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Fecal expression of *Escherichia coli* lysine decarboxylase (LdcC) is downregulated in E-cadherin negative lobular breast carcinoma

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ABSTRACT

Breast cancer is characterized by oncobiosis, the abnormal composition of the microbiome in neoplastic diseases. The biosynthetic capacity of the oncobiotic flora in breast cancer is suppressed, as suggested by metagenomic studies. The microbiome synthesizes a set of cytostatic and antimetastatic metabolites that are downregulated in breast cancer, including cadaverine, a microbiome metabolite with cytostatic properties. We set out to assess how the protein expression of constitutive lysine decarboxylase (LdcC), a key

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enzyme for cadaverine production, changes in the feces of human breast cancer patients (n = 35). We found that the fecal expression of *Escherichia coli* LdcC is downregulated in lobular cases as compared to invasive carcinoma of no special type (NST) cases. Lobular breast carcinoma is characterized by low or absent expression of E-cadherin. Fecal *E. coli* LdcC protein expression is downregulated in E-cadherin negative breast cancer cases as compared to positive ones. Receiver operating characteristic (ROC) analysis of LdcC expression in lobular and NST cases revealed that fecal *E. coli* LdcC protein expression might have predictive values. These data suggest that the oncobiotic transformation of the microbiome indeed leads to the downregulation of the production of cytostatic and antimetastatic metabolites. In E-cadherin negative lobular carcinoma that has a higher potential for metastasis formation, the protein levels of enzymes producing antimetastatic metabolites are downregulated. This finding represents a new route that renders lobular cases permissive for metastasis formation. Furthermore, our findings underline the role of oncobiosis in regulating metastasis formation in breast cancer.

KEYWORDS

breast cancer, microbiome, *Escherichia coli*, LdcC, cadaverine

INTRODUCTION

Breast cancer is characterized by an altered gut [1–7], breast [8], oral [9], and urinary [9] microbiome. The majority of studies reported suppressed microbiome diversity (reviewed in Ref. [10]). Animal [11, 12] and human studies [13] suggest that antibiotic administration that reduces the biomass and diversity of gut flora, increases the risk for breast cancer development. Probiotic treatment has an inverse effect by decreasing the incidence of breast cancer [14]. These observations suggest a pathophysiological role for the oncobiome in breast cancer.

Changes to the microbiome can affect the immune functions of the host [15] and the microbes can modulate the behavior of the host by secreting pleiotropic metabolites. These metabolites get to cancer cells through the systemic circulation [10]. The majority of the studies report lower diversity in the breast cancer oncobiome as compared to the healthy eubiome, resulting in a restricted biosynthetic capacity [3, 6, 7]. This lower biosynthetic capacity probably leads to lower production of bacterial metabolites in both the serum and the tumor. A number of antineoplastic bacterial metabolites were identified in breast cancer, including short chain fatty acids [10], lithocholic acid [7, 16] and cadaverine [6].

Cadaverine can be synthesized from lysine through decarboxylation by bacterial enzymes (LdcC and CadA) [17, 18], although the human body can also produce cadaverine. *Shigella flexneri*, *Shigella sonnei*, *Escherichia coli*, and *Streptococcus* possess enzymes for cadaverine biosynthesis [19]. The main role of cadaverine is to buffer the pH of the environment [20]. The receptors for cadaverine are members of the trace amino acid receptor (TAAR) family [6, 21]. High TAAR1 expression correlates with better survival in breast cancer [22]. Furthermore, TAAR1, TAAR2, TAAR3, TAAR5, TAAR8, and TAAR9 can repress breast cancer [6]. Cadaverine suppresses epithelial-to-mesenchymal transition, cellular movement, chemotaxis, diapedesis, and metastasis formation in breast cancer cells, but has no effect on non-transformed cells [6].

There is an ample set of metagenomic studies in breast cancer cases [10]. However, to our best knowledge, no protein studies are available due to difficult sample preparation and lack of

antibodies for analysis. In the current study, we assessed the expression of a bacterial enzyme, LdcC, which produces the cytostatic bioactive metabolite, cadaverine [6]. The aim of this study was to assess the fecal protein expression of *E. coli* LdcC in breast cancer patients.

MATERIALS AND METHODS

Patient cohort

Fecal protein expression was assessed in a female breast cancer patient cohort composed of 35 participants with a mean age of 57 years. Samples from stage I–III and Nottingham grade 1–3 patients were used in the study. The average stage and grade were not statistically different amongst the groups. Fecal samples were obtained from patients with no special type (NST) and lobular tumors; tumors of other histological types were excluded.

The collection and biobanking of feces were authorized by the Hungarian national authority (ETT). Patients and healthy volunteers meeting the following criteria were excluded from the study: 1) has a previous history of breast cancer or had been operated due to neoplasia, 2) has a disease of unknown origin, 3) has a chronic contagious disease, 4) had contagious diarrhea 6 months prior to enrollment, 5) taken antibiotics within the 6 months prior to enrollment, 6) had chemotherapy, biological therapy, or immunosuppressive therapy 6 months prior to enrollment, 7) used intravenous drugs 12 months prior to enrollment, 8) had piercing, tattooing, acupuncture, or other endangering behavior or action 12 months prior to enrollment, 9) exposure to an allergen to which the enrolled individual had been sensitized to, or 10) underwent colonoscopy 12 months prior to enrollment. The first morning feces was sampled; samples were frozen and deposited in the biobank within 2 h after defecation. Samples were stored at −70 °C until subsequent use. We obtained informed consent from study participants. The patient's routine pathological data were assessed in the study and were compared to the fecal expression of *E. coli* LdcC.

Fecal protein sample preparation

Fecal proteins were isolated as described in Ref. [6]. Fecal samples (100 mg) were lysed in 500 μL RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM NaF, protease inhibitor cocktail) and sonicated (Qsonica Q125 Sonicator, Newtown, Connecticut) 3 times for 30 s with 50% amplitude. After centrifugation, 8 μL β-mercaptoethanol and 25 μL 5× SDS sample buffer (50% glycerol, 10% SDS, 310 mM Tris HCl, pH 6.8, 100 mM DTT, 0.01% bromophenol blue) were added to each 100 μL extract. Then, fecal protein samples were heated for 10 min at 96 °C and held on ice until loading. Protein extract (40 μL) was loaded on 8% SDS-PAGE gels and proteins were separated and transferred onto nitrocellulose membrane. Ponceau-red staining took place after transfer, but before blocking. Ponceau-stained membranes were photographed and used in subsequent analyses. Membranes were cut at the 70 kDa standard and the top part was blotted for LdcC (*E. coli* LdcC, 1:100, Abcam (ab193351)). Membranes were blocked in TBST containing 5% BSA for 1 h and incubated with anti-LdcC primary antibody overnight at 4 °C. After washing with 1× TBS-TWEEN solution, the membranes were probed with IgG HRP-conjugated peroxidase secondary antibodies (1:2000, Cell Signaling Technology, Inc, Beverly, MA,

USA). Bands were visualized by enhanced chemiluminescence reaction (SuperSignal West Pico Solutions, Thermo Fisher Scientific Inc., Rockford, IL, USA). Blots were evaluated by densitometry using Image J software and antibody signals were normalized to total protein stained by Ponceau-red (Sigma-Aldrich). A sample LdcC blot is published in Ref. [6].

Statistical analysis

All data are represented as average ± SEM. Statistical tests are mentioned in figure captions. For statistical analysis, Graphpad 8.1 software was used. The receiver operating characteristic (ROC) curve was re-calculated using R (version v 3.6.3 (2020-02-29)) [23] and the pROC package [24].

RESULTS

Fecal expression of LdcC is low in the E-cadherin negative, lobular subtype of breast cancer

First, we assessed the fecal protein expression of *E. coli* LdcC as a function of the histological subtype of breast cancer. We found that the fecal expression of *E. coli* LdcC enzyme was lower in the lobular subtype compared to the invasive carcinoma of no special type (NST) of breast cancer (Fig. 1A).

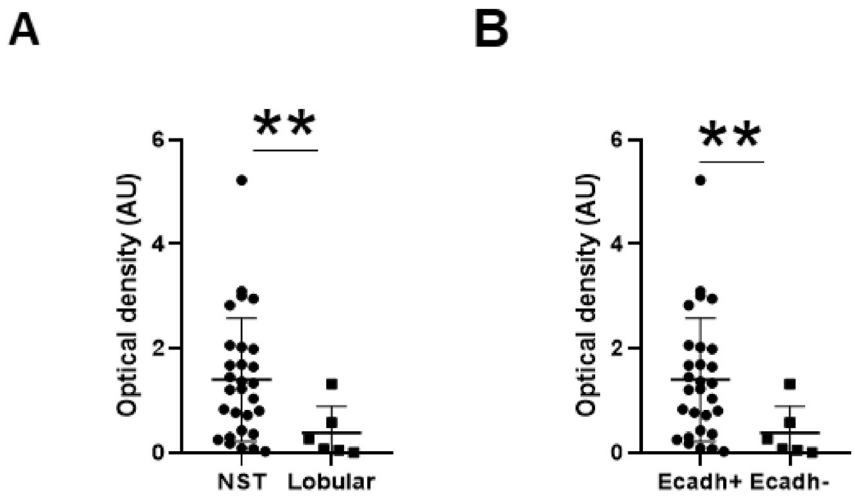

Fig. 1. Fecal expression of *E. coli* LdcC enzyme is lower in lobular, E-cadherin negative than in NST, E-cadherin positive breast cancer cases.
(**A**) Fecal samples of 29 NST and 6 purely lobular cases were assessed by Western blotting using an anti-LdcC antibody. Protein content-normalized values are plotted. (**B**) Fecal samples of 29 E-cadherin positive (Ecadh+) and 6 E-cadherin negative (Ecadh−) breast cancer cases were assessed by Western blotting using an anti-LdcC antibody. Protein content-normalized values are plotted. Data are presented as average ± SEM. Significance between groups was analyzed by two-tailed Student's *t*-test using Welch's correction. ** indicates statistically significant differences between groups at $P < 0.01$

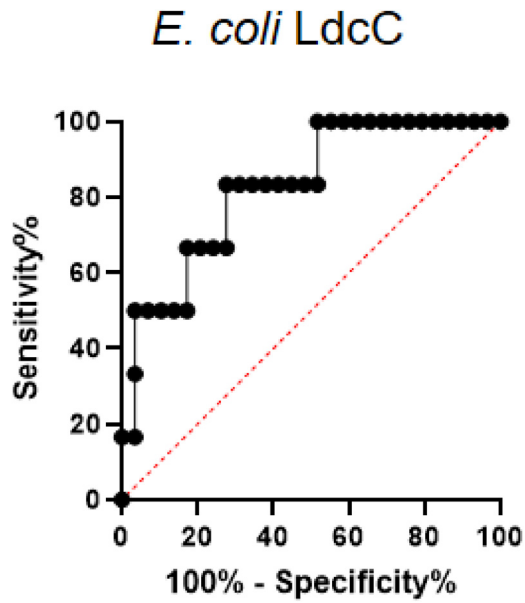

Fig. 2. ROC analysis of fecal *E. coli* LdcC expression to distinguish E-cadherin positive and E-cadherin negative breast cancer cases.
Fecal *E. coli* LdcC protein expression values were entered into an ROC analysis using the Graphpad 8.1 statistical software

Lobular breast carcinoma is characterized by lower or absent E-cadherin expression [25]. Furthermore, the absence of E-cadherin is a predictive factor for metastasis formation, and therefore, predicts poorer clinical outcomes [25–27]. We compared the LdcC expression in E-cadherin positive and negative tumors. The fecal expression of *E. coli* LdcC was significantly lower in E-cadherin negative cases as compared to E-cadherin positive cases (Fig. 1B).

The striking difference in the fecal expression of *E. coli* LdcC in E-cadherin+ versus E-cadherin− cases prompted us to assess whether it would be possible to discriminate between E-cadherin+ and E-cadherin− cases using LdcC expression. To that end, we performed a ROC analysis. According to the ROC analysis, fecal *E. coli* LdcC expression is a good assay to classify E-cadherin+ and E-cadherin− cases (Fig. 2). The area under the curve for the ROC analysis was 0.8276 (Std error: 0.0864, 95% confidence interval [CI]: 0.6583–0.9969). The threshold of the curve was 0.642 (corresponding specificity 83.33% (CI: 50–100) and sensitivity was 72.41% (CI: 55.17–86.21)). The *P* value for the curve was $P=0.0126$.

DISCUSSION

All previous work on the breast cancer oncobiome applied nucleic acid-based techniques [10]. The bacterial metabolome was not assessed in dedicated studies, only tangentially in general metabolomic studies [7, 28, 29]. General bacterial proteomic studies are missing. Due to these

caveats, we do not have a comprehensive picture of the metabolic function of the oncobiome and its pathophysiological role in breast cancer. Our aim was to provide an initial, rudimentary study on fecal bacterial protein expression in breast cancer patients, as a proof of principle study. The major technical drawback we faced was the lack of antibodies against relevant bacterial enzymes in diverse species. In this study, we used an antibody specific to the LdcC enzyme that produces cadaverine, which has cytostatic properties in breast cancer [6]. The anti-LdcC antibody was specific for *E. coli* and there were no available antibodies against the same enzyme in other species. LdcC was downregulated at the DNA level, when comparing breast cancer patients to healthy controls [6]. In support of this observation, breast cancer is characterized by suppressed diversity [10] and suppressed biosynthetic capacity of the gut microbiome [3, 6, 7].

Fecal *E. coli* LdcC enzyme expression was downregulated in lobular E-cadherin negative breast cancer cases. E-cadherin is a cell adhesion molecule, which is crucial in the suppression of invasion [26]. Epigenetic reprogramming or mutations that downregulate E-cadherin expression support tissue invasion and metastasis formation [25–27, 30]. Re-expression of E-cadherin reprograms mesenchymal cells towards epithelial morphology [30]. Lobular breast cancer has a higher potential for metastasis formation, marked by a higher frequency of peritoneal metastases (e.g., Krukenberg tumors). Increased peritoneal metastases is probably linked to low or absent E-cadherin expression. The finding that fecal *E. coli* LdcC expression was lower in E-cadherin negative cases is in good agreement with the observation that oncobiosis supports metastasis formation in breast cancer [6, 7, 15]. Cadaverine, produced by LdcC, suppresses metastasis formation [6].

Earlier, metagenome-based reports have suggested that the breast cancer oncobiome changes as a function of stage [6, 7], estrogen, and HER2 receptor status [1, 2, 10]. Therefore, the association of oncobiome changes with lobular breast cancer or with E-cadherin status is not surprising. However, to the best of our knowledge, breast cancer oncobiome changes have not associated with E-cadherin expression in breast cancer.

What do these data suggest to us from a functional perspective? The literature suggests that the biosynthetic or "bioconversion" capacity of the oncobiome in breast cancer is drastically suppressed [10, 31–35]. Bacterial estrogen reactivation, through the bacterial estrobolome, is upregulated in breast cancer patients. Increased estrogen reactivation and reuptake supports the proliferation of breast cancer cells [3, 4, 33, 35]. Cytostatic bacterial metabolites were also identified, such as short chain fatty acids [10], lithocholic acid [7, 16, 36] and cadaverine [6]. LdcC plays role in cadaverine biosynthesis. LdcC is downregulated in E-cadherin negative cases that have a higher risk for metastasis formation. These observations support the idea that oncobiotic transformation in breast cancer downregulates the production of cytostatic and antimetastatic metabolites and, hence, supports metastasis formation in humans [6, 7, 15]. The bacterial metabolome-driven metastatic events are likely to be parallel mechanisms to the pathways regulated by the loss of E-cadherin or other adhesion proteins.

We are approaching a better understanding of the pathological role of the oncobiome and the oncobiotic transformation in breast cancer. This study provides evidence that the low bacterial production of cytostatic/antimetastatic metabolites in human breast cancer supports metastasis formation in breast cancer. Furthermore, the results of the DNA-based studies suggesting differences in the microbiome in subtypes of breast cancer [1, 2, 6, 7, 35] can be translated into changes in the proteome and, most probably, to changes in the bacterial metabolome as well. From a therapeutic perspective, these data support the applicability of bacterial metabolites in suppressing or preventing breast cancer metastasis.

PERSPECTIVES

Oncobiosis characterizes most cancers [37, 38]. Breast cancer oncobiosis affects the distal gut [1–7], oral [9], urinary [9] and the breast's own microbiome [8]. Apparently, changes to these microbiome compartments can increase the risk for developing breast cancer. Breast cancer cases in women peak around the menopause, hence, aging is a risk factor for breast cancer. Aging comes along with dysbiosys [39, 40] suggesting a likely causative relationship between changes to the microbiome and increased risk to breast cancer. Aging-associated, low grade inflammation is held responsible for the deterioration of multiple organs and organ systems [39–43]. Inflammation may in one hand damage cells and lead to mutations and cellular dysfunction, eventually to cell death [44]. Furthermore, constant low grade inflammation may be a pathway for the aging-associated simplification of the microbiome [39]. These changes are then translated to changes to the microbial metabolome [45] that may in turn give an age-related pathway for breast carcinogenesis.

DATA AVAILABILITY

All primary data are available at https://figshare.com/s/bc183edf92224c293f46 (DOI: 10.6084/m9.figshare.12017550).

Conflict of interest: The authors declare no conflict of interest.

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