Corp., Irvine, CA, USA). The converted DNA was analyzed with an in-house HOXA9 methylation specific assay and control assay (Aluminiun) [25] using the BioRad® (Hercules, CA, USA) Droplet Digital PCR system QX200 according to the manufacturer’s instructions. The sense and antisense probes specific for methylated bisulfite-converted DNA were labeled with FAM or a mixture of FAM/CAL Fluor orange fluorophores, resulting in separated fluorescent signals to investigate the correlation and sensitivity of the sense-antisense ddPCR analysis (Supplementary Fig. 1). Details on primer and probe sequences are listed in Table 1.

Primers and HOXA9 probes were purchased from LGC Biosearch technologies, Aarhus, Denmark and the albumin probe from Thermo Fisher Scientific (Waltham, MA, USA). Human methylated control DNA (Hercules, CA USA) Droplet Digital PCR system QX200 according to the manufacturer’s instructions. The sense and antisense probes specific for methylated bisulfite-converted DNA were labeled with FAM or a mixture of FAM/CAL Fluor orange fluorophores, resulting in separated fluorescent signals to investigate the correlation and sensitivity of the sense-antisense ddPCR analysis (Supplementary Fig. 1). Details on primer and probe sequences are listed in Table 1. Primers and HOXA9 probes were purchased from LGC Biosearch technologies, Aarhus, Denmark and the albumin probe from Thermo Fisher Scientific (Waltham, MA, USA). Human methylated control DNA

![Cohort 1](https://via.placeholder.com/150)

![Cohort 2](https://via.placeholder.com/150)

![Cohort 3](https://via.placeholder.com/150)

**Fig. 1.** The different cohorts used in the study for determination of limit of blank and cutoff value (Cohort 1), method validation (Cohort 2) and analysis of the diagnostic potential of meth-HOXA9 (Cohort 3).
Regression coefficient (R²) of 0.99 (p < 0.001) implying a linear correlation between sense-antisense analysis and antisense alone using meth-HOXA9 control DNA at various dilutions (undiluted, x2, x4, x8, x16, x32, x64, and x128) to validate the correlation between the number of copies measured by simultaneous sense-antisense assays and the antisense assay alone. QuantaSoft™ version 1.7.4 (BioRad®, Hercules, CA, USA) was used to analyze the data and quantify HOXA9 methylation.

2.5. CA125 analysis

Plasma samples for CA125 analysis were collected before treatment (surgery or neoadjuvant chemotherapy) and analyzed using Cobas e801 and the Elecsys® CA125 II kit (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer’s instructions.

2.6. Statistical analysis

Categorical and continuous variables are presented as frequencies and means, respectively. Assumption of normality was checked for the continuous variables. Comparison between groups was made with Wilcoxon rank-sum test for numeric non-parametric variables and Student’s t-test for numeric parametric data. Fischer’s exact test or chi-squared test was used for binary parametric data to compare two unpaired groups.

Meth-HOXA9 was assessed both as a continuous (percentage) and a binary (detectable or undetectable) variable.

Linear regression models were applied for evaluation of linear correlations. All reported p-values were two-sided, and p-values < 0.05 were considered statistically significant. Results were analyzed using Stata/IC 16.0 (StataCorp LLC, College Station, Texas, USA).

3. Results

3.1. Methylated HOXA9 cfDNA validation, LOB and cutoff value for a positive sample

The comparison of sense-antisense and antisense alone measurement using purchased human meth-HOXA9 control DNA is shown in Supplementary Fig. 2. The slope was 1.99 (95% CI 1.77–2.21) and the regression coefficient (R²) 0.99 (p < 0.001) implying a linear correlation between sense-antisense analysis and antisense alone using meth-HOXA9 control DNA at various dilutions.

Plasma from 64 self-reported healthy women were analyzed (Cohort 1) to establish the LOB. The mean amount of cfDNA in the samples was 1860 B2M/mL (range: 519–5155). The number of meth-HOXA9 positive droplets was ranked and a line at the 95% percentile was drawn. An LOB of 4 positive droplets was found in both the antisense and sense-antisense measurement and the cutoff level was set at < 5 positive droplets [22,26]. The LOB was set to 4 droplets in both measurements and a cutoff for a positive sample was ≥ 5 meth-HOXA9 droplets per test accepting a false positive rate per test up to 5%. Since the number of meth-HOXA9 droplets in 61 of the 64 normal samples was < 5, the exact specificity of the meth-HOXA9 analysis was 95.3%. The cutoff value was used as reference value to dichotomize all plasma samples into meth-HOXA9 detectable and undetectable.

HOXA9 data were normalized (after LOB establishment) to the level of albumin and reported as the ratio between meth-HOXA9 and albumin molecules (copies/mL) in order to minimize analytical variation. The fraction of meth-HOXA9 = [meth-HOXA9 copies] / [Albumin copies] × 100 with the 95% confidence interval (CI) based on the Poisson distribution of the molecules in the droplets.

Twenty-six patients with recurrent OC (Cohort 2) were used to validate the simultaneous sense-antisense ddPCR analysis. A linear correlation was found between simultaneous sense-antisense and antisense alone with meth-HOXA9 expressed as a percentage of total, isolated DNA (Fig. 2). Log-transforming of data for parametric analysis yielded a regression coefficient (R²) of 0.96 (p < 0.001).

3.2. Patient characteristics of Cohort 3 (newly diagnosed OC)

Plasma for meth-HOXA9 analysis was obtained from 79 newly diagnosed OC patients of which 40 had localized (FIGO stage I-II) and 39 had advanced disease (FIGO stage III-IV). Patient characteristics are shown in Table 2. Age at diagnosis varied with FIGO stage with a mean age of 56, 60, 75 and 68 years for FIGO stage I, II, III and IV, respectively (p < 0.001). Histopathologically, the majority of the patients (69.6%) had high-grade serous adenocarcinoma (69.6%). The mean level of cfDNA in the whole cohort was 4882 B2M/mL whereas in FIGO stage I-II and III-IV disease it was 4538 B2M/mL and 5235 BM2/mL, respectively (p = 0.014).

3.3. Meth-HOXA9 in newly diagnosed OC patients applying sense-antisense measurement

Meth-HOXA9 cfDNA was detected in the plasma of 47/79 patients (59.5%) with newly diagnosed OC using sense-antisense measurement (Cohort 3), given a positive predictive value of 94% (47/50) when including Cohort 1. Divided into FIGO stage meth-HOXA9 cfDNA was detected in 15 patients (37.5%) with stage I-II and in 32 patients (82.1%) with stage III-IV. A tendency towards a difference in meth-HOXA9 status between histologic subtypes was found (p = 0.054) with mucinous tumors having the highest fraction of undetectable meth-HOXA9 (8/11) cases.
3.4. Sense-antisense measurement as compared to antisense only to detect meth-HOXA9 and the correlation with CA125

Simultaneous sense-antisense assays were investigated and compared to antisense assay alone. Applying the antisense assay only 41/79 (51.9%) newly diagnosed OC patients had detectable meth-HOXA9 ctDNA (Table 3). The sense-antisense measurement detected three additional patients with localized disease, i.e. 12 patients (30%) with FIGO stage I-II disease had detectable meth-HOXA9 ctDNA using the antisense assay compared to 15 patients using simultaneous sense-antisense assays. Correspondingly, 29 patients (74.4%) with FIGO stage III-IV disease had detectable meth-HOXA9 ctDNA using the antisense technique only and three additional patients were positive for meth-HOXA9 using the sense-antisense measurement, which increases the fraction of positive samples to 82.1% (32/39). The use of simultaneous sense-antisense measurement increased the overall sensitivity by 14.6% (51.9% to 59.5%), whereas in patients with FIGO stage I-II disease it was increased by 25% (30% to 37.5%) (Fig. 3).

The combination of meth-HOXA9 ctDNA and CA125 status at diagnosis according to FIGO stage (Table 4) demonstrated that 44 patients had detectable meth-HOXA9 and CA125 > 35 kUI/L (double positive), the majority with advanced disease. Three patients (3.8%) had undetectable CA125 and detectable meth-HOXA9 as opposed to 23 patients (29.1%) with undetectable meth-HOXA9 and detectable CA125.

In nine patients (11.4%) with localized disease both meth-HOXA9 and CA125 was undetectable. If meth-HOXA9 ctDNA and CA125 was combined as a biomarker algorithm, 88.6% of all patients (70/79) could be diagnosed.

4. Discussion

Analysis of ctDNA methylation markers in the blood holds potential for early detection of cancer, but there is a major issue of sensitivity, especially in relation to low stage disease.

In this study, we developed HOXA9 methylation specific PCR assays targeting both the sense and the antisense DNA strand after bisulfite conversion. The simultaneous sense-antisense ddPCR analysis increased the overall sensitivity of meth-HOXA9 as a biomarker by 14.6%, i.e. the number of meth-HOXA9 copies detected increased by targeting both DNA strands. As expected, the sensitivity was higher in patients with advanced disease (FIGO stage III-IV) with detectable meth-HOXA9 ctDNA in 82.1% of the patients. In patients with localized disease, however, the sensitivity increased by 25% using simultaneous sense-antisense ddPCR measurement as compared to antisense assay only.

To our knowledge, no other studies have investigated and quantified meth-HOXA9 ctDNA in plasma using dual-strand assays targeting both DNA strands after bisulfite conversion. A study by Singh et al. [13] reports a sensitivity of 62.2% using serum samples and MethyLight assay targeting one DNA strand of the HOXA9 gene. The study included 44 serum samples from patients with malignant disease, only 10 of which had FIGO stage I-II.

One of the potential limitations incorporating ctDNA for diagnostic use is the so-called ‘non-shedders’, i.e. patients in which meth-HOXA9 ctDNA cannot be detected in the plasma. This could be due to a low amount of DNA shed by asymptomatic tumors or the biology of OC tending to spread from the fallopian tube/ovaries into the abdomen escaping the bloodstream. This results in low numbers of ctDNA copies presented in the blood, as ctDNA only accounts for 0.1% or less of the total cfDNA [27]. Considering the low numbers of ctDNA copies, a significant variability must be anticipated as to how many targets will be subsampled in a 9 mL EDTA tube relative to the expected number [22]. The number of ctDNA molecules in patients with early stage cancer may thus be very low and whether the collected blood volume contains tumor DNA may be a question of chance. Consequently, the current literature is not supportive of using ctDNA for detection of early stage cancer in asymptomatic individuals [27].

To increase the sensitivity the volume of the samples could be extended. Another alternative is to collect liquid biopsies from other sites of origin for screening or diagnostic purposes. A study by Valle et al. [28] investigated DNA promoter methylation in tissue and biofluids of...
control cases. High levels of meth-HOXA9 in the endometrial biopsy observed high levels of HOXA9 methylation in the OC cases compared to endometrial tissue from premenopausal women only (methylation status than serum/plasma indicating that detection of urine DNA was a better indicator of HIST1H2BB and MAGI2 DNA tissue from cervical swabs, serum/plasma and urine. The study found membrane-associated guanylate kinase inverted 2 (MAGI2) in paired OC using cervical swabs, plasma/serum, and urine suggesting that these

Table 4

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<th>Table 4 Combined Meth-HOXA9 ctDNA and CA125 status at diagnosis according to FIGO stage.</th>
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<td>FIGO stage</td>
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<td>FIGO stage I-II disease (N = 40)</td>
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<td>FIGO stage III-IV disease (N = 39)</td>
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Fig. 3. The proportion of patients with detectable meth-HOXA9 using antisense assay alone as compared to the use of simultaneous sense-antisense assays.

clinical validation.

The issue of ‘non-shedders’ also applies to the currently used biomarker CA125 [11]. In our study, the level of CA125 in 12 patients (15.2%) was within normal range (<35 kU/L); all but one had localized disease (FIGO stage I-II). This addresses the inherent difficulties in early cancer detection with normal CA125 in 27.5% of stage I-II patients and emphasizes the need for combining biomarkers for screening purposes and improved early detection.

Methylated ctDNA in plasma appears to be a promising biomarker for diagnostic and/or prognostic purposes in OC [34–36]. Our study suggests that meth-HOXA9 ctDNA has potential as a biomarker in an algorithm for OC diagnostics. Its feasibility as a tool for screening, diagnostic work-up, prognostic and predictive purposes, and monitoring needs to be investigated further [37]. In relation to survival, recent studies have suggested meth-HOXA9 ctDNA in OC to be valuable as a prognostic marker [38–40].

There is an obvious need for new analytical approaches with a better sensitivity to enable screening and early diagnosis of OC. Randomized controlled trials have not yet demonstrated an impact on disease mortality [5,41]. Given the increased sensitivity obtained using ddPCR and simultaneous assays targeting both DNA strands, the potential to detect small asymptomatic tumors by means of ctDNA comes closer.

In conclusion, this study showed that simultaneous measurement of both DNA strands after bisulfite conversion increases the sensitivity of HOXA9 meth-ctDNA and that the marker had high efficiency in discriminating OC from non-malignant samples. The analytical approach improves the sensitivity, which may be valuable for liquid biopsies and should be considered in the screening and early diagnosis of OC.

CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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