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GLIKOZILACIJA HAPTOGLOBINA U KOLOREKTALNOM KARCINOMU

DOKTORSKI RAD

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HAPTOGLOBIN GLYCOSYLATION IN COLORECTAL CANCER

DOCTORAL DISSERTATION

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SAŽETAK

Kolorektalni karcinom drugi je uzročnik smrti među karcinomima, kako u svijetu, tako i u Hrvatskoj. Najčešće se javlja sporadično, ali može imati i nasljednu komponentu. Razvoj novih, specifičnijih, učinkovitijih i manje invazivnih metoda za otkrivanje kolorektalnog karcinoma, unaprijedilo bi dijagnostičke i prognostičke mogućnosti u kliničkoj praksi. Prethodna istraživanja pokazala su promjene u N-glikozilaciji proteina plazme u kolorektalnom karcinomu. Jedan od potencijalnih nositelja tih promjena je haptoglobin, protein akutne faze, koji ima četiri glikozilacijska mjesta u svojoj strukturi. Cilj ovog doktroskog rada bio je razviti analitičke metode za analizu glikozilacije haptoglobina iz seruma ili plazme velikog broja uzoraka na razini glikopeptida i ukupnih N-glikana. Primarno istraživanje napravljeno je na 185 uzoraka seruma ispitanika s kolorektalnim karcinomom i 185 kontrola prikupljenih u biobanci LUMC (engl. *Leiden University Medical Center*) u Nizozemskoj. Analiza je napravljena specifično za svako glikozilacijsko mjesto upotrebom spregnutog sustava tekućinske kromatografije sa spektrometrijom masa. Validacijsko istraživanje provedeno je na 224 uzorka plazme ispitanika s kolorektalnim karcinomom i 269 kontrola. Ovi uzorci prikupljeni su u Kliničkom bolničkom centru Sestre milosrdnice u Zagrebu u Hrvatskoj. Ukupni N-glikom haptoglobina analiziran je na uzorcima validacijskog istraživanja metodom tekućinske kromatografije ultravisoke djelotvornosti s fluorescentnom detekcijom. Primjenom metode logističke regresije ispitana je značajnost povezanosti promjena u glikozilaciji haptoglobina i stanja kolorektalnog karcinoma. Statistički značajne promjene uočene su u povećanoj razini manje razgranatih glikanskih struktura, ukupnoj fukozilaciji i sijalinizaciji na razini određenih glikozilacijskih mjesta i ukupnih N-glikana. Iako su navedene promjene specifične za glikozilacijsko mjesto, rezultati primarnog istraživanja potvrđeni su i u validacijskom istraživanju. Promjene u fukozilaciji i sijalinizacji oslikavaju promjene prethodno objavljene na ukupnim proteinima plazme.

Ključne riječi: kolorektalni karcinom, haptoglobin, N-glikozilacija, tekućinska kromatografija, spektrometrija masa

SUMMARY

Introduction: Colorectal cancer is the second leading cause of cancer-related deaths both worldwide and in Croatia. It most commonly develops through the transformation of an adenomatous polyp, a process that takes approximately 10 years to progress into cancer. The diagnosis is performed through histopathological analysis of colon tissue following a colonoscopy. Currently, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) are used as serum tumor markers for colorectal cancer. There is a need for new, more effective, specific, and less invasive procedures to enhance diagnostics and prognostics, especially if they enable the detection of cancer in the early stages of the diseases.

Increased concentration of acute-phase proteins, along with alterations in protein plasma glycosylation, a common co- and post-translational modification, were previously described as characteristics of colorectal cancer. Haptoglobin is an acute phase plasma glycoprotein, mainly synthesized in hepatocytes. During synthesis in hepatocytes, two chains, the light α and heavy β chain, are formed and connected via disulfide bonds. The haptoglobin structure includes two α chain variants, leading to the distinction of three phenotypes: Hp 1-1, Hp 2-1 and Hp 2-2. On the other hand, the β chain is the only glycosylated chain in haptoglobin and has four N-glycosylation sites: Asn 184, Asn 207, Asn 211 and Asn 241.

Protein glycosylation analysis can be performed at the level of whole proteins, glycopeptides or glycans. Various methodologies are available for studying site-specific protein glycosylation or glycans enzymatically cleaved from proteins. Here, we developed and applied high-throughput methods to explore haptoglobin glycosylation in colorectal cancer, aiming to address whether haptoglobin is one of the carriers of changes in plasma glycosylation in colorectal cancer.

Material and Methods: In this thesis, we developed a high-throughput method for site-specific analysis of haptoglobin glycosylation using liquid chromatography-mass spectrometry (LC-MS).

Haptoglobin was isolated from serum using *Capture Select Haptoglobin Affinity Resin*. Subsequently, it underwent reduction with dithiotreitol and alkylation with iodoacetamide followed by digestion with trypsin. Tryptic glycopeptides were separated into three clusters employing gradient analysis and detected on mass spectrometer in the range between 550 m/z and 1800 m/z.

Additionally, a method for haptoglobin N-glycome analysis using ultra-high performance liquid chromatography with fluorescence detection (UHPLC-FLR) was established. Briefly, haptoglobin was enriched using a monolithic plate immobilized with anti-haptoglobin. The isolated protein was denatured at 60 ºC for 10 minutes with sodium dodecyl sulfate and after that another detergent, Igepal CA-630 was added. N-glycans were enzimatically released by PNGaseF, followed by labeling with procainamide through a reductive amination reaction and subsequently separated into 28 chromatographic peaks using UHPLC-FLR.

Both methods were optimized for application in a 96-well format. Method for site specific analysis was applied to the discovery cohort, consisting of 370 participants, 185 colorectal cancer patients and 185 controls. Samples were obtained from the biobank Leiden University Medical Center in the Netherlands. Furthermore, haptoglobin N-glycome analysis using UHPLC-FLR was performed on samples from the validation cohort. The validation included 224 plasma samples from colorectal cancer patients and 269 controls collected at the Sestre milosrdnice University Hospital Center in Zagreb, Croatia.

The abundance of glycopeptides within the glycosylation sites and individual chromatographic peaks were relatively quantified. Glycopeptides per glycosylation site and individual chromatographic peaks were grouped into derived traits based on their structural similarities. Logistic regression was used to test for associations of glycopeptide and labeled N-glycan features with case-control status. Statistical analysis was also performed on derived traits.

Results: Analysis of serum and plasma samples of participants from both cohorts by using orthogonal analytical methods was additional confirmation that the biological effects measured in the study originate from differences between colorectal cancer patients and controls and not from potential technical effects of the method.

The discovery cohort showed some changes specific for each glycosylation site. Fucosylation within diantennary and triantennary sialylated glycans at Asn 184 was significantly elevated in CRC patients groups compared to controls, while fucosylation within dinatennary sialylated glycans on Asn 241 had opposite direction. Analysis of haptoglobin N-glycome reflected significant change in fucosylation of triantennary sialylated glycans observed at Asn 184. Furthermore, total fucosylation of haptoglobin N-glycans was also significantly increased.

Increased levels in sialylated triantennary glycans were significant at Asn 184 and Asn 241 in colorectal cancer and this these findings were replicated in the validation cohort at the level of

haptoglobin N-glycome. Besides that, elevation in low branched glycans observed on Asn 241 were also confirmed in haptoglobin N-glycome analysis.

Conclusions: The development of analytical methods through this study enables efficient and rapid immunoaffinity isolation and enrichment of haptoglobin from plasma or serum, followed by site-specific glycosylation analysis and at the level of total haptoglobin using nanoLC-MS and UHPLC-FLR techniques, respectively These developed methods can be applied in large scale to study different pathological condition or in population studies.

In this study, observed changes in haptoglobin glycosylation in colorectal cancer were an increase in low branched glycans, fucosylation and sialylation. The majority of changes observed in the discovery cohort were replicated in the validation cohort. Increases in fucosylation and sialylation reflects changes reported previously in total plasma and serum N-glycome.

Keywords: colorectal cancer, haptoglobin, N-glycosylation, liquid chromatography, mass spectrometry