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Human neutrophil peptides 1, 2 and 3 are biochemical markers for metastatic colorectal cancer

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ABSTRACT

Colorectal cancer (CRC) patients have increased levels of human neutrophil peptides 1–3 (HNP1–3) in tumour tissue and plasma. The aim is to study whether the amount of HNP1–3 in tumour and plasma from CRC patients correlate with Dukes' stages A–D. The amount of HNP1–3 in tumour tissue, normal colonic mucosa, and plasma was determined with mass spectrometry. Plasma levels of HNP1–3 were determined with enzyme-linked immuno-sorbent assay (ELISA). The amount of HNP1–3 determined with mass spectrometry was increased in tumours compared to normal colonic tissue in CRC patients in Dukes' stage A–D, whereas HNP1–3 in plasma was only elevated in Dukes' stage D compared to healthy individuals. HNP1–3 plasma concentration determined with ELISA was increased in Dukes' stages C and D, but not in A and B. It is concluded that HNP1–3 is a potential marker for prognostic assessment, surveillance of patients, and monitoring chemotherapy in CRC patients with advanced disease.

1. Introduction

Colorectal cancer (CRC) is the fourth most common cancer worldwide. The clinical stage of CRC at diagnosis determines the survival rate. Patients diagnosed with early stage of CRC have a 75 percent chance of 5-year survival, whereas patients diagnosed with metastatic CRC only have a 5-10 percent chance.[1] The implementation of screening programs for the early detection of CRC could have beneficial impact on the survival rate.[2] Furthermore, improved monitoring of patients with CRC following treatment may increase the survival.[3] In recent years several serum markers have been described for CRC including carcinoembryonic antigen (CEA), CA 19-9, CA 242, tissue polypeptide antigen (TPA), and tissue inhibitor of metalloproteinases-1 (TIMP-1).[4] Only CEA, which is the oldest marker, is routinely used in preoperative assessment of CRC and monitoring of therapy of advanced disease.[5]

Recently, three reports have described that human neutrophil peptides (HNP1–3), also known as α-defensins 1, 2 and 3, as well as human defensin α6 (HD6), are elevated in plasma and tumour tissue from patients with CRC. Using surface enhanced laser desorption/ionisation time-of-flight (SELDI-TOF) mass spectrometry (MS), we reported that HNP1–3 levels are elevated in serum and tumour extracts from CRC patients compared with serum from healthy individuals and normal colonic tissue, respectively.[6] Melle et al.[7] analysed colon cancer epithelium microdissected from colon tumours with SELDI-TOF MS and found that HNP1–3 are more highly

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expressed in the tumour than in the normal epithelium. Furthermore, enzyme-linked immuno-sorbent assay (ELISA) of HNP1–3 in serum showed increased levels in CRC patients compared with healthy individuals.[7] Finally, analysis of microarray data from various tumours and normal tissues showed that HD6 was maximally expressed in colon cancer, and ELISA of serum showed that the concentration of HD6 was increased in CRC patients compared with healthy donors.[8]

HNP1–3 are part of the α-defensin family of peptides, which in humans include six known members: HNP 1, 2, 3 and 4, and HD5 and HD6.[9,10] α-defensins are part of the innate immune response and are active against a variety of bacteria, fungi, parasites and some viruses. HNP1–3 are synthesised in neutrophil precursor cells, and mature circulating neutrophils release HNP1–3 in inflammatory sites. The gastrointestinal tract is a prominent site of defensin expression, where β-defensins are expressed at multiple sites, whereas α-defensins are largely confined to the small intestine.[10] HD5 and HD6 are expressed by Paneth cells in the duodenum, jejunum and ileum, and possible in other parts of the gastrointestinal tract and by other cell types.[11] In the gastrointestinal tract, HD5 and HD6 are believed to play a role in the mucosal defence against microbes and HD5 is upregulated in inflammatory bowel disease.[11,12] HNP1–3 have been observed in intestinal epithelial cells during active inflammatory bowel disease.[11,13,14]

In the present study, we have used SELDI-TOF MS to determine the amount of HNP1–3 in tumour tissue and plasma from patients with CRC in Dukes' stage A–D in order to analyse the correlation between the HNP1–3 level and the clinical stage. HNP1–3 is upregulated in colon tumour tissue compared with normal colonic tissue from patients in all stages of CRC, whereas HNP1–3 is increased in plasma only from CRC patients with metastatic disease. This finding was confirmed by ELISA of HNP1–3 concentration in plasma, and we suggest that plasma HNP1–3 can be used for assessing the prognosis of CRC, and monitoring chemotherapy in CRC patients with advanced disease. However, HNP1–3 cannot be applied as plasma markers for the early detection of CRC.

2. Materials and methods

2.1. Study population and samples

The project was reviewed and approved by the Scientific Ethical Committee of Copenhagen County (Københavns Amts Videnskabsetiske komite: KA 02140g) and the Danish Data Protection Agency (Datatilsynet). With the patients' informed consent, colon tumour and normal colonic tissue and plasma samples were obtained from 32 CRC patients undergoing surgery at the Department of Gastrosurgery, Glostrup Hospital, and University of Copenhagen. The demographic and pathological features of the patients are summarised in Table 1. The tissue and plasma samples from 32 CRC patients were used for SELDI-TOF MS analysis of HNP1–3 (see below). Furthermore, plasma samples were obtained after informed consent from 119 CRC patients (including the group of 32 CRC patients). The demographic and pathological features are summarised in Table 2. These plasma samples were used for HNP1–3 analysis by ELISA (see below). Finally, plasma samples were collected anonymously from 34 healthy individuals after informed consent. The gender (15 females and 19 males), and age range (53–75 years) matched the CRC patients.

2.2. Pathology of CRC

The pathological features of CRC patients included the localisation of the tumour in cecum, ascending colon, tranverse colon, descending colon, sigmoid or rectum. CRC was classified according to the modified Dukes' staging system: Dukes' A (the tumour has grown through the mucosa into the submucosa and muscularis propria of the bowel wall), Dukes' B (the tumour has grown through the muscularis propria of the bowel wall into other nearby tissues or organs), Dukes'

Table 1 – Demographic and pathological features of 32 CRC patients selected for SELDI-TOF MS analysis of colon tumours and plasma samples

Feature	Number of colorectal cancer patients (n = 32)					
Age	43–50:3	50–60:4	60–70:10	70–80:6	80–86:9	
Sex	Females:12			Males:20		
Tumour localisation	Cecum:5	Ascending:3	Transverse:2	Sigmoid:9	Descending:2	Rectum:11
Dukes' stage	Stage A:8		Stage B:8	Stage C:8		Stage D:8
Histological tumour grade	Low:3		Intermediate:27	High:1		Unknown:1

Table 2 – Demographic and pathological features of 119 CRC patients used for ELISA of plasma HNP1–3

Feature	Number of colorectal cancer patients (n = 119)					
Age	43–50:5	50–60:20	60–70:39	70–80:37	80–86:18	
Sex	Females:53			Males:66		
Tumour localisation	Cecum:16	Ascending:8	Transverse:10	Sigmoid:35	Descending:6	Rectum:44
Dukes' stage	Stage A:15		Stage B:46	Stage C:38		Stage D:20
Histological tumour grade	Low:11		Intermediate:99	High:4		Unknown:5

C (the tumour has grown through the bowel wall into other nearby tissues or organs and has spread to 1 to 3 nearby lymph nodes), or Dukes' D (the tumour has spread beyond the confines of the lymph nodes to distant sites such as the liver, lung, peritoneum, or ovary). The histologic tumour grade (or differentiation) was determined by microscopy of tumour sections and ranged from low (well-differentiated), intermediate (moderately differentiated) to high (poorly or undifferentiated).

2.3. Sample preparation

The tissue samples were collected fresh and stored at −80 °C. Tumour and normal colonic tissue (100 mg) extracts were prepared as previously described.[6] Blood samples from fasting CRC patients were obtained immediately before anaesthesia. Blood samples from 34 healthy individuals were collected anonymously from 18 males and 16 females, 50 to 80 years. Blood was drawn into a BD Vacutainer® EDTA Tube, 3 ml, evacuated sterile blood collection tube for whole blood haematology determination, translucent lavender BD Hemogard™ safety closure, Plus (medical grade PET) tube, 13 × 75 mm, K2EDTA (spray), product no. 368856. The tube was kept at room temperature for 30 min before centrifugation at 3000 rpm for 10 min at 4 °C. Plasma was collected, separated into aliquots, and immediately stored in 2 ml plastic tubes at −80 °C.

2.4. Sample analysis

Protein extracts of colonic tissue as well as plasma samples were analysed by SELDI-TOF MS on the PBS II instrument (Ciphergen Biosystems, Inc. Freemont, California) using IMAC30 (Ni) protein arrays as previously described in detail.[6]

The HNP 1–3 ELISA kit was obtained from HyCult Biotechnology, The Netherlands. Plasma samples were analysed in duplicate together with standard measurements, according to the manufacturer's instructions. ELISA plates were measured on a MAXline Microplate Reader (Molecular Devices) at 450 nm. Concentration of HNP1–3 in plasma was calculated according to a standard curve.

2.5. Statistical analysis

Data was analysed with the SAS Analyst 8.02 software. Average values and standard deviation was calculated using descriptive statistics and *p*-values were calculated using *t*-test for means. Receiver operating characteristics curve (ROC) analysis was done with the MedCalc software version 8.2.1.0.

3. Results

3.1. SELDI-TOF MS analysis of HNP1–3 in colonic tissue

The expression of HNP1–3 was measured in protein extracts of tumour tissue and normal colonic tissue from 32 CRC patients using SELDI-TOF MS and IMAC30 (Ni) protein arrays. The analysis of a representative sample of CRC tumour is shown in Fig. 1A. Three protein peaks with mass over charge ratios (m/z) 3372, 3443 and 3486 were observed in the tissue extract (Fig. 1B), corresponding to the oxidised state of HNP2, 1 and 3 with three intact disulfide bridges.[6] The peak intensity of HNP1–3 was relatively strong in all tissue extracts, forming a characteristic pattern, where the middle peak, HNP1 with m/z 3443, produced the strongest signal and HNP2 and 3 produced peaks of lower intensity (Fig. 1B).

The amount of HNP1–3 in protein extracts of normal colonic tissue and tumour tissue was determined with SELDI-TOF MS. Tumour samples from 32 CRC patients with eight in each of Dukes' stage A, B, C and D (Table 1) were analysed and compared with samples of normal colonic tissue from eight CRC patients. In Fig. 2A the peak intensity of HNP1–3

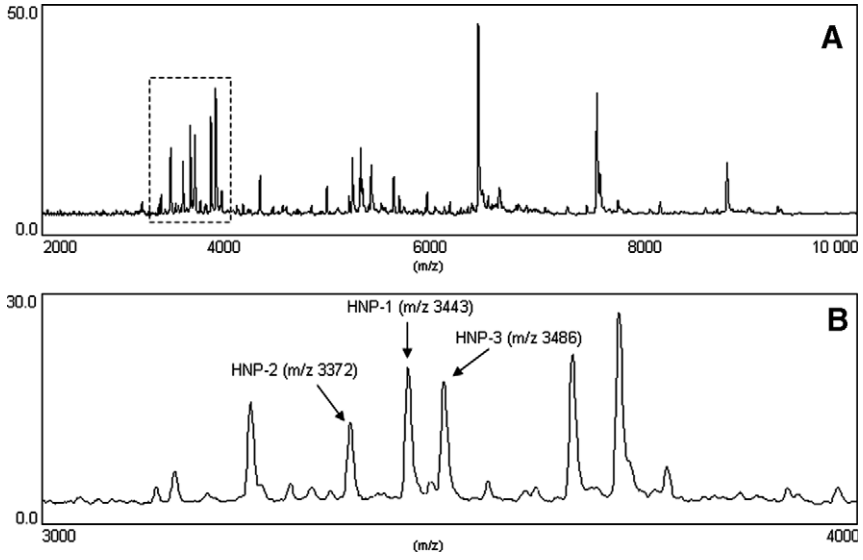

Fig. 1 – Protein pattern in colon tumour tissue determined with SELDI-TOF MS. (A) The intensity of protein peaks is shown as a function of the m/z value in the range from 2 to 10 kDa. (B) The intensity of protein peaks is shown in the range from 3 to 4 kDa. The protein peaks with m/z 3372, 3443 and 3486 represent HNP2, HNP1 and HNP3, respectively.

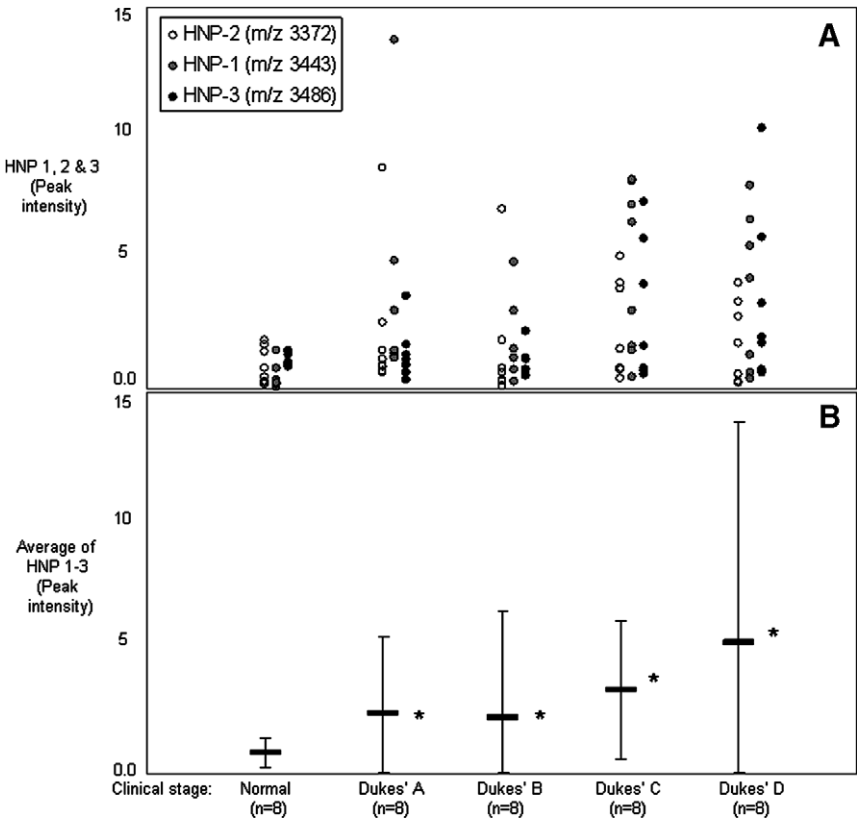

Fig. 2 – HNP1–3 levels in extracts of tumour and normal colonic tissue from CRC patients. (A) The peak intensity of HNP 1, 2 and 3 (m/z 3443, 3372 and 3486) was determined with SELDI-TOF MS in normal colonic tissue and tumours from 32 CRC patients in Dukes' stage A, B, C, and D. (B) The mean value and SD of peak intensities of HNP1–3 in normal colonic tissue and tumours in Dukes' stage A, B, C, and D was calculated. * denotes that the mean value is statistically different from normal colonic tissue with *p*-values < 0.005.

was plotted for each group of normal colonic tissue and CRC tissue from patients in Dukes' stage A-D. In Fig. 2B the average peak intensity and SD for HNP1–3 was plotted versus normal colonic tissue and tumour tissue from CRC patients in Dukes' stage A, B, C and D. The expression of HNP1–3 was significantly upregulated in tumour tissue from patients with CRC in Dukes' stage A ($p < 0.004$), B ($p < 0.002$), C ($p < 0.002$) and D ($p < 0.005$) tumours compared to normal colonic tissue (Fig. 2B and Table 3). However, there was no statistically significant difference between the peak intensity of HNP1–3 in tumour tissue when CRC patients in Dukes' stage A–D were compared (data not shown).

3.2. *SELDI-TOF MS analysis of HNP1–3 in plasma*

The peak intensity of HNP1–3 in plasma from 32 CRC patients (Table 1) and eight healthy individuals was measured with SELDI-TOF MS using IMAC30 (Ni) protein array. Plasma samples from 32 CRC patients with eight in each of Dukes' stage A–D were analysed and compared with plasma samples from eight healthy individuals. The peak intensity of HNP1–3 was low in plasma and was not detected in all samples from healthy individuals and CRC patients. In Fig. 3A the peak intensity of HNP1–3 in plasma was plotted for each group of healthy individuals and CRC patients in Dukes' stage A–D.

Table 3 – HNP1–3 levels in tumour tissue and plasma from CRC patients determined with SELDI-TOF MS and ELISA

Sample type Clinical stage	SELDI-TOF MS (Peak intensity of HNP 1–3)		ELISA (ng/ml of HNP 1–3)
	Tissue Average (SD)	Plasma Average (SD)	Plasma Average (SD)
Normal	0.8 (0.6) ($n = 8$)	0.4 (0.3) ($n = 8$)	96.6 (36.2) ($n = 34$)
Dukes' stage A	2.4 (3.0)[a] ($n = 8$) ($p < 0.004$)	0.5 (0.1) ($n = 8$) ($p > 0.1$)	105.4 (80.6) ($n = 15$) ($p > 0.3$)
Dukes' stage B	2.2 (4.2)[a] ($n = 8$) ($p < 0.002$)	0.5 (0.2) ($n = 8$) ($p > 0.1$)	102.2 (34.9) ($n = 46$) ($p > 0.2$)
Dukes' stage C	3.3 (2.7)[a] ($n = 8$) ($p < 0.002$)	0.6 (0.2) ($n = 8$) ($p > 0.1$)	141.5 (48.5)[a] ($n = 38$) ($p < 0.0001$)
Dukes' stage D	5.2 (8.7) ($n = 8$) ($p < 0.005$)	0.8 (0.2)[a] ($n = 8$) ($p < 0.003$)	244.3 (63.9)[a] ($n = 20$) ($p < 0.000001$)

a Significantly upregulated compared to normal ($p < 0.05$).

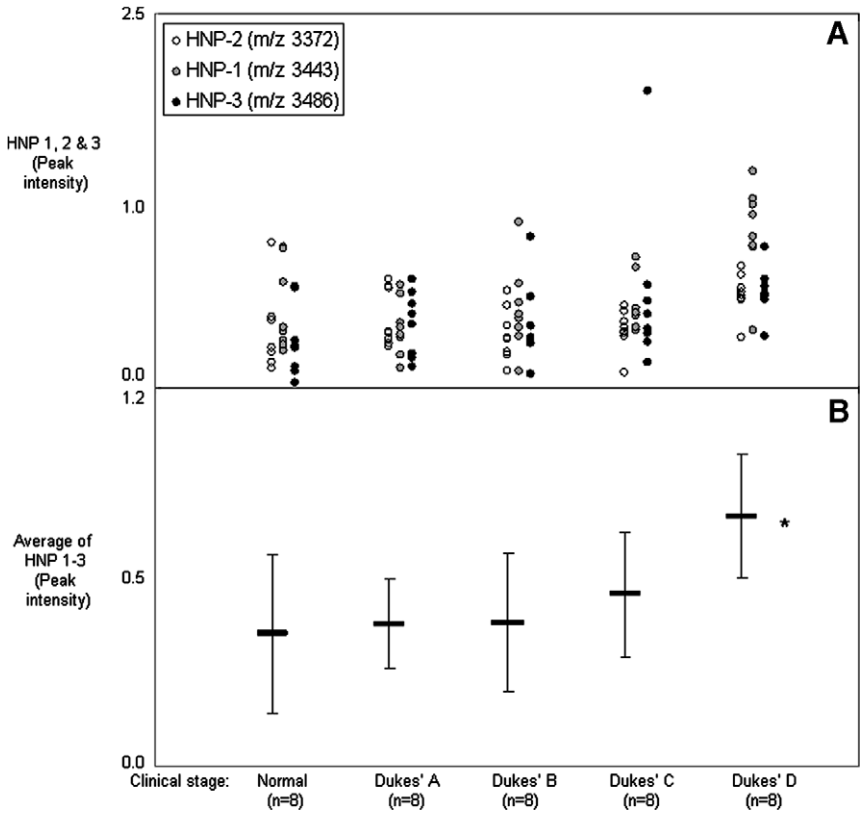

Fig. 3 – HNP1–3 levels in plasma from CRC patients and healthy individuals determined with SELDI-TOF MS. (A) The peak intensity of HNP 1, 2 and 3 (m/z 3443, 3372 and 3486) in plasma was determined in 34 healthy individuals and 32 patients with CRC in Dukes' stage A, B, C and D. (B) The mean value and SD of peak intensities of HNP1–3 in plasma in healthy individuals and patients with CRC in Dukes' stage A, B, C and D was calculated. * denotes that the mean value is statistically different from normal colonic tissue with *p*-value < 0.003.

In Fig. 3B the average peak intensity and SD of HNP1–3 was plotted for healthy individuals and CRC patients in Dukes'-stage A–D. The peak intensity of HNP1–3 was not increased in plasma from CRC patients in Dukes' stage A, B or C compared to plasma from healthy individuals. However, the peak intensity of HNP1–3 was significantly increased in plasma from patients with CRC in Dukes' stage D compared to plasma from healthy individuals ($p < 0.003$) (Fig. 3B and Table 3).

3.3. *ELISA of HNP1–3 in plasma*

The plasma concentration of HNP1–3 was measured with ELISA in samples from 119 CRC patients in Dukes' stage A–D (Table 2), and from 34 healthy individuals. In Fig. 4A the plasma concentration of HNP1–3 was plotted for each group of CRC patients in Dukes' stage A–D and healthy individuals. In Fig. 4B the average plasma concentration and SD of HNP1–3 was plotted for each group of CRC patients and healthy individuals. The average concentration and SD of HNP1–3 in plasma is shown in Table 3. The plasma concentration of HNP1–3 was significantly increased in CRC patients in Dukes' stage C and D compared to healthy individuals ($p < 0.0001$ and $p < 0.000001$, respectively). There was no significant difference between the plasma HNP1–3 levels in CRC patients in Dukes' stage A or B and healthy individuals ($p > 0.1$) (Fig. 4B and Table 3).

ROC analysis of HNP1–3 levels in plasma from CRC patients versus healthy individuals was used to calculate the sensitivity and specificity of discriminating CRC patients from healthy persons. First, ROC analysis of HNP1–3 levels was used on all CRC patients in Dukes' stage A–D ($n = 119$) versus healthy persons ($n = 34$) resulting in a sensitivity of 79.8% and a specificity of 52.9% using a cut-off point of HNP1–3 at 86 ng/ml. Next, ROC analysis of HNP1–3 levels in CRC patients, Dukes' stage C and D ($n = 58$) versus healthy persons ($n = 34$) gave a sensitivity of 77.6% and a specificity 76.5% using a cut-off point of HNP1–3 at 117 ng/ml. Using a lower cut-off point of HNP1–3 at 95 ng/ml the sensitivity increased to 91.4%, whereas the specificity decreased to 55.9% for Dukes' stage C and D. Finally, ROC analysis of HNP1–3 levels in plasma from CRC patients in Dukes' stage D ($n = 20$) versus healthy individuals ($n = 34$) gave a sensitivity of 100.0% and a specificity of 82.2% with a cut-off point of HNP1–3 at 144 ng/ml.

4. Discussion

We have previously reported that the amount of HNP1–3 is increased in protein extracts of colon tumour tissue and in serum from CRC patients using SELDI-TOF MS analysis.[6] In agreement with our findings, Melle et al.,[7] showed that HNP1–3 are more highly expressed in tumour tissue than in normal colonic epithelium from 39 CRC patients using

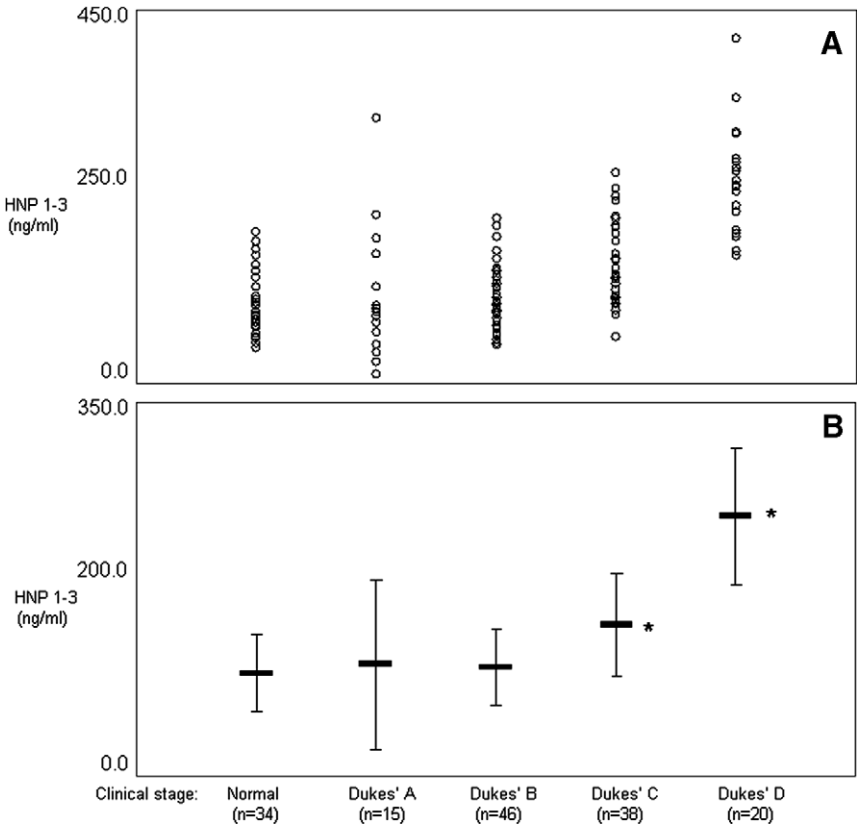

Fig. 4 – HNP1–3 plasma concentration in CRC patients and healthy individuals determined with ELISA. (A) The concentration of HNP1–3 in plasma (ng/ml) was determined in 34 healthy individuals and 119 patients with CRC in Dukes' stage A, B, C and D. B: The mean value and SD of plasma concentration of HNP1–3 in healthy individuals and patients with CRC in Dukes' stage A, B, C and D was calculated. * denotes that the mean value is statistically different from normal colonic tissue with *p*-value < 0.00001.

SELDI-TOF MS analysis of microdissected tumour tissue as well as immunohistochemistry. In their study tumour specimens were categorised according to the WHO classification, and most of the tumours were classified as pT2 and pT3, corresponding to Dukes' stage B and C. Furthermore, Melle et al.[7] showed that the serum level of HNP1–3 determined with ELISA was significantly elevated in serum from 26 CRC patients compared with 22 healthy donors, and that CRC could be detected with a sensitivity of 69% and a specificity of 100% using HNP1–3 as a marker in serum. Based on these findings Melle et al.[7] proposed that HNP1–3 may be used as a serum marker for detection of CRC.

In the present study, we have extended these studies and asked whether the increased level of HNP1–3 in tumour tissue and plasma from CRC patients correlates with the clinical stage of disease using Dukes' classification. Our results are summarised in Table 3. Using SELDI-TOF MS analysis of tumour tissue and plasma from CRC patients we found that HNP1–3 is elevated in tumour tissue from all patients with CRC in Duke's stages A–D, whereas HNP1–3 in plasma is only elevated in patients with metastatic CRC in Dukes' stage D. ELISA of the HNP1–3 in plasma is increased in CRC patients in Dukes' stage C and D.

Based on our findings we suggest that determination of the plasma concentration of HNP1–3 in patients with CRC could be performed before surgery in order to identify the patients with metastatic CRC in Dukes' stage C and D versus patients with localised CRC in Dukes' stage A and B. This information could be used to plan the treatment of these patients including surgery, chemotherapy and irradiation. Furthermore, the level of HNP1–3 in plasma could be used in monitoring CRC patients during chemotherapy and irradiation treatment in order to observe disappearance of metastases. Finally, measurements of HNP1–3 in plasma could be applied in follow-up of patients after treatment in order to observe recurrence of metastases. Accordingly, we conclude that HNP1–3 in plasma may be used as a marker for the diagnosis of CRC with metastases and for monitoring the disease during and after treatment.

Currently, the best available serum marker for CRC is carcinoembryonic antigen (CEA).[5] Although CEA cannot be used in screening of asymptomatic individuals or diagnosis of early or low-stage CRC due to inadequate sensitivity of 30–40%, CEA is commonly used as diagnostic aid in advanced disease, for assessment of prognosis of CRC, surveillance of CRC patients after surgery, and monitoring chemotherapy in CRC patients with advanced disease.[5] However, 20–30% of patients with CRC fail to produce elevated serum levels of CEA, despite advanced disease, and consequently other markers are necessary for follow-up of these patients.[4] Several other serum markers for CRC including CA 19-9, CA 242, TPA, and TIMP-1 have been

identified, but none of these have yet been established in clinical use due to insufficient data.[4] Our results suggest that HNP1–3 in plasma can be applied as a marker for CRC together with CEA. However, the application of HNP1–3 as marker of CRC requires studies of larger groups of CRC patients before it is implemented.

HNP1–3 is not a specific marker for CRC, since HNP1–3 is elevated in other cancers, including renal cell carcinoma,[15] bladder cancer,[16] squamous cell carcinomas of the human tongue,[17] oral cancer,[18] and breast cancer.[19] HNP-1 is elevated in lung cancer[20] and HNP-3 is elevated in lymphoma.[21] HNP1–3 in serum is also elevated in other diseases like idiopathic pulmonary fibrosis[22] and acute respiratory distress syndrome.[23] HNP1–3 expression is increased in intestinal epithelial cells during active inflammatory bowel disease.[13]

It is not clear whether the increased HNP1–3 in tumours is localised to malignant cells, or neutrophil leucocytes.[11] Previous studies have shown that HNP1–3 expression in tumours primarily originate from eosinophil and neutrophil leucocytes invading the tumour.[15,17,20] However, the elevated amounts of HNP1–3 observed in urine from bladder cancer patients were often produced by the cancer cells.[16] Furthermore, CRC cell lines LoVo, Caco2, HCT-15, SW480, and SW620 showed significantly higher level of HD6 expression than non-cancer cell lines.[8] Thus, the increased amount of HNP1–3 in tumours may primarily originate from invading inflammatory cells, but could also be produced by cancer cells.

The level of HNP1–3 in plasma in patients and control groups of healthy individuals varied significantly between five different studies suggesting inter-assay and inter-laboratory variations. In two studies the plasma concentration of HNP1–3 was determined by radioimmunoassay (RIA).[20,21] In patients with idiopathic pulmonary fibrosis, plasma HNP1–3 concentration was 768.2 ng/ml compared with 323.3 ng/ml in the control group,[22] and in patients with acute respiratory distress syndrome, HNP1–3 in plasma was 2012 ng/ml in comparison with 271 ng/ml in the control group.[23] Three studies including our own used ELISA from HyCult Biotechnology for HNP1–3 measurements.[7,19] In one study, the concentration of HNP1–3 in commercial pooled standard serum was determined as 41 ng/ml.[19] In CRC patients the serum HNP1–3 concentration was 29.4 ng/ml compared with 5.6 ng/ml and 8.1 ng/ml in healthy individuals.[7] In our study, plasma HNP1–3 in CRC patients varied from 105.4 to 244.3 ng/ml, compared with 96.6 ng/ml in healthy individuals. Currently we have no explanation for the discrepancy between the levels of HNP1–3 determined by different laboratories. The ten-fold difference in the HNP1–3 concentration determined by ELISA in healthy individuals between the study of Melle et al.[7] and our study may be due to the matrix effect of serum versus plasma causing interference in the immunoassay.[24–27]

To conclude, the expression of HNP1–3 is increased in tumour tissue from CRC patients in Dukes' stages A–D. The plasma concentration of HNP1–3 is elevated in CRC Dukes' stages C and D, but not in A and B. It is unlikely that HNP1–3 could function as marker for early detection of CRC. However, plasma HNP1–3 may be used for assessing prognosis, surveillance of patients with diagnosed CRC, and monitoring chemotherapy in patients with advanced disease.

Conflict of interest statement

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