Title: **A pilot evaluation of prognostic molecular and serum biomarkers of disease recurrence after chemoradiation in patients with locally advanced cervical cancer from EMBRACE study group (BIO-EMBRACE-1)**

Short Title   BIO-EMBRACE-1: translational research pilot study in locally advanced cervical cancer

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TABLE OF CONTENTS

ABSTRACT

The aim of this translational molecular pilot study is to investigate the relationship between markers of epithelial mesenchymal translation, stemness and immune response (systemic and local) with disease recurrence in patients treated with definitive radio (chemo)therapy for locally advanced cervical cancer recruited within either RetroEMBRACE or EMBRACE studies. The tests will be performed in available material from 15 centres in upto 300 patients with data available details on patient, tumor, treatment characteristics and follow-up.

1   BACKGROUND INFORMATION AND RATIONALE

1.1   Introduction

Cervical cancer is one of the most frequent cancers worldwide with 5,28000 new cases and 2,66000 deaths worldwide.(1) Cervical cancer is particularly common in lower and middle-income countries in India, Latin America and Africa. Though there is a relative reduction worldwide, by the year 2030 the absolute incidence is expected to increase to 710000 with an expected mortality of 3,83000 indicating that despite advances in treatment almost 50% of women would continue to die of cervical cancer. Especially since the peak incidence of cervical cancer is among women in the age group 40-50 years, the mortality and morbidity has great societal impact.

Definitive radiotherapy, consisting of external beam radiotherapy followed by brachytherapy, in combination with weekly Cisplatin based chemotherapy (chemoradiation) is considered the standard of care for locally advanced cervical cancer. The introduction of image guided adaptive brachytherapy (IGABT) has further improved local control with a simultaneous decrease of morbidity. After publication of recommendations for the target concept and dose reporting for IGABT by the GEC ESTRO GYN Working Group, the group initiated a series of EMBRACE studies.(2) EMBRACE I is a prospective multicenter observational study on MRI based IGABT and included 1416 patients between 2008 and 2015. RetroEMBRACE was a retrospective study on outcome after IGABT among 852 locally advanced cervical cancer patients from 12 centers between 1998 and 2012, showing excellent local control rates at 5-years of 98% for stage IB, 91% for stage IIB and 75% for stage IIIB. The 5-year cancer specific survival was 73%, with distant metastasis being the most frequent cause of disease recurrence and cancer related death. Despite gains in pelvic control, these are not necessarily reflected in disease free or overall survival as a significant proportion of women develop distant metastasis. Both FIGO stage and nodal involvement are established risk factors for distant metastasis. Mono-institutional publications indicate that tumour volume reduction or changes in functional imaging characteristics during treatment may predict

response to treatment and overall outcomes.(3-8) A systematic review in cervical cancer shows prognostic importance of various biomarkers (9) however, they are not incorporated in clinical decision making as data from well annotated prospective series that have both tissue and imaging biomarkers is lacking. The Cancer Genome atlas has put forth a molecular classification for cervical cancer in 2017 and highlights the possible role of PI3AKT pathway and epithelial mesenchymal transition (EMT) pathway however is based on small cohort of 115 patients and 12 events.(10)

Despite multiple studies investigating the role of tissue or functional biomarkers, FIGO stage and lymph node metastasis currently remain the strongest prognostic factors. So clearly there is a need to further investigate biological factors, which allow accurate risk stratification and response prediction in a well-annotated cohort of uniformly treated patients.

Patients treated in the EMBRACE studies represent a patient cohort that's treated with high quality assurance and optimal uniform chemoradiation and brachytherapy, including extensive annotation and prospective follow-up including patient report quality of life in EMBRACE I. As the study involved baseline MRI imaging for all, it has the strength to better stratify patients on the basis of MRI based disease extent, nodal positivity and actual treatment delivered. As there is complete clinical prognostic information available in this cohort, it is appropriate to further study molecular factors that may have independent prognostic value in predicting disease recurrence.

1.2   Relevant Literature and Data

Translational studies within selected EMBRACE-I centres indicate that expression of hypoxia gene signature, stemness-related proteins, and epithelial mesenchymal translation markers and systemic inflammation-immune index are prognostic factors in cervix cancer.(11-15)

Multiple studies have indicated that hypoxia markers may predict tumour response. Studies at Toronto have focused on HIF 1 alpha, GLUT 1 and CA IX. While CA IX expression was not reported to be correlated with outcomes in a study at Princess Margaret Hospital, a study at National Cancer Centre, Korea tumors with high expression of CAIX in primary tumor tissues were associated with a higher incidence of lymph node metastasis and poor prognosis.(12, 16) When expression of CA IX was examined in primary cervical tumor tissues and their surgically-dissected matching lymph nodes (LN), the primary tumors that showed high CA IX expression also showed high CA IX expression in the matching metastatic LNs, which suggests that the CA IX-expressing clones in tumors are metastatic clones.(12) Recently investigators from Oslo have also reported the ability to classify patients for risk of chemoradiotherapy failure on the basis of hypoxia gene signature that is also identified on Dynamic Contrast Enhanced MRI.(17)

A recently completed randomized study from National Cancer Centre Korea that tailored treatment according to CA IX status in baseline biopsies demonstrated excellent paraaortic control rates in patients with hypoxic tumours that were randomized to prophylactic paraaortic RT arm however this did not impact overall survival.(16) While hypoxia has a prognostic and predictive factor heterogeneous results are available from multiple studies. A negative Phase III NCIC /GOG study that added hypoxic modifier (Tirapazamine) to concurrent chemoradiation in locally advanced cervical cancer highlights the need of investigating other biomarkers that may be driving response and distant metastasis within hypoxic microenvironment.(18) Recent study from head and neck cancers by the Danish group in 158 patients demonstrates the ability of risk stratification on the basis of risk groupings combining stem cell expression (CD44), hypoxia gene signature and HPV positivity.(19) The investigators further demonstrated that risk stratification may be feasible on the basis of p16 and CD44 status and an interplay was identified with expression of CD44 and p16 and risk stratification.(20)

A study from Tata memorial Centre that included 138 patients with locally advanced cervical cancer reported a difference in 3 year disease free survival (DFS) on the basis of coexpression of stem cell markers and CD44 with patients with ESC+/CD44- having a reduced DFS as compared to ESC+/CD44+ population (60% vs 37%).(15) In the same cohort investigators have also observed a difference in DFS on the basis of baseline systemic inflammation immune index. High inflammation immune index at baseline was associated with reduced DFS. On multivariate analysis that included standard prognostic factors ESC+/CD44- population and high systemic inflammation immune index independently predicted for reduced disease free survival. Further investigation into CD44 biology and immune response may therefore be needed.

These findings are also relevant as CD44 links with epithelial mesenchymal pathways that have been identified to be or prognostic value in The Cancer Genome Atlas. Research from Leiden university has demonstrated that risk stratification may be feasible on the basis of L1CAM and PDL1 expression.(14) L1CAM is a molecule indicative of epithelial to mesenchymal transition and is upregulated in squamous cervical cancer; its upregulation was recently shown by us to be associated with improved recurrence free survival. In addition, upregulation of PD-L1 in cervical tumours was shown to also be linked to poor patient survival, specifically when taking into account the expression patterns. This molecule can bind to PD1 on T cells causing anergy and immune escape.(21)

The recently published results of The Cancer Genome Atlas study also revealed distinct molecular subtypes with differences in methylation, copy number variation and upregulated resistance pathways, and results of the BioRAIDS study are awaited.(22)

While various studies have investigated different pathways related to hypoxia, stemness, epithelial mesenchymal transition and immune response there is a lack of integrated analysis of all these relevant pathways in risk prediction in patients with cervical cancer.

The present study is therefore being proposed to undertake an integrated analysis through immunohistochemistry of these 3 distinct pathways (stemness, epithelial mesenchymal transition and immune modulation) on outcomes of patients treated with EMBRACE Studies

1.3 Compliance Statement

This study will be conducted in accordance to institutional review board policies that are prevailing in participating institutions.

2 STUDY OBJECTIVES

This pilot study will focus on identifying markers of disease recurrence through immunohistochemistry performed on formalin fixed paraffin embedded tissue samples that the participating institutions will contribute.

Aim 1: To quantify expression of molecular markers of stemness, epithelial mesenchymal transition and tumour and systemic immune response.

Aim 2: To evaluate prognostic impact of these markers on disease recurrence.

Aim 3: To evaluate the relation between molecular markers and radiomics and their respective prognostic impact.

3 INVESTIGATIONAL PLAN

3.1 General Schema of Study Design

This pilot study will be performed using available paraffin embedded tumor material and available information from baseline blood tests that have been performed prior to initiating chemoradiation in the participating institution to determine the systemic inflammation/immune index.

The study intends to investigate immunohistochemical expression of SOX-2, CD44, L1CAM, CD163 / PDL-1, CD3/CD8/FOX-P3, on baseline paraffin embedded tumor

tissue. Furthermore the study will also investigate relationship between the systemic immune response (quantified by systemic inflammation/immune index, neutrophil lymphocyte ratio and platelet lymphocyte ratio) and local immune response (tumour infiltrating macrophages and lymphocytes)

All biomarker studies will be performed at Leiden University Medical Centre, Netherlands at a central pathology lab. Participating centres from Europe are expected to obtain Material Transfer Agreement (MTA) and ship a total of 5 unstained slides and 1 H&E slide to the central pathology lab. An example MTA is provided (Appendix B). Centres outside Europe (Tata Memorial Centre and PGI Chandigarh) that have difficulty in obtaining permissions for material transfer to Europe, will have the same tests performed at Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Tata Memorial Centre, India. The patient material should be coded using the individual EMBRACE identification code prior to dispatch to preserve patient identity. For EMBRACE studies only the local investigator has access to the key to link the study identification code to the patient identity.

The detailed immunohistochemistry protocol for each of the markers and scoring system for expression quantification is attached as Appendix A.

3.2   Study Duration, Enrollment and Number of Sites

3.2.1   Date Range of Study

The study will collect 1 H&E slide and 5 unstained paraffin slides from patients included in the RetroEMBRACE or EMBRACE studies.. The study period will therefore range for patients recruited from 1998 onwards.

3.2.2   Total Number of Study Sites/Total Number of Subjects Projected

A total of 15 institutions from the first EMBRACE study have expressed willingness to collaborate towards this joint initiative with vast majority of institutions also expressing feasibility of shipping unstained slides to a centralized facility.

We expect a maximal total of 200 patients to be recruited towards this pilot study. It is expected that centers that have agreed to participate may not be able to retrieve material for all patients included in the original studies.

3.3 Study Population

The study population will include patients recruited in the RetroEMBRACE and EMBRACE I studies that have completed treatment and have follow up information available.

4 STUDY PROCEDURES

A. On Tissue Material

The study procedure involves identification of tumor tissue material that can be used for translational research. It will be mandatory that stringent quality assurance is maintained to ensure yield. It is important the tumors are as homogenous as possible and biopsies should meet the minimum requirement to be considered for molecular work.

Centres are expected to provide a total of 5 unstained slides and 1 H & E slide. Tumour sample inclusion for the molecular study necessitates that at least >40% of the sample is composed of viable tumour cells. A Standard Operating Procedure (SOP) for sectioning of formalin fixed paraffin embedded tissue is included as Appendix A.

Institutions are requested to review the material transfer requirements of their institution and choose 1 of the following option

1. To ship 5 unstained and 1 H & E slide per patient under unique EMBRACE study identification code to the central facility (dept. of Pathology Leiden University Medical Center).

For centres where shipping out material is not a possibility, IHC will be performed at Advanced Centre for Treatment Research and Education in Cancer (ACTREC),

Tata Memorial Centre, India, using the described procedure. The IHC standardization slides should be scanned for review by central committee. In addition 10% samples will be reviewed for reproducibility of IHC based scoring.

Following putative prognostic markers will be tested in the slides that have been provided

1. SOX-2, CD-44
2. L1CAM
3. PDL-1/CD163
4. CD3/CD8/FOX-P3

The details of the IHC staining protocol is listed in Appendix B.

The scoring for Sox-2, CD44, will be performed by both intensity of staining and proportion of tumor cells scored positive. As only a small proportion of cells may stain positive for stem cell markers hence both intensity and proportion of cells staining positive is recommended.

Following are the proposed scores

A) Intensity based scoring:

Score 0: No staining; Score 1: weak staining; Score 2: modest staining; Score 3:strong staining.

B) Proportion based scoring

No uptake:

Score 0=Less than 10% of cells:

Score 1= 10-25% positive cells:

Score 2= 26-50% of cells:

Score 3= and 51-75% of cells

Score 4=>75% cells.

For L1CAM the proportion of tumor cell positivity will be scored. The most informative cutoff value that has been described for different cancer types including cervical cancer is >10% L1CAM positive tumor cells.

With regard to PD-L1 apart from proportion based scoring, scoring will include separation between PD-L1 positive tumor cells and immune infiltrate cells.

The scoring for the different subpopulations of T cells and macrophages (CD163+ and CD163+PD-L1+) will be performed by an automated software algorithm using the Vectra PerkinElmer scanner and the Inform software. The quantity of immune cell subtypes will be scored in both tumor and stroma.

B. On Baseline Blood Samples

For patients entering into this study the study database will be retrieved to find the following information on pretreatment blood tests

1. Absolute Neutrophil Count
2. Absolute Lymphocyte Count
3. Absolute Platelet count

These metrics will be used to determine systemic immunoinflammation index, neutrophil lymphocyte ratio, Platelet lymphocyte ratio.

4.1 Data Sources

4.1.1 Case ascertainment

As per proposed inclusion criteria in section 3.3

4.1.2 Data sources

The EMBRACE study database will be used to retrieve clinical characteristics and outcomes of the included patients.

5 STATISTICAL CONSIDERATIONS

5.1 Statistical Methods

After the Immunohistochemistry has been performed the scores of each of the markers will be converted into categorical scores that will represent 2 distinct patient populations i.e. those expressing stem cell markers, epithelial mesenchymal transition markers and immune response markers vs not. Furthermore, combination categories of the biologically linked pathways will be made (i.e. CD44 and L1CAM or SOX2 and CD44). Similarly the relationship of these pathways with immune markers on IHC and systemic immune response will also be tested. The correlative study will focus on evaluating each of these markers and their combinations for their ability to predict disease recurrence, in addition to standard prognostic information that is available. Similarly impact of systemic inflammation immune response in predicting disease recurrence will be tested. The predictive factors identified will be tested using both univariate and multivariate analysis also using correction for multiple testing when applicable.

5.2 Sample Size and Power

The proposed sample size of 300 is a convenience based sampling that is based on estimate of paraffin embedded material likely to be available from the participating institutions. Based on the number of events in the RetroEMBRACE study, the estimated event rate is 30% in this population. The sample size of 300 patients will allow for a hypothesis generating exploratory analysis in this pilot study. Most of the molecular research studies in cervical cancer that have been published till date are based on less than 300 patients and it is likely that the EMBRACE cohort will represent the first large cohort of molecular study in cervical cancer patients treated with contemporary chemoradiation and IGABT.

6   STUDY ADMINISTRATION

6.1   Data Collection and Confidentiality.

Each patient's slides will be identified by a unique EMBRACE study identification code prior to shipping to the central pathology facility. The clinical and other prognostic information will be obtained from the EMBRACE database using the study identification code. In each case patients identity will not be revealed at any step. The data will be coded using the unique assigned EMBRACE study code and identifying data is accessible only to the local study investigators at each participating center.

6.2   Risk Assessment

As participants have been treated several years prior to this study there is no potential direct risk or benefit to the study participants. However the aim is to use the available information to evolve treatment in future.

6.3   Informed Consent

RetroEMBRACE was a retrospective study that did not obtain informed consent from individual patients. EMBRACE I was a prospective observational study that obtained informed consent for the use of patient, tumor, treatment characteristics, and information on prospectively assessed quality of life and clinical follow-up under EMBRACE study code. At the time use paraffin embedded tissue material was not envisioned and not consented for on an individual basis for the purpose of this study. EMBRACE-II has both informed consent for study participation and specifically for the use of paraffin embedded tissue material in position.

For this pilot study each participating individual center is required to obtain IRB approval for a waiver of consent for this study. Several centers may have obtained consent for use of tissue from these patients for use in other related studies or may have a departmental or institutional waiver in place for use of tumor material for these purposes.

7 SAFETY MANAGEMENT

7.1 Clinical Adverse Events

As this pilot study entails immunohistochemical staining of markers on collected paraffin embedded tumor material we do not expect any clinical adverse events or requirement for adverse event reporting.

8 PUBLICATION POLICY

Authorship will be on the basis of direct contribution towards the proposed work.

First author will be the person who did the major part of the research work and wrote the manuscript. The senior author will be the supervising expert of the research work. The BIO-EMBRACE study chairs will have a senior author position,

Co-Authorship will be on the basis of direct contribution towards the proposed work. Each contributing center will have one co-author listed with the authorship sequence based on the contribution of samples towards the research project.

The EMBRACE study chairs will be co-authors, which should include usually two coordinators (including the PI).

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APPENDIX

Appendix A

SOP: Sectioning Formalin Fixed Paraffin Embedded Tissue

1.   Treat all tissue as potentially infectious. Sectioning is performed by the laboratory or histology technician/technologist or personnel trained to use a microtome and cut histological sections.

2.   Have materials and equipment ready. Have as many slides as needed labelled and ready.

3.   Pre-cool paraffin blocks, tissue side down, on a tray of ice. In some cases this may facilitate sectioning. Using a steel microtome knife or disposable blade cut sections that are 4-5 microns and label slides serially. Use slides that are suitable for antigen retrieval treatment at high temperatures (e.g. Strafrost adhesive slides).

4.   Dry paraffin sections at 37° C overnight. Remove the sections from the oven and allow cooling at room temperature.

5.   The sections are stored in slide mailers or stored in slide holder boxes most often at room temperature. Extended storage (usually more than 3 days) of unstained FFPE slides should be avoided as this may result in the loss of antigens or slides should be vacuum sealed and refrigerated (4°C) which may help preserve some unstable antigens.

Appendix B

Protocol for Immune histochemistry

I.SOX-2:

    Antibody Suggested: Abcam laboratories Monoclonal antibody

    Suggested Dilution: 1:250

    Expected Staining pattern: Nuclear

II.CD 44

    Antibody suggested: Abcam Laboratries, Monoclonal antibody

    Suggested Dilution: 1:100

    Expected staining pattern: Cytoplasmic.

III. L1CAM

    Incubate slides overnight at room temperature with mouse monoclonal anti-L1CAM (1:500, IgG1, clone 14.10, BioLegend), and subsequently for 30 min with PowerVision-Poly/HRP (Immunologic, Duiven, the Netherlands). Visualize immunoreactions using 0.5% 3.30-diamino-benzidine-tetrahydrochloryde (DAB) and 0.002% H_2O_2 in Tris-HCl, and counterstain with hematoxylin.

III. Multiplex fluorescent IHC T cells

    CD3CD8FoxP3 staining

1.   Cut 4-6 μm sections and deparaffinize
2.   Perform standard Tris-EDTA antigen retrieval
3.   wash slides in 1xPBS incubate ON with 100μl of FoxP3 (1:100, mouse monoclonal IgG1, clone 236A/E7; Abcam) + CD3 (1:100, ab828 rabbit polyclonal antibody; Abcam) + CD8 (1:100, mouse monoclonal IgG2b, 4B11; Novocastra) in 1% BSA/PBS

4.   wash 3x in PBS

5.   incubate for 1h with 100μl of ALEXA antibodies from Molecular probes:

Goat-anti-mouse IgG1 Alexa 488 (green) +

Goat-anti-rabbit Alexa 546 (red)

Goat-anti-mouse IgG2b Alexa 647 (blue)

diluted 1:200 in 1%BSA/PBS

9.   wash 3x in PBS.

10.   cover slides with 1 drop of MOWIOL/Dapi or Vectashield/Dapi and with a coverslip

11.   store at 4°C

IV. Multiplex fluorescent PDL1/CD163 staining

Follow the same protocol as described for the T cell staining using 1:100 mouse IgG1 anti-CD163 (clone 10D6; Novocastra, Milton Keynes, UK) and PDL1 1:100 rabbit anti-PD-L1 (clone SP142; Spring Bioscience, Pleasanton, CA, USA).