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Evaluation of serum lysyl oxidase as a blood test for colorectal cancer

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Abstract

Aims: Lysyl oxidase (LOX) expression is elevated in colorectal cancer (CRC) tissue and associated with disease progression. A blood test may form a more acceptable diagnostic test for CRC although LOX has not previously been measured in the serum. We therefore sought to determine the clinical usefulness of a serum LOX test for CRC in a symptomatic population.

Methods: Adult patients referred to a hospital colorectal clinic with bowel symptoms completed a questionnaire and provided a blood sample for serum LOX measurement. Associations between presenting symptoms, serum LOX concentrations and outcomes of investigations were tested by univariate and multivariate analyses to determine if serum LOX was clinically useful in the prediction of CRC. LOX expression in CRC and adjacent colon biopsies was evaluated by ELISA and immunohistochemistry.

Results: Thirty-one cases of colorectal cancer and 16 high-risk polyps were identified from a total of 962 participants. There was no association between serum LOX concentration and the presence of CRC, high-risk polyps or cancers at any site. LOX expression was significantly increased in CRC tissue compared to adjacent colon.

Conclusion: Despite overexpression of LOX in CRC tissue, elevated serum levels could not be demonstrated. Serum LOX measurement is therefore not a clinically useful test for CRC.

Keywords: Colorectal cancer; Diagnosis; Serum; Lysyl oxidase

Introduction

There are over 40,000 new cases of colorectal cancer (CRC) diagnosed each year in the UK and the disease accounts for 10% of all cancer deaths.[1] Early diagnosis is associated with improved survival rates and the NHS Bowel Cancer Screening Programme aims to identify early stage disease. Screening, in the form of faecal occult blood tests on a 2-yearly basis from the age of 60 years, has been shown to reduce the incidence of colorectal cancer.[2] Other screening tests are in the process of being introduced following clinical trials of their effectiveness.[3] Approximately half of patients decline screening. Research has found that barriers to screening uptake include fear of the outcome and lack of time, and crucially, many patients may decline screening due to the nature of the test, which requires patients to come into close contact with faecal matter.[4] There is therefore an urgent need for alternative procedures to detect tumour markers that could be used to diagnose bowel cancer at an early stage.

The majority of bowel cancer diagnoses are made following presentation with bowel symptoms to primary care.[5] Only a minority of patients referred to secondary care with bowel symptoms are subsequently diagnosed with bowel cancer.[6] A blood test that could reliably exclude bowel cancer would therefore reduce the morbidity and mortality associated with performing colonoscopy in a

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healthy population, and might be more acceptable to patients than a stool test.

Lysyl oxidase (LOX) is an extra-cellular matrix-modifiying enzyme that has been linked to CRC cell proliferation, metastasis and angiogenesis.[7,8] High expression of LOX correlated with poor prognosis in a variety of different solid tumours[9] and LOX-inhibition reduces metastasis in mice.

LOX is a copper-dependent amine oxidase that oxidises lysine residues in collagen and elastin resulting in cross-linking of these fibres, and this modulation of the extracellular matrix is thought to play an important role in the process of metastasis.[10] LOX has been demonstrated in the circulation by way of serum enzyme activity measurement.[11] However, no published studies to date have directly measured serum LOX protein concentration.

Given the overexpression of LOX in CRC tissue, an evaluation of serum LOX levels as a potential diagnostic marker is warranted. This study measured serum LOX levels in a large cohort of patients who had been referred to secondary care with bowel symptoms to determine whether serum LOX levels, either alone, or in combination with bowel symptoms and patient factors, could predict the incidence of CRC. Tissue LOX levels in biopsies from CRC and matched adjacent colon were also evaluated by ELISA and immunohistochemistry.

Patients and methods

Patients and samples

We had access to stored serum samples from a historical cohort of adults who had been referred to the colorectal clinic at the University Hospital Birmingham NHS Foundation Trust. All patients had been recruited into a previous study evaluating serum MMP-9 levels in colorectal cancer, the details of which have been reported in full elsewhere.[12,13] Patients had given consent for serum samples to be stored and used in the future for the purpose of evaluation of diagnostic tests and new potential biomarkers. Patients were aged 18 or over and had completed a questionnaire detailing bowel symptoms, recent injuries and chronic illnesses experienced in the 3 months prior to questionnaire completion, and personal or family history of bowel cancer. Following the completion of this questionnaire, patients were seen in the clinic and a blood sample taken. All details of clinical examination and investigations together with their outcomes were recorded.

Justification of sample size

The sample size calculation for the MMP9 study was based on the precision with which the sensitivity of MMP9 levels for predicting the presence of colorectal cancer could be estimated. Pilot work had shown MMP9 to have a sensitivity of 99%, thus 60 cases of colorectal cancer were required within the study population to estimate this sensitivity with 95% confidence. A sample size of 1000 patients was therefore chosen, assuming a 6% prevalence of colorectal cancer in the population. In our study, serum LOX sensitivity was unknown, but a conservative assumption that serum LOX sensitivity may be as low as 0.5 was made. Therefore, 32 cases of colorectal cancer would be sufficient to estimate a serum LOX sensitivity of 75% at 90% confidence.

Serum LOX measurement

Blood samples were taken into a red-topped Vacuette tube (Greiner Bio-One Ltd., Gloucester, UK) with no additive and kept on ice until the end of the colorectal clinic. The samples were transported on ice to the laboratory where they were centrifuged and the serum fraction separated and stored at −80 °C until further use. Serum samples were diluted with an equal volume of phosphate-buffered saline and duplicate diluted samples were assayed for serum LOX concentration using an ELISA kit (USCN Life Science Inc., China). ELISA assays were conducted at the same time by 3 individuals who were blinded to all patient and sample details. Linearity of the assay has been demonstrated over a concentration of range of 0.156–10 ng/ml with a sensitivity of 0.053 ng/ml.

Tissue LOX measurement

Tissue samples were obtained from patients undergoing resection for colorectal cancer having given informed consent at the University Hospital Birmingham NHS Foundation Trust, Birmingham, UK. The study was approved by the Local Research & Ethics Committee (South Birmingham 2003/242, renewed 2012). Fresh pieces of tumour tissue and strips of colonic tissue, a minimal distance of 10 cm from the tumour, were taken with the help of an experienced pathologist and snap frozen in liquid nitrogen. Protein was extracted from snap-frozen tissue by incubation in ice-cold lysis buffer (CellLytic MT, 20 μL/mg tissue, Sigma–Aldrich, UK) containing a proteinase inhibitor cocktail (Sigma–Aldrich, UK) and 5 U/mL DNase I (Roche Diagnostics, UK). The tissue was mechanically dissociated in the buffer solution using the GentleMACS tissue dissociator (Miltenyi Biotech, UK). Samples were pelleted and the supernatant collected. Protein concentrations were determined against a bovine serum albumin standard using a bicinchoninic acid assay and sample protein concentrations adjusted to 2 mg/mL by dilution in cell lysis buffer. Protein samples were diluted 1 in 10 parts phosphate-buffered saline and tissue LOX concentration was then measured using the LOX ELISA kit as above.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were deparaffinised and rehydrated by passing the sections through fresh solutions of Clearene (Leica Biosystems, UK) and