Clinical aspects of circulating tumor specific methylated DNA in epithelial ovarian cancer

PhD thesis

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Introduction

Denmark has the second-largest incidence of Ovarian Cancer (OC) in the world (15 per 100,000 women) with a lifetime risk of 2%[1]. The 5-year survival in Denmark is only 40%[2], primary caused by advanced-stage disease at diagnosis (Stadium II-IV), recurrence and resistance to chemotherapy. The few vague symptoms explain why the majority of patients are diagnosed in advanced stage.
Epithelial ovarian cancer (EOC) accounts for more than 90% of all OC cases, with a multifactorial etiology.

The treatment of OC is primary surgical debulking followed by adjuvant chemotherapy or neoadjuvant chemotherapy and interval debulking surgery followed by chemotherapy in around 50%.
By this approach 60-80% of patients will achieve clinical complete or partial response. However, 70-80% of the patients will experience recurrence of disease, most within two years after completion of chemotherapy.
Recurrent OC is an incurable disease in almost all cases and leaves the patients with few or no treatment options, often after several chemotherapy regimens.
Long-term survival is dependent on the result after surgery as well as stage at time of diagnosis and varies in Denmark from 25 – 87 % (FIGO stage IV–II respectively)[1].

In OC, there is a lack of effective biomarkers for early detection, prognosis of clinical outcome and response to treatment, which contributes to maintain the low survival rate. The Cancer Antigen 125 (CA125) is used in pre-operative diagnostics, for measuring tumor response to therapy and for detection of recurrent disease. Increasing CA125 can detect recurrence of disease with a lead time of around 5 months[3].
CA125 does not however, meet the criteria for an ideal tumor marker as both the sensitivity and specificity of CA125 is known to be poor[4] and 20% of OCs have little or no expression of CA125[5], which is why the interest in new markers specific for OC ranks high.

Aberrant methylation occurs in almost all malignant tumors. Methylation of the promoter region silences the gene and methylation of tumor suppressor genes is an important step in carcinogenesis as well as disease progression[6,7].
Part of the methylated DNA is shed in the circulation, as cell-free DNA (cfDNA) circulates at high concentrations in peripheral blood of cancer patients. Circulating tumor DNA (ctDNA) represents a small percentage of cfDNA and is released by the primary tumor cells and carries the molecular alterations corresponding to the tumor including methylated DNA.
Clinical evaluation and application has been facilitated by development of techniques for rapid, deep sequencing of ctDNA in small quantities of plasma, which gives potential to analyse DNA methylation from blood samples. Analysis of plasma, a liquid biopsy, has several advantages compared to tissue biopsies. It overcomes the intratumoral heterogeneity, it is timely correct, convenient to the patients and sufficient material is easily accessed.

In OC a number of mutated genes have been found and investigated, but it is a cumbersome technic to sequence the whole genome and it has not yet been possible to find a single mutated gene specific for OC. A method to detect mutated tumor DNA in a simple blood test specific for OC is of great clinical interest.
Detection of a methylated gene carries a potential advantage compared to sequencing tumor DNA, especially if it is present in most OC cases and does not occur in normal individuals. This can thereby be used as a surrogate for mutated ctDNA instead of the more extensive work of targeting several tumor specific mutated genes in order to detect cancer present in the body.

Homeobox genes

Homeobox genes (HOX) were first discovered in *Drosophila* by the ability of mutations within these genes to cause inappropriate formations of body parts. The majority of our current understanding of the functions of HOX genes has come from studies of knock-out mice. In humans HOX genes regulate tissue regeneration and play critical roles in controlling self-renewal and differentiation of hematopoietic progenitors[8].

HOX genes constitute a gene superfamily, where the mammalian HOX family is the largest and comprises 39 genes that are tandemly organized in 4 clusters (HOXA, HOXB, HOXC, HOXD) located on different chromosomes. Overexpression or down-regulated expression of many HOX genes has been observed in a wide variety of malignancies, due to their influence in apoptosis, receptor signaling, differentiation and angiogenesis[9,10].
The mechanisms that cause HOX gene deregulation in solid tumors are however less understood, as the vast majority of the studies deal with hematologic malignancies.

Ovarian tumors express a family of HOX genes that are normally expressed during development of the reproductive tract[11]. The role and mechanisms of HOX genes in the clinical behavior of OC remains unknown[12], but theories include 'reactivating' or overexpression of HOX genes that are normally expressed during embryonic development[13].
The HOX gene HOXA9 has especially been associated with OC. This gene is normally expressed during differentiation of the Müllerian ducts into the female reproductive tract during embryogenesis.
Studies have demonstrated that HOXA9 enables OC cells to adapt to their peritoneal environment and 'educates' different types of stromal cells to become permissive for tumor growth[12]. High HOXA9 expression in OC has therefore been strongly associated with aggressive behavior of tumor cells, reduced survival and promotion of ovarian tumor growth[14].
The mechanism thought to be involved is aberrant methylation of the HOXA9 gene[15,16].

Common to most of the above-mentioned studies is that they use tumor tissue to detect HOXA9. Studies regarding quantification of methylated ctDNA, more specific HOXA9, in blood are only marginally investigated. Small studies from our laboratory suggest, that methylated HOXA9 in blood may serve as both prognostic marker and as a biomarker to predict treatment efficacy in BRCA-mutated OC[17].
Consequently, it seems well motivated to investigate if HOXA9 methylated ctDNA can be applied as a circulating biomarker, with prognostic or predictive importance in the clinical settings.

Purpose
The aim of the present thesis is to investigate the clinical potential of circulating HOXA9 methylated ctDNA with respect to prognosis, prediction and treatment monitoring. It will also focus on early detection of progression.

Hypotheses

- Methylated HOXA9 is present in >95% of all malignant ovarian tumors. It is not expressed in normal ovarian tissue or borderline tumors but can be detected in tissue from OC patients.
- Methylated HOXA9 is found in the plasma of 80% of patients with advanced OC and in 40% of patients with localized tumors. It does not occur in the blood from healthy individuals.
- HOXA9 methylation can be used for monitoring the effect of neoadjuvant chemotherapy and select patients for radical operation.
- HOXA9 methylation can be used as a prognostic factor during palliative treatment in patients with recurrence.
- Patients without or with low levels of methylated HOXA9 in plasma do better compared to patients where HOXA9 converts to a methylated status while undergoing treatment or patients with unchanged high levels of HOXA9 during treatment.

Material and Methods
The following describes the three sub-studies in the PhD thesis. The studies are based on blood samples, except study one, where tissue is included in order to perform a methodological study to test the hypothesis that methylated HOXA9 fulfills the criteria of a valid biomarker.

Study 1:

A methodological study to characterize methylated HOXA9 levels in tissue and blood in patients with OC.

Main objective:
This study will evaluate the presence of methylated HOXA9 in OC tumor tissue as compared with that of borderline and benign tumors. Secondly, investigate and compare HOXA9 methylation in plasma from healthy women with that of patients with OC. The investigations will establish the limit of blank (LOB) and limit of detection (LOD) for HOXA9 as a biomarker both in tissue and in plasma.

Material:
The material for the study has already been collected. The tissue has been collected from 2005-2008 as part of a translational biomarker protocol (S-VF-20050009). The material was collected at the time of surgery and consists of malignant, benign, borderline and normal ovaries. HOXA9 analysis will be performed from paraffin embedded tumor tissue. A pathologist will select representative tumor tissue from each section before analysis. To achieve a wide distribution throughout stages, 20 patients with FIGO stage I-II, 20 patients with stage III and 20 patients with stage IV disease will be included – all high-grade serous histology. For comparison between histology types 10 patients with clear cell, 10 patients with mucinous, 10 patients with low-grade serous and 10 patients with endometrioid OC will also be examined.
The HOXA9 levels in the tissue samples with OC will also be compared with HOXA9 methylation in 20 normal ovaries, 20 ovaries with borderline ovarian tumors and 20 tissue samples with benign ovarian tumors.
The blood samples for this study are derived from three different clinical protocols (CaToRoc, VeliBRCA, ToCoOvar) where blood has been drawn at baseline prior to chemotherapy and includes 86 patients with OC. All studies included patients with advanced disease, and therefore, 40 patients with early stage disease will be enrolled from the Danish Cancer Biobank to achieve representation from all disease stages.
Blood samples from 100 healthy controls (without cancer) have already been collected for comparison.
As the material is already collected the study can be performed at the beginning of the PhD study.

Method:
DNA from Formalin-Fixed Paraffin-Embedded (FFPE) tissue is isolated using the Maxwell 16 FFPE Tissue DNA purification kit.
Plasma is separated by centrifugation at 2000 g for 10 minutes within 4 hours after collection and stored at minus 80 degree Celsius until use. Plasma is centrifuged again10 minutes at 10000 g before analysis. DNA is extracted from 4 mL plasma with the QiaSymphony purification system (Qiagen).
After these steps plasma and tissue DNA is analysed in the same way.
DNA is bisulfite converted as recommended by the manufacturer (Zymo research). The converted DNA is analysed with methylation specific assay (HOXA9) and control assay (Albumin) using the BioRad® Droplet Digital PCR system QX100. Water and a pool of lymphocyte DNA from non-cancer individuals is included in each round of analyses as negative controls, and human methylated control DNA (Zymo research) as positive control.

Study 2:

The potential of HOXA9 methylation for prediction of interval debulking surgical outcome after neoadjuvant chemotherapy.

Main objective:
To monitor methylated HOXA9 in blood samples during neoadjuvant treatment with the purpose of investigating if HOXA9 methylated ctDNA can predict the effect as indicated by the results of the operation.

The potential better selection of patients based on HOXA9 status before the comprehensive operation is of major clinical interest as residual tumor is the greatest prognostic factor[18] .
Patients with insufficient treatment effect of neoadjuvant chemotherapy will not achieve major benefit from interval debulking surgery. A preoperative marker to select which patients should be offered interval surgery would therefore have major clinical impact and spare some patients for surgical complications.

Material:
The material is currently being collected from an existing protocol (OC-Neo, S-20160051).
The OC-Neo protocol includes patients with advanced primary inoperable disease allocated for neoadjuvant chemotherapy before interval debulking surgery. Blood samples are drawn before start of chemotherapy, 15 days after the first cycle and prior to each of the three planned neoadjuvant chemotherapy cycles with a further sample one month after surgery.
The study will include 60 patients.

Method:
The method used to analyse the plasma to detect methylated HOXA9 is the same as described in study 1.
In this study consecutive blood samples will be analysed, and we will follow the individual levels of methylated HOXA9 before chemotherapy and compare it with the outcome of surgery.

Study 3:

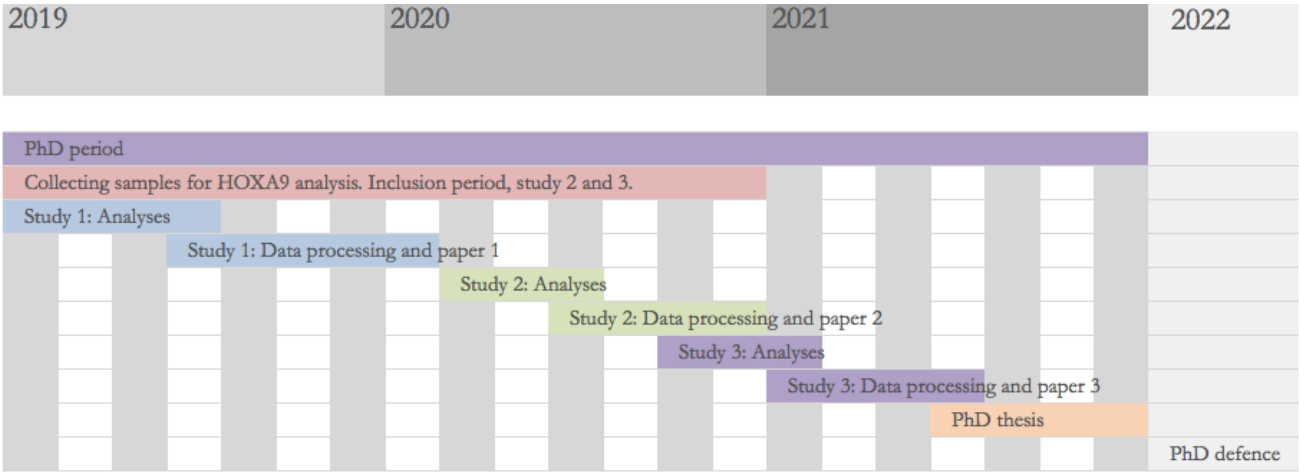
The predictive value of HOXA9 methylation in patients undergoing palliative treatment for recurrence of disease.

Main objective:
To analyse and monitor HOXA9 methylation in patients with recurrence of disease, with the perspective to identify patients who can benefit from palliative chemotherapy.
The study will challenge the hypothesis, that change from methylated to unmethylated status of HOXA9 in plasma is an important prognostic factor. The study will also investigate the prognostic importance of quantitative methylated HOXA9 ctDNA.

Material:
The material is based on an already on-going translational biomarker protocol (OC-Recur, S-20160049).
The protocol includes patients with relapse of OC (all stages) who are offered palliative chemotherapy of any kind.
Blood samples will be drawn before start of chemotherapy, 15 days after the first cycle and at every cycle until progression or stop of treatment for other reasons. The sampling schedule follows the standard procedure in the department.
It will be investigated whether stagnation or elevation of HOXA9 will indicate lack of response or progression of disease, which will be compared to the general clinical condition, CA125 and imaging
The study will include 80 patients.

Methods:
The method used to analyse plasma to detect methylated HOXA9 is the same as described in study 1 and 2.
In this study several blood samples during palliative chemotherapy for recurrence of OC will be analysed, as we will follow the individual level of methylated HOXA9 during treatment to detect response to therapy.

Timeline



Statistics

Analyzes for correlation between HOXA9 levels and clinico-pathologic characteristics of patients will be performed using the chi-squared test or Fishers Exact test when feasible. Univariate Progression Free Survival (PFS) and Overall Survival (OS) analyzes will be performed using Kaplan-Meier survival plots and log-rank equal weighting and multivariate analyzes using the Cox Regression Model. Professional statistical advice will be acquired for finalization of the protocols.
The studies based on materials 1-3 are hypothesis generating and descriptive studies and therefore power of the studies cannot be calculated beforehand without available knowledge of methylated levels of HOXA9.

Ethics

The three studies are combined retrospective and prospective studies. The material in the studies consists of already collected tissue samples and of prospective blood samples only and do not affect the patient's current treatment, according to standard guidelines.
The blood samples consist of 30 ml extra blood per blood test and will be collected along with the already scheduled standard blood tests at each cycle of chemotherapy. The included patients will therefore not be affected regarding this thesis and both written and oral informed consent will be obtained.

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