Original Article

Evaluation of Carcinoembryonic Antigen, Vascular Endothelial Growth Factor, Neutrophil Elastase as Serological Biomarkers in Patients with Colorectal Cancer

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Key Words
Colorectal cancer;
Biomarker;
Carcinoembryonic antigen (CEA);
Vascular endothelial growth factor (VEGF);
Neutrophil elastase (NE)

Background. Tumor microenvironment contains tumor cells and other non-tumor cells such as inflammatory cytokines and immune cells. With the high proliferation of tumor cells, VEGF is secreted by tumor cells for exacerbating angiogenesis. Immune cells such as neutrophils recruit to inflammatory tumor tissues and secrete neutrophil elastase (NE) to enhance tumor proliferation and angiogenesis.
Aims. We intended to evaluate conventional CEA and high abundant VEGF and NE as the serological biomarkers in patients with colorectal cancer (CRC).
Methods. Cancer tissues (n = 3) were confirmed by methylene blue stain. Neutrophils were detected in the CRC tissues by naphthol AS-D chloroacetate esterase stain and MALDI-TOF MS targeting HNP-1 (m/z 3442). In addition, CEA, VEGF, and NE were measured by ELISA in the sera of CRC patients. The correlation statistical analysis and ROC curve were used to evaluate CEA, VEGF, and NE as the serological biomarkers in the CRC patients (n = 27 vs. n = 14 from the healthy volunteers).
Results. We found that neutrophils highly accumulated in the CRC tissues compared to the adjacent normal tissues. Meanwhile, CEA, VEGF, and NE all increased in the sera of CRC patients compared to the healthy volunteers, which are independently expressed. Area under ROC curve of CEA, VEGF, and NE was 0.76 (p = 0.008), 0.57 (p = 0.39), 0.76 (p = 0.0077), respectively, indicating that CEA and NE were reliable as the biomarker of CRC patients.
Conclusions. We demonstrated that immune cell neutrophils accumulated in the CRC tissues and its secreted enzyme NE may be a serological biomarker of CRC. The study warrants further investigation for the functions of neutrophils in the CRC tumor microenvironment.

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It has been demonstrated that tumorigenesis recruits immune cells in the tumor microenvironment, causing tissue inflammation.[1] The production of cytokines majorly attracts and recruits immune cells, including macrophages, neutrophils, dendritic cells, natural killer cells, T and B cells, homing to the tumor microenvironment. The accumulation of immune cells in the tumor microenvironment is considered capable of pro-

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moting tumor progression and development.[2-5] Circulating neutrophils are a short half-life of 7-10 hours but capable of modulating tumor progression when located in tumor tissues being N2 tumor-associated neutrophils (TANs). The main function of N2 TANs is demonstrated to stimulate angiogenesis and inhibit other anti-tumor immune cells.[6,7]

Literature has found that infiltrated human neutrophils in the cancerous tissues and suggested that the number of neutrophils may be positively associated with tumor severity.[8] Particularly a high neutrophil to lymphocyte ratio is correlated to the poor clinical survival rate in patients with several cancers being a prognostic factor.[9,10] TANs in CRC were defined by the expression of $CD45^{+}Lin^{-}HLA\text{-}DR^{-}CD33^{+}CD66b^{+}CD11b^{+}$, producing arginase 1 (ARG1) and reactive oxygen species (ROS) higher than autologous neutrophils.[11] A previous study has revealed neutrophils highly express in the CRC tissues from 271 patients.[12] In the tumor microenvironment, tumor cells highly proliferated with simultaneous recruitment and activation of TANs which are abundant higher than MDSCs by 80-fold.[11] The results support tumor- and neutrophil-secreted factors may be highly expressed in the serum of patients with cancer as reliable diagnostic biomarkers.

The endoscopic and histological examinations are considered the gold standard for CRC diagnosis in clinical practice.[13-15] To widely diagnose CRC in the clinic, serum biomarkers are useful and necessary. Several non-invasive biomarkers for evaluating CRC have been reported and used, such as carcinoembryonic antigen (CEA).[16-18] The high level of CEA in CRC patients is associated with poor therapeutic efficacy. Besides, tumor cells express VEGF to enhance blood angiogenesis by supplying enough nutrients and oxygen to support tumor progression. The secreted VEGF is considered a therapeutic target in clinical practice, a high level of that may also be a diagnostic candidate in patients with cancer. Meanwhile, with high recruitment of TANs in tumor tissue, it revealed that TNAs-secreted factors may be increased as a diagnostic biomarker in CRC patients, such as neutrophil elastase (NE).

In this study, we intended to evaluate neutrophils accumulation in the CRC tissues compared to the adjacent normal tissues. In addition, we evaluate CEA, VEGF, and NE as the serological biomarkers of CRC patients. This study demonstrated that neutrophils accumulated in the CRC tissues and its secreted enzyme NE may be a serological biomarker of CRC patients.

Material and Methods

Serum and tissue from the patients with colorectal cancer

The clinical samples including sera and tissues were collected from Cheng Hsin General Hospital, Taiwan, which was approved by the Institutional Review Board (CHGH-IRB-(240) 100-01). The type of colorectal adenocarcinoma was collected and analyzed. The pairs of tissues including tumors (T) and adjacent non-tumors (NT) from the CRC patients were acquired by surgery. Total 3 pairs of clinical tissues from the enrolled patients were stained using methylene blue staining and distinguished by a pathologist. Tumor histopathology and severity were determined according to the rules of the American Joint Commission on Cancer Staging (AJCCS) system. The serum was collected from the CRC patients (n = 27, age: 61 ± 12. Male: 13; Female: 14) and the healthy volunteers (n = 14, age: 52 ± 12. Male: 12; Female: 2). The healthy volunteer enrolled in this study had no evidence of known CRC.

Methylene blue staining and neutrophils detection in colorectal cancer tissues

The pairs of 10 μm-thick tissues from CRC patients cut by a cryostat (HM525, Thermo Scientific Microm, Germany) at -20 °C were attached to the general glass slides for methylene blue staining and for naphthol AS-D (3-hydroxy-2-naphthoic-o-toluidide) chloroacetate esterase staining. In methylene blue staining, the slides were fixed using 37% of formaldehyde for 15 mins. The fixed tissues were then immersed in 0.1% of methylene blue for 1 min after PBS buffer (10 mM sodium phosphate, pH 7.4, and 0.9%

sodium chloride) washing. For neutrophils staining, the naphthol AS-D (3-hydroxy-2-naphthoic-o-toluidide) chloroacetate esterase staining method (Sigma, USA) was performed according to the manufacturer's instruction.

CEA, VEGF, and NE measurement

CEA, VEGF, and NE levels were measured using an individual enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Massachusetts, USA). Measurements were performed according to the manufacturer's instructions and quality control was ensured.

MALDI-TOF MS

The tissue samples were spotted with the fresh sinapinic acid (SA) matrix solution (20 mg/ml SA, 50% acetonitrile, and 0.1% Trifluoroacetic acid in deionized water) onto the tissue surfaces. The MALDI-TOF MS instrument (UltraFlexIII, Bruker Daltonics, Germany) was used to enforce the main processing with a standard 337 nm N_2 laser at 1000 Hz and a laser spot size of 50 μm. The instrument was operated in positive linear mode at 26 to 28% laser power.

Statistical analysis

Statistical analysis was performed using GraphPad Prism V5.01 software (GraphPad Software, Inc., California, USA). Unpaired student's t-test was used to compare the significance of CEA, VEGF, and NE between the healthy volunteers and CRC patients. Data are expressed as mean ± SD. Pearson's correlation was used to calculate the correlation coefficient between the markers in healthy volunteers and CRC patients, in which r = -0.3~0.3: poor correlation; r: 0.3~0.6 and -0.3~-0.6: media correlation; r = 0.6~0.9 and -0.6~-0.9: high correlation; r = 1 and -1: complete correlation. Meanwhile, the ROC curve was used to analyze the sensitivity and specificity of CEA, VEGF, and NE. The significant difference was acceptable as $p < 0.05$.

Results

Highly neutrophils in the tissue of colorectal cancer compared to the paired adjacent normal control

The tumor tissues (n = 3) were first collected by surgical operation and then stained using methylene blue dye for distinguishing cancerous tissue (T) compared to the adjacent non-tumor tissues (NT) in the individual clinical biopsy specimens (Fig. 1A, only shown by 2 specimens). The numbers of the infiltrated neutrophils in the CRC tissue were measured using naphthol AS-D (3-hydroxy-2-naphthoic-o-toluidide) chloroacetate stain which was used to specifically detect neutrophil esterase. The results indicated that the numbers of the infiltrated neutrophils significantly increased in the CRC tissues compared to the adjacent non-tumor regions (Fig. 1B). Meanwhile, the CRC tissue was also investigated by MALDI-TOF MS, which revealed that neutrophil secreted peptides, HNP-1 (m/z 3442),were overexpressed in the CRC tissues compared to the adjacent non-tumor tissue (Fig. 1C).

CEA, VEGF, and NE levels in the serum from CRC patients

To evaluate the expression of the selected biomarkers, an individual ELISA kit was used to measure the serum concentration of CEA, VEGF, and NE in the CRC patients (n = 27) and the normal individuals (n = 14). The serological levels of CEA, a well-known biomarker that positively correlated to CRC progression, were significantly overexpressed in the CRC group higher than in the healthy group (Fig. 2A, 20.86 ± 6.004 ng/dL vs. 1.548 ± 0.3276 ng/dL, $p < 0.05$). The serum concentration of VEGF and NE were also significant in the CRC specimens higher than that in the healthy controls (Fig. 2B and 2C; VEGF: 184.5 ± 56.9 pg/mL vs. 341.5 ± 63.19 pg/mL, $p < 0.05$; NE: 0.56 ± 0.08 μg/mL vs. 0.22 ± 0.03 μg/mL, $p < 0.05$). Here, we found that there was no correlation between the serum CEA, VEGF, and NE (Fig. 2D-F), indicating that CEA, VEGF, and NE were independently regulated in CRC patients.

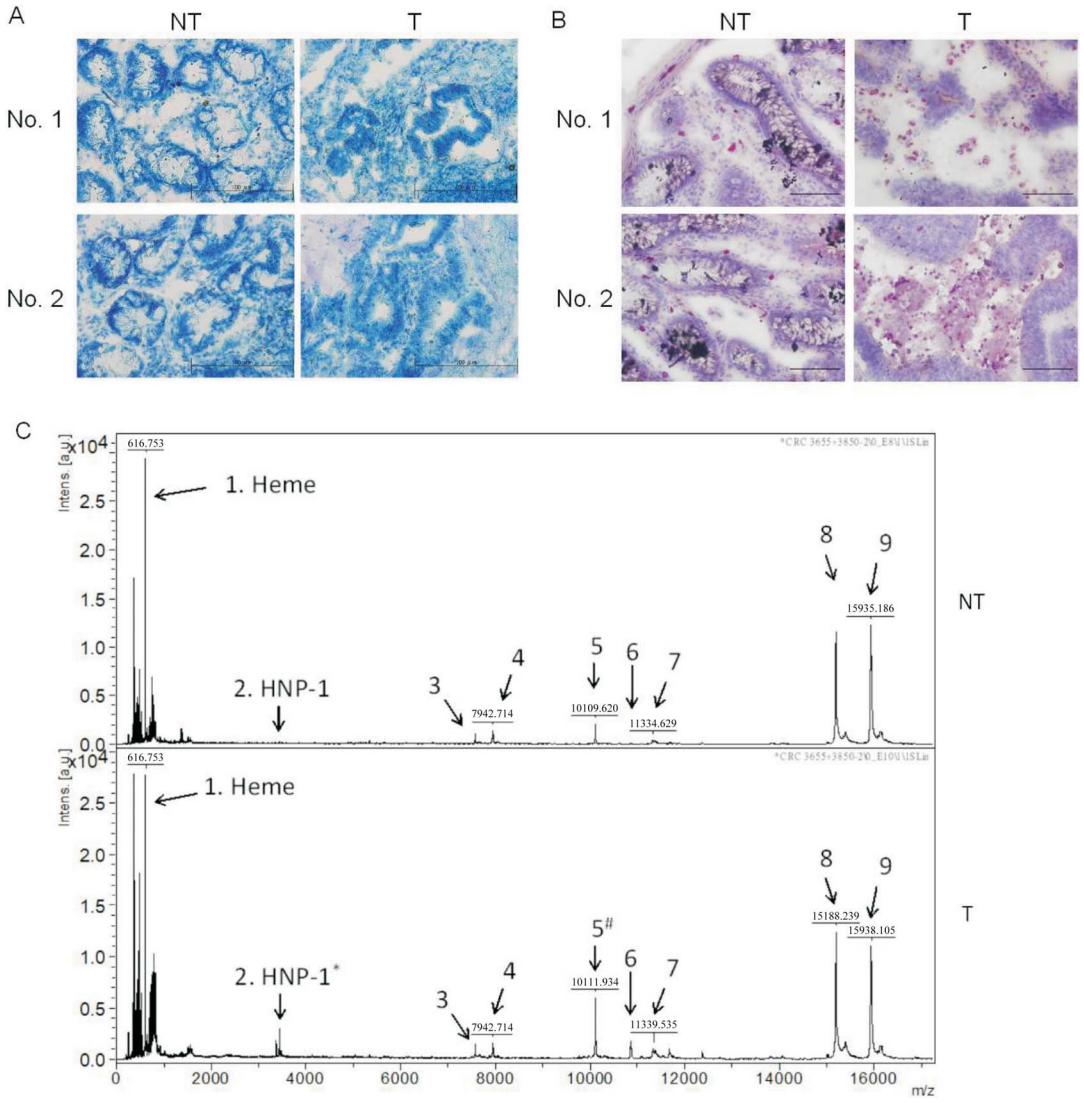

Fig. 1. Neutrophils accumulated in cancerous tissues of colorectal cancer (CRC) with elevated expression of human neutrophil peptide-1 (HNP-1). (A) The cancerous tumor (T) and the adjacent non-tumor (NT) tissues of CRC specimens were figured out using methylene blue staining. Scale bar, 100 μm. (B) Tumor-infiltrated neutrophils were detected using naphthol AS-D chloroacetate esterase staining. (C) MALDI-TOF MS was used to detect the protein profile in the tumor and non-tumor tissues. Heme and HNP-1 were predicted by the accurate m/z, whereas Heme was m/z 616 and HNP-1 was m/z 3442. * Specifically expressed in the tumor tissues.

Serum CEA and NE were putative biomarkers for CRC patients

To evaluate the discrimination ability of CEA, VEGF, and NE for CRC diagnosis, the sensitivity and specificity were determined according to the analysis of the receiver operating characteristic curve (Fig. 2G-I). The results indicate that the sensitivity and specificity of CEA were 59.26 and 85.71, respectively, with cut-off values of 2.78 ng/dL. The sensitivity and specificity of VEGF were 55.81 and 62.5, respectively, with cut-off values of 131.3 pg/mL. The sensitivity and specificity of NE were 81.48 and 57.14, respectively, with cut-off values of 0.19 μg/mL. Moreover, the area under curve (AUC) of CEA, VEGF, and NE were 0.76 ($p = 0.008$), 0.57 ($p = 0.39$) and 0.76 ($p = 0.0077$), respectively. The approximated AUC value between CEA and NE indicated that NE was a reliable biomarker for diagnosing CRC.

For improving the diagnostic accuracy in patients

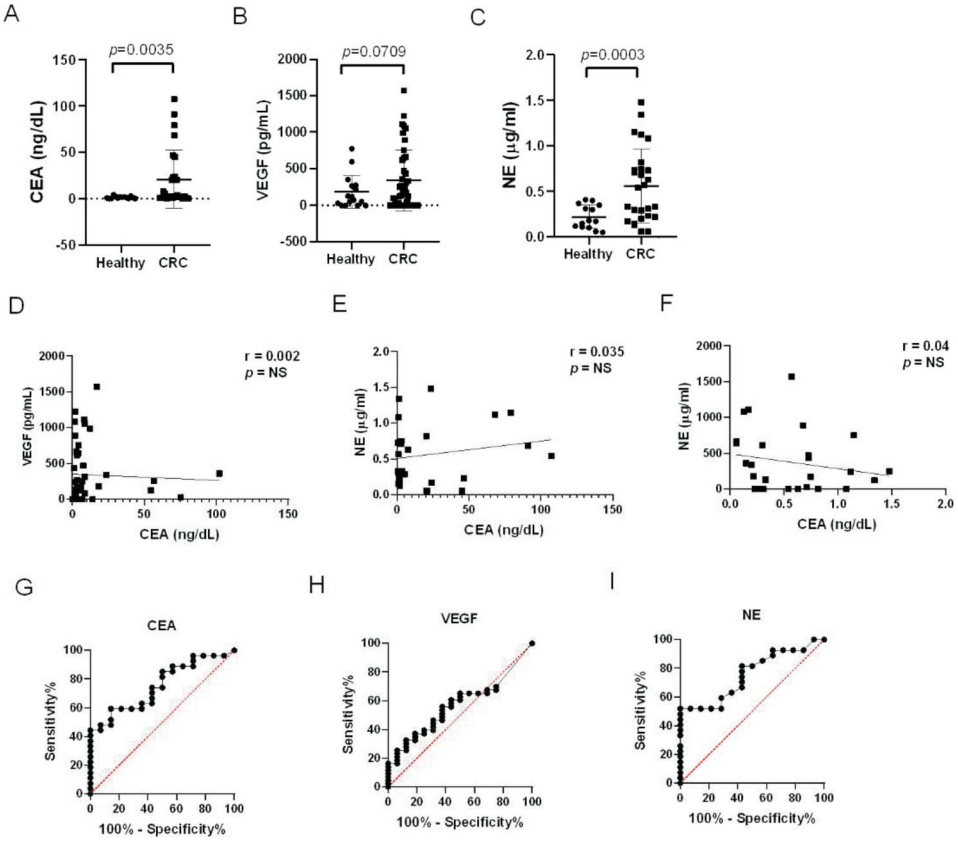

Fig. 2. Increasing NE level in the sera of CRC patients as a putative diagnostic marker. (A-C) Serum concentrations of CEA, VEGF, and NE in the CRC patients (n = 27) and the healthy donors (n = 14) were detected using ELISA kits. (D-F) The correlation between the expression level of CEA, VEGF, and NE was individually investigated. (G-I) The sensitivity and specificity of CEA, VEGF, and NE were determined by the receiver operating characteristic curve (ROC): CEA were 59.26 and 85.71, respectively, with cut-off values of 2.78 ng/dL; VEGF were 55.81 and 62.5, respectively, with cut-off values of 131.3 pg/mL; NE were 81.48 and 57.14, respectively, with cut-off values of 0.19 μg/mL. NS, non-significant.

with CRC, CEA and NE were combined. Based on the average value in the healthy volunteers using CEA > 1.58 ng/dL and NE > 0.22 μg/mL, there were 74% and 81% of tumor patients diagnosed with CEA and NE, respectively. The diagnosis by combined CEA and NE was 96% (Table 1). However, there were still 64% of healthy volunteers who exhibited false positives by combined CEA and NE. Furthermore, when we used a 2-fold average value in the healthy volunteers using CEA > 3.09 ng/dL and NE > 0.44 μg/mL, the diagnosis of combined CEA and NE in the patients with CRC was 74%, which was better than CEA (48%) and NE (52%) alone. Meanwhile, the false-positive ratio decreased to 14% in the healthy volunteers (Table 1). The results revealed that combined CEA and NE were better for diagnosing the patients with CRC.

Discussion

In this study, we evaluated neutrophils accumulated in the tumor tissues of CRC patients by directly detecting esterase and HNP-1. We also demonstrated that CEA, VEGF, and NE all overexpressed in the sera of CRC patients. In addition, CEA and NE possessed a better area under the ROC curve: CEA was 0.76 (p = 0.008) and NE was 0.76 (p = 0.0077), revealing that CEA and NE may be reliable as serological biomarkers in CRC patients. Compared to CEA, NE had similar diagnostic accuracy. Since there was no correlation between NE and CEA, we suggest that a combination of NE with CEA may benefit the diagnostic accuracy of CRC.

Previous studies have demonstrated that neutro-

Table 1. Combined CEA and NE for diagnosing patients with colorectal cancer

Average in the healthy volunteers			Healthy volunteers			Tumor patients		
			CEA (n)	NE (n)	CEA + NE (n)	CEA (n)	NE (n)	CEA + NE (n)
CEA (ng/dL)	Sensitivity	Specificity	6/14 (43%)		9/14 (64%)	20/27 (74%)		26/27 (96%)
> 1.58	70.37	57.14						
NE (μg/mL)	Sensitivity	Specificity		6/14 (43%)			22/27 (81%)	
> 0.22	74.07	57.14						
2-fold average in the healthy volunteers			CEA (n)	NE (n)	CEA+NE (n)	CEA (n)	NE (n)	CEA+NE (n)
CEA (ng/dL)	Sensitivity	Specificity	2/14 (14%)		2/14 (14%)	13/27 (48%)		20/27 (74%)
> 3.09	48.15	87.71						
NE (μg/mL)	Sensitivity	Specificity		0/14 (0%)			14/27 (52%)	
> 0.44	51.85	100						

phils participate in the development and metastasis of tumors,[19-23] such as $CCl2^{+}CCL17^{+}$ TANs majorly playing a role in suppressing anti-tumor T cells.[11] Thus, suppression of neutrophils is also a proposed strategy for tumor therapy. Several strategies are investigated currently targeting neutrophils in patients with cancers, including NE inhibitor sivelestat (NCT01170845).[7] Overproduction of NE in tumor tissue elucidates a character of neutrophils in promoting tumor growth, which is considered a prognostic marker.[24,25] NE can trigger tumor proliferation via degrading IRS-1 in tumor cells.[19] Therefore, NE is a target for anti-tumor therapy, sivelestat targeting NE is demonstrated to inhibit neutrophils-mediated tumor promotion and metastasis.[26,27] In this study, we further validated the overexpression of NE in the sera of CRC patients, which was independently expressed and presented similar diagnostic sensitivity and specificity to CEA as a potential biomarker for diagnosing CRC.

Meanwhile, we found neutrophil secreted peptide, human neutrophil peptide (HNP) alpha defensin 1 (HNP-1) in the CRC tissues compared to the adjacent normal tissues. HNP-1 is highly expressed in the inflammatory tissues responsible for bacterial infection, which is also capable of triggering macrophage activation and phagocytosis.[28] HNP-1 is reported up-regulated in a variety of cancers, including metastatic colorectal cancer, bladder cancer, renal cell carcinoma, squamous cell carcinoma, and breast cancer[29-34] as a potential biomarker.[8,35] The high expression of HNP-1 has been demonstrated to correlate with the severity of the tumor stage.[8] The overexpressed HNP-1 in cancer tissue seemingly implies the accumulation of neutrophils, providing a rapid strategy to detect neutrophils in tumor tissues using MALDI-TOF MS.

Conclusion

We investigated and evaluated neutrophils accumulated in the tumor tissue in the CRC patients compared to the adjacent normal tissues. Compared to the conventional CEA biomarker in clinical practice, we also investigated VEGF and NE levels in the sera of CRC patients compared to healthy volunteers. We found that CEA, VEGF, and NE all overexpressed in the sera of CRC patients with independent correlation. Moreover, CEA and NE presented better AUC than VEGF in ROC curve analysis, indicating that CEA and NE were potential as serological biomarkers for diagnosing CRC.

Competing Interest

The authors have declared that no competing interests exist.

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原　著

驗證癌胚胎抗原，血管內皮生長因子，嗜中性白血球彈性蛋白酶作為大腸癌病人的血清生物標記

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背景　腫瘤微環境包含腫瘤細胞和其他非腫瘤細胞例如發炎因子和免疫細胞。隨著腫瘤細胞的高度增殖，腫瘤細胞分泌 VEGF 加劇血管生成。嗜中性白血球細胞等免疫細胞遷移到發炎腫瘤組織並分泌 neutrophil elastase (NE) 以增強腫瘤增殖和血管生成。

目的　評估常規 CEA，VEGF，NE 作為大腸直腸癌 (CRC) 患者的血清生物標誌。

方法　臨床大腸癌症組織 (n = 3) 通過亞甲藍 (methylene blue) 染色確認。在 CRC 組織中通過萘酚 AS-D 氯乙酸酯酶 (naphthol AS-D chloroacetate esterase) 染色和 MALDI-TOF MS 靶向分析 HNP-1 (m/z 3442) 檢測嗜中性白血球。此外，通過 ELISA 測量 CRC 患者血清中的 CEA、VEGF 和 NE。相關統計分析和 ROC 曲線用於評估 CEA、VEGF、NE 作為 CRC 患者的血清學生物標誌物 (n = 27 vs. n = 14 來自健康志願者)。

結果　我們發現大腸癌組織有大量嗜中性白血球聚集，相對於健康受試者，病人血清中大量表現 CEA、VEGF、NE，但彼此之間並不具有相關性。CEA、VEGF、NE 的 ROC 曲線面積分別為 0.76 (p = 0.008)，0.57 (p = 0.39)，0.76 (p = 0.0077)，顯示 CEA 及 NE 可能為大腸癌生物標誌。

結論　我們證明在大腸直腸癌組織中積累嗜中性白血球細胞，其分泌的 NE 可能是大腸直腸癌的血清學生物標誌。該研究值得進一步研究嗜中性白血球在 CRC 腫瘤微環境中的功能。

關鍵詞　結直腸癌、生物標誌物、癌胚抗原 (CEA)、血管內皮生長因子 (VEGF)、中性粒細胞彈性蛋白酶 (NE)。