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Plasma Protein Profiling Reveal Osteoprotegerin as a Marker of Prognostic Impact for Colorectal Cancer

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Abstract

BACKGROUND: Due to difficulties in predicting recurrences in colorectal cancer stages II and III, reliable prognostic biomarkers could be a breakthrough for individualized treatment and follow-up. *OBJECTIVE:* To find potential prognostic protein biomarkers in colorectal cancer, using the proximity extension assays. *METHODS:* A panel of 92 oncology-related proteins was analyzed with proximity extension assays, in plasma from a cohort of 261 colorectal cancer patients with stage II-IV. The survival analyses were corrected for disease stage and age, and the recurrence analyses were corrected for disease stage. The significance threshold was adjusted for multiple comparisons. *RESULTS:* The plasma proteins expression levels had a greater prognostic relevance in disease stage III colorectal cancer than in disease stage II, and for overall survival than for time to recurrence. Osteoprotegerin was the only biomarker candidate in the protein panel that had a statistical significant association with overall survival ($P = .00029$). None of the proteins were statistically significantly associated with time to recurrence. *CONCLUSIONS:* Of the 92 analyzed plasma proteins, osteoprotegerin showed the strongest prognostic impact in patients with colorectal cancer, and therefore osteoprotegerin is a potential predictive marker, and it also could be a target for treatments.

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Background

Since the detection of carcinoembryonic antigen (CEA) in 1965 [1] a large number of biomarker candidates have been proposed to have a potential prognostic impact in colorectal cancer (CRC). However, CEA is still the only serologic marker recommended in surveillance for CRC by experts groups of American society of colon and rectal surgeons [2] and European society for medical oncology [3].

Due to the lack of sensitivity or specificity of the biomarker candidates and due to the polymorphism of the CRC and the tested cohorts none of the suggested biomarker candidates have shown superiority to CEA. The field is extensively expanding due to new analytic techniques such as next generation sequencing, which adds to the complexity of the information.

The present cohort has previously been used for several studies that have improved our understanding on both soluble and tissue prognostic biomarkers [4–14]. In this study, in search for prognostic biomarkers, the samples were assessed using the proximity extension assays (PEA) [15,16], and a protein panel consisting of 92 highly oncology-related protein biomarker candidates. In the multiplex PEA, each target protein is recognized by a pair of DNA-conjugated affinity binders such as poly- and monoclonal antibodies. Upon simultaneous target recognition the DNA arms on the antibodies are brought in proximity and hybridized to each other allowing an enzymatic DNA polymerization. The newly synthesized DNA

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molecule is then amplified using real-time qPCR. A combination of duel recognition and subsequent signal amplification results in detection of proteins with high specificity and sensitivity. The technology has now been widely used and is demonstrated to be suitable for multiplex and high throughput analyses of panels of proteins in large numbers of samples. The technology has, for instance, been used to identify novel biomarker candidates for small intestinal neuroendocrine tumor [17] to demonstrate the strong effect of genetic and lifestyle factors on protein biomarker levels [18] to identify circulating protein markers predicting of incident heart failure in the elderly [19] to reveal lower levels of several peripheral inflammatory protein biomarkers in women with antenatal depression [20]. The PEA has also been used to characterize exosomal proteome and to trace the exosomes to their originating cells and tissues [21].

The aim of this study was to investigate whether any of the selected biomarker candidates allow prediction of death or disease recurrence in patients with CRC.

Materials and Methods

Patient Samples

The study was prospective and the cohort included patients treated for CRC at the Department of Surgery, Central District Hospital, Västerås, County of Västmanland, Sweden, with a population of 260,000. The study period was between August 2000 and December 2003, and the inclusion criterion was a histologically verified adenocarcinoma of the rectum or colon. The total number of this patients cohort is 324, but for the present study samples from a subgroup of 270 patients were analyzed with disease stages II-IV, excluding disease stage I due to good prognosis with only one recurrence in that group.

Blood samples were collected into endotoxin-free tubes with EDTA one day prior to the planned resection of the CRC. For plasma preparation, the blood samples were centrifuged at 2,000×*g* for 10 min at room temperature, and plasma was transferred to a new tube and stored at −70°C until use. All assays were performed in a blinded manner.

Surveillance was according to national guidelines with computed tomography scan of thorax and abdomen after 1 and 3 years, and colonoscopy every 5 years up to 75 years of age for all patients. Patients with rectal cancer underwent rectoscopy or palpation of perineum every 6 months up to 3 years and then after 4 and 5 years from the operation. Additional radiological examinations outside the surveillance program were made if patients sought with symptoms suspecting recurrence of the CRC.

Information about disease stage, tumor differentiation grade, mucinous histology, death and cancer recurrence were collected from the histopathological, surgical and oncology records.

The latest update on the database was in May 2015 with new recurrences and the exact date of deaths recorded, which were available from the computerized hospital record system.

Protein detection

The PEA was performed using Olink Oncology I panel (Olink Proteomics, Uppsala, Sweden), according to the manufacturer's instructions and as described previously [15,21]. The list of the 92 oncology-related proteins included in the panel is summarized in Table 1. Briefly, 1 µl plasma sample was mixed with 3 µl incubation mix, containing a mixture of 92 probe pairs, in a 96-well plate. Each probe consisting of an antibody conjugated to a unique DNA oligonucleotide. The mixture was incubated at 4°C overnight, allowing recognition of target proteins by a pair of probes. Thereafter, 96 µl extension mix, containing PEA enzyme and PCR reagents, was added, the mixture was incubated for 5 min at room temperature before the DNA extension was initiated in a thermal cycler for 20 min at 50°C, followed by 17 cycles DNA amplification. A new mixture was prepared by adding 2.8 µl of the PCR products to 7.2 µl detection mix in a new 96-well plate from which 5 µl was transferred to a 96.96 Dynamic Array IFC (Fluidigm, South San Francisco, CA, USA) that was in advanced prepared and primed according to the manufacturer's instructions. The unique pair of primers for each protein was loaded in the other side of the array chip and the expression program was performed in a BioMark™ HD real-time PCR platform (Fluidigm, South San Francisco, CA, USA).

The CEA determination was on serum with a commercially available ELISA kit. The analysis is based on the principle of a Solid-Phase-Enzyme-Linked immunosorbent assay. According to the manufacturer's instructions, this assay has a detection limit of 1 ng/ml and the standard range is 5 to 75 ng/ml. (IBL; Immuno Biological Laboratories; http://www.ibl-hamburg.com).

The study was approved by the Regional Ethics Committee in Uppsala, Sweden (Dnr. 2000:001 and Dnr. 2009:345). Written study information was given to the patients, and all patients participating in the study gave a verbal consent. The verbal consent was approved by the ethical committee, and was documented in a questionnaire filled in by the patient or the researcher.

Statistical Analyses

Of the 92 measured proteins, the 78 proteins with less than 20% of the measured values below limit of detection (LOD) were included in the data analyses (Table 1).

Values for CEA measured using the ELISA kit were log-transformed before analysis. To avoid log of zero the transform $\log_2$ (CEA+ 1) was used.

The association between biomarkers and clinical parameters were measured univariately using Mann–Whitney test (gender, mucinous) or Spearman's correlation test (age, disease stage, tumor differentiation grade and CEA levels).

The association between levels of proteins and overall survival or time to recurrence was studied using Cox regression. For each protein a univariate Cox model is performed and summarized using the hazard ratio (HR) with 95% confidence interval and p-value. In addition, multivariate models with both protein level and clinical parameters as independent variable are computed and the association between survival/recurrence and protein level, adjusted for clinical parameters is assessed using the likelihood ratio test (p.lr). The clinical parameters included in the models are age and disease stage for the outcome overall survival and only disease stage for time to recurrence. Bonferroni's method for multiple testing correction was applied.

The recurrence or survival was illustrated with Kaplan–Meier curves, where the patients were divided into two groups with high or low protein levels using the median biomarker level as cut-off.

To investigate whether combination of more than one protein biomarker candidate did increase the prognostic significance, the most promising proteins were combined in a Cox regression model and a permutation test was adopted to check if the achieved association was stronger than expected by random.