Neutrophil extracellular traps in gastrointestinal cancer

Zi-Qiang Chu, Ke-Cheng Zhang, Lin Chen

Abstract

Gastrointestinal (GI) cancer is a high-risk malignancy and is characterized by high mortality and morbidity worldwide. Neutrophil extracellular traps (NETs), a weblike structure consisting of chromatin DNA with interspersed cytoplasmic and granule proteins, are extruded by activated neutrophils to entrap and kill bacteria and fungi. However, accumulating evidence shows that NETs are related to the progression and metastasis of cancer. In clinical studies, NETs infiltrate primary GI cancer tissues and are even more abundant in metastatic lesions. The quantity of NETs in peripheral blood is revealed to be associated with ascending clinical tumour stages, indicating the role of NETs as a prognostic markers in GI cancer. Moreover, several inhibitors of NETs or NET-related proteins have been discovered and used to exert anti-tumour effects in vitro or in vivo, suggesting that NETs can be regarded as targets in the treatment of GI cancer. In this review, we will focus on the role of NETs in gastric cancer and colorectal cancer, generalizing their effects on tumour-related thrombosis, invasion and metastasis. Recent reports are also listed to show the latest evidences of how NETs affect GI cancer. Additionally, notwithstanding the scarcity of systematic studies elucidating the underlying mechanisms of the interaction between NETs and cancer cells, we highlight the potential importance of NETs as biomarkers and anti-tumour therapeutic targets.

Key Words: Neutrophil extracellular traps; Gastric cancer; Colorectal cancer; Biomarkers; Therapeutic targets

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Neutrophil extracellular traps (NETs) are extruded by activated neutrophils into the extracellular environment and have three-dimensional lattices, which are composed of decondensed chromatin with interspersed proteins such as neutrophil elastase (NE), cathepsin G, myeloperoxidase (MPO), histones and some other antimicrobial peptides. Once the tumour progresses and spreads to distant organs (e.g., liver and lung), neither conventional surgery nor targeted therapies can reverse the dismal outcomes. Therefore, it is of high importance in all future studies to understand the mechanisms of deterioration of GI cancer, by measuring not only the tumour itself, but also the surrounding cells that can be modified by the tumour to generate a supportive microenvironment; these cells include macrophages (tumour-associated macrophages), fibroblasts (cancer-associated fibroblasts) and neutrophils (tumour-associated neutrophils).

Neutrophil extracellular traps (NETs) are extruded by activated neutrophils into the extracellular environment and have three-dimensional lattices, which are composed of decondensed chromatin with interspersed proteins such as neutrophil elastase (NE), cathepsin G, myeloperoxidase (MPO), histones and some other antimicrobial peptides. NETs were originally discovered to ensnare and kill extracellular bacteria and fungi and may act as a physical barrier to impede the dissemination of microbes. However, recent studies have revealed that NETs, if dysregulated, can contribute to the development or progression of some inflammatory or immune-related diseases, such as atherosclerosis, systemic lupus erythematosus (SLE), diabetes, vasculitis, wound healing, and coronavirus disease 2019 (COVID-19). COVID-19 is still spreading worldwide, and clinical evidence shows that increased NET formation after the COVID-19 infection can be a potential biomarker for disease severity; thus, targeting NETs may alleviate the condition of patients.

More work is needed before NETs can be used as reliable biomarkers in these diseases. In addition to the roles of NETs mentioned before, studies have expanded their biological scope and suggest that elevated numbers of neutrophils and levels of NETs in peripheral circulation are a hallmark of cancer. Recently, NETs have been suggested to participate in the biological process of cancer. Several studies have shown that NETs can cause hypercoagulability, accumulate in peripheral blood vessels and impair organ function, promote cancer development and metastasis, sequester circulating tumour cells, and even stimulate dormant cancer cells. Therefore, more work needs to be done to elucidate the mechanism between NETs and cancer.

In this review, we will discuss the role of NETs in GC and CRC according to the latest findings and consider their potential of NETs as biomarkers and therapeutic targets.
NETs

The formation of NETs, known as NETosis, was first observed by Takei et al.[34] via stimulation with phorbol-12-myristate-13-acetate (PMA) and is regarded as a novel form of neutrophil death that is different from apoptosis and necrosis. The antibacterial role of NETs was confirmed in 2004 and is dependent on the web-like structure [10]. The core scaffold of NETs is the nuclear DNA extruded from neutrophils and that forms a three-dimensional meshwork with a number of interspersed specific cytoplasmic and granular proteins[35]. Notwithstanding the lack of mechanistic or interventional studies investigating NET formation in peripheral circulation, initial studies suggest that NETs are released by dying neutrophils. This phenomenon of NET formation can be mainly described by at least two mechanisms. One mechanism requires the lytic suicide of neutrophils, while the other mechanism is independent of this process[36,37]. Neubert et al.[38] demonstrated that entropic chromatins swelling is the major physical driving force of NETosis. In addition, other myeloid cells, such as human blood monocytes[39], mast cells[40] and eosinophils[41], have been discovered to release DNA networks. However, neutrophils seem to remain the main source of these networks because of their high efficiency of secretion.

The functions of NETs in defending against infection depend on the composition of the network, including the DNA itself, which is a chelator of divalent cations that possesses antibacterial ability. The citrullination of histones loosens their grip on DNA and provides a chance for interaction between DNA and bacteria[42]. Moreover, highly toxic modified histones, which constitute the major part of NET-associated proteins, are also mediators of bactericide[43]. Moreover, NE synergizes with MPO to assist the formation and antibacterial role of NETs[44]. More generally, NETs prevent dissemination of infection by entrapping microbes and facilitate their killing by bacterial proteins which have been shown to be involved in both direct and indirect mechanisms. In addition, NETs are revealed to be vital factors in non-infectious diseases. Hakkim et al.[45] suggested that the persistence of NETs exacerbates the autoimmune response and forms a vicious cycle in SLE. Similarly, studies on diabetes[15], vasculitis[16], wound healing[17] and COVID-19[18-20] have described the pathophysiological role of NETs.

Recently, an emerging role of NETs in promoting cancer progression has been described. Solid tumours are prone to generating a leukemoid reaction and pre-educate neutrophils to form extracellular DNA traps, and intratumoural NET formation is associated with thrombosis[46]. The accumulation of tumour-induced NETs in the peripheral circulation drives the systemic inflammation and vascular dysfunction in mice with cancer[26]. Leal et al.[47] indicates that NETs serve as scaffolds and collaborate with tumour-derived procoagulant exosomes in the establishment of a prothrombotic phenotype in cancer. CXCL8 (interleukin-8, IL8) is a chemokine that can be produced by tumour cells and its role in promoting angiogenesis in cancer has been well discussed[48]. Furthermore, the presence of IL8 activates the extrusion of NETs with potential involvement in cancer progression and promotes cancer cell metastasis partially through AKT and STAT3 signalling pathways[49,50]. NETs produced by tumour-infiltrating neutrophils mediate the crosstalk between the tumour microenvironment and deterioration by regulating the HMGB1/RAGE/IL8 axis[51]. IL17 is a cytokine described as a protumourigenic factor involved in the initiation and development of cancer[52]. A recent study demonstrated that IL17 can induce NETosis through epithelial cell signalling to favour tumour growth[53]. Rocks et al.[54] associated NETs with cancer cells and confirmed that the release of NETs favour tumour cell dissemination and colonization in organs.

Since the discovery of NETs, the role of neutrophils and NETs has attracted increasing attentions. Additional studies have reported on numerous stimuli that affect NET formation in vitro and in vivo. PMA, a potent artificial activator of the neutrophil respiratory burst, has been broadly applied to identify the mechanism of NETosis. Cytokines, such as the aforementioned IL8, IL17 and tumour necrosis factor alpha[55], have been investigated with respect to their role in expediting NET formation. Nicotine, a major addictive component of tobacco, is reported to induce NETosis in a dose-dependent manner, implying potential threats to human health[56]. Moreover, sulfasalazine, a drug used to treat inflammatory bowel disease and rheumatoid arthritis, has been found to significantly promote NETosis by accelerating lipid oxidation[57]. Most recently, Yasuda et al.[58] established the relationship between NETosis and epigenetics and demonstrated that DNA demethylation enhances spontaneous NET formation by reinforcing PAD4 expression and histone citrullination.
NETS IN GC

GC is the fifth most common cancer and ranks third in terms of mortality worldwide. Several studies have tried to confirm the characteristics of NETs in GC and clarify the underlying mechanisms in vitro and in vivo (Table 1).

Notwithstanding advances in diagnosis and therapy, recurrence and metastasis are still common as a result of its high molecular and phenotypic heterogeneity[59], which contributes to a coagulable state that causes the proliferation and invasion of GC cells [60-63]. In GC patients, NET formation is significantly upregulated and its increased level is consistent with advanced TNM stage. Moreover, a procoagulant role of NETs in GC has been confirmed. When NETosis is inhibited by DNase I, the levels of TAT and D-dimers are downregulated. Furthermore, NETs obtained from GC patients can also stimulate control plasma to generate thrombin and fibrin[64].

A common target of metastatic gastrointestinal cancer is the peritoneal cavity[65]. The invention of heated intraperitoneal chemotherapy[66] and postoperative chemotherapy[67] has indeed reduced the rate of peritoneal recurrence and metastasis. However, limitations still exist with regard to the benefit of these treatments, and severe general toxicity has always been an unavoidable side effect. To elucidate the underlying mechanisms, Kanamaru et al[68,69] collected the peritoneal lavages from GC patients before and after radical surgery. Then NET formation emerged after short term culture of purified low-density neutrophils (LDNs) separated from the lavages fluids. Moreover, the majority of NET-like structures were discovered on the surface of omental tissue. In a in vivo study, GC cells lines such as MKN45, OCUM-1 and NUGC-4 were found attached to the NETs and remained healthy; this interaction was entirely prevented upon treatment with DNase I, which can degrade NETs.

Notwithstanding the advances of therapeutic strategies in GC, surgical resection remains the mainstay treatment. Therefore, the subsequent surgical stress, which suppresses immunity, constitutes one of the key factors that influence the prognosis of GC patients[70,71]. The percentage of LDNs in the peripheral circulation of GC patients who underwent abdominal surgery was elevated and in vitro studies observed the adhesion of cancer cells to NET structure, suggesting that partial adverse effects of surgical stress may be explained by the formation of NETs[72].

According to a pulmonary metastasis model of GC, accumulation of neutrophils in the adjacent vascular vessels has been found during the colonization of GC cells. Extracts from the root of *Salvia miltiorrhiza* (*Danshen*)[73], a medicinal plant used for cancer therapy, have been observed to prevent neutrophil trafficking to the metastatic sites and obstruct the formation of NETs via inhibitory activities on MPO and NADPH oxidase (NOX)[74]. A recent study has proposed that obstruction of NETosis by Cl-amidine or DNase I significantly suppresses the progression of GC cells by regulating the expression of apoptosis-associated genes, among which Bcl-2[75] levels were downregulated and the expression of Bax[76] and NF-kB p65[77] increased significantly[78].

NETS IN CRC

CRC is the third most frequent malignancy and second leading cause of cancer-related mortality worldwide. To date, metastasis and cancer-associated thrombosis are still the main causes of death in CRC patients. To address this issue, much work has been done or is currently in progress; recently, more attention has been drawn to the establishment of tumour microenvironment. Of note, recent accumulating evidence has shown that NETs may play a pivotal role in the progression of CRC (Table 2).

CRC patients are at high risk of venous thrombosis as a result of hypercoagulable state and the levels of NETs in the peripheral blood are positively related to cancer progression. A comparison between 60 newly diagnosed CRC patients and 20 healthy controls revealed that neutrophils in CRC patients can release more NETs, and this tendency parallels increased TAT levels and fibrin formation, which is indicative of a hypercoagulable state[79]. Thus, a vicious cycle can be established between activated platelets and neutrophils. Additionally, in vitro studies show that NETs can convert endothelial cells to a procoagulant phenotype[80]. Another study observed that NETs were concentrated in the centre of CRC tissues and reduced gradually towards adjacent normal tissues, and this characteristic could help surgeons determine a better surgical area through pathological examination of the tumour margins. Furthermore, tissue factor, a stimulus of coagulation and a pro-angiogenic factor, was discovered in NETs from the primary tumour and metastatic lymph nodes[81]. Altogether, the
NETs: Neutrophil extracellular traps; GC: Gastric cancer.

### Table 1 Role of neutrophil extracellular traps in gastric cancer

| Ref. | Cell lines | Model (in vitro, in vivo, human) | Conclusion |
|------|------------|---------------------------------|------------|
| Yang et al [64], 2015 | - | Human | NETs contribute to the hypercoagulable state in GC patients with stage III/IV |
| Kanamaru et al [65], 2018 | MKN45, NUGC-4, OCUM-1 | In vitro, in vivo | NETs on peritoneal surface assist the clustering and growth of GC tumor cells disseminated in abdomen |
| Tao et al [76], 2018 | BGC-823 | In vitro, in vivo | NET formation is inhibited by Sal B and DHT I at the earlier stage |
| Kumagai et al [72], 2020 | MKN45, NUGC-4, OCUM-1 | In vitro, human | NETs formation is enhanced under surgical stress and can effectively trap circulating tumor cells |
| Li et al [78], 2020 | BGC-823, SGC7901, MKN28 | In vitro, human | NETs destruction promotes the apoptosis and inhibits the invasion of gastric cancer cells by regulating the expression of Bcl-2, Bax and NF-κB |
| Zhang et al [94], 2020 | - | Human | NETs have novel diagnostic, therapeutic predictive, and prognostic value in GC patients |

### Table 2 Role of neutrophil extracellular traps in colorectal cancer

| Ref. | Cell lines | Model (in vitro, in vivo, human) | Conclusion |
|------|------------|---------------------------------|------------|
| Arelaki et al [81], 2016 | Caco-2 | In vitro, human | NETs concentration is gradually reduced from the tumor mass to the distal margin |
| Tohme et al [83], 2016 | MC38 | In vitro, human | Surgical stress and intratumoral hypoxia induce NETs formation and NETs can trigger HMGB1 release and activate TLR9-dependent pathways to promote adhesion, proliferation, migration, and invasion in CRC |
| Richardson et al [84], 2017 | - | Human | NET production in the later postoperative period appears to coincide with surgical recovery |
| Arelaki et al [88], 2018 | MC38, H59 | In vitro, human | PolyP is present in human colon cancer and increase NETosis |
| Yazdani et al [89], 2019 | MC38, HCT116, Hepa 1-6, Huh7 | In vitro, in vivo, human | NETs facilitate the growth of stressed cancer cells by altering their bioenergetics |
| Zhang et al [80], 2019 | HUVECs | In vitro, human | NETs are involved in the progression of CRC and act as potential agonists in CRC-related hypercoagulability |
| Rayes et al [113], 2020 | HT-29, MC38, A549 | In vitro, in vivo, human | NET-associated CEACAM1 acts as a putative therapeutic target to prevent the metastatic progression of colon carcinoma |
| Shang et al [92], 2020 | DKS-8, DKO-1, PMN | In vitro, in vivo, human | Exosomes may transfer mutant KRAS to recipient cells and trigger increases in IL-8 production, neutrophil recruitment and formation of NETs, eventually leading to the deterioration of CRC |
| Xia et al [105], 2020 | MC38, HepG2 | In vitro, in vivo | AAV-mediated DNase I liver gene transfer is a safe and effective modality to inhibit metastasis and represents a novel therapeutic strategy for CRC |
| Yang et al [91], 2020 | HT29, MC38 | In vitro, in vivo, human | A novel positive feedback between elevated tumorous IL-8 and NETs can promote CRC liver metastasis |
| Yang et al [93], 2020 | HCT116, MDA-MB-231, MCF-7, 4T1, HEK293T, HeLa, I0771 | In vitro, in vivo, human | CCDC25 mediates NET-dependent metastasis and is suggested to be a therapeutic target for the prevention of cancer metastasis |

NETs: Neutrophil extracellular traps; CRC: Colorectal cancer; AAV: Adeno-associated virus.

By analysing large-scale human data, Rayes et al [82] demonstrated that NETs can not only bind tumour cells but also promote metastasis. In addition, established preclinical models of gastrointestinal cancer revealed that tumours induce neutrophils to extrude NETs into the extracellular environment in the absence of surgical stress. Furthermore, NET formation could promote CRC metastasis after surgical stress [83].
In a cohort study, increased postoperative NET formation was associated with disease-free survival in patients undergoing hepatic surgical resection, and the mouse model of surgical stress has obtained similar results. Further studies demonstrated that the hypoxic environment in solid tumours provides a favourable condition for NETosis and that NETs could in turn promote CRC progression by releasing HMGB1 [85], a DNA-binding protein that participates in the activation of TLR9[86]. Additionally, the protumourigenic effects of NETs are abolished by DNase and PAD4 inhibition[83]. Another clinical trial suggested that increased preoperative NETosis is related to an increased hospital stay and complications of CRC patients who undergo colorectal resection, suggesting a promising therapeutic benefit of surgery focused on the relationship between CRC and NETs[84].

In malignant and premalignant colon tissues, CD68+ polyP-expressing cells and NETosis were detected, suggesting a possible interaction between them in the tumour microenvironment. Further investigation indicates that polyP[87], which is secreted by activated platelets and could induce inflammation and thrombosis in CRC, may promote the formation of NETs[88]. A PAD4-KO mouse model, which is genetically incapable of NETosis, showed that inhibition of NETs halted CRC growth through its negative regulation of mitochondrial biogenesis-associated genes. Further studies show that CRC cells subjected to hypoxia have upregulated expression of HMGB1[85], which acts as an inducer of NETosis, and NET released from NETs could assist tumour proliferation as a result of inducing mitochondrial biogenesis by activating the TLR4-p38-PGC1α pathway in CRC[89].

In CRC, IL8 has been reported to participate in growth, angiogenesis and metastasis [90]. High expression of IL8 also implicates adverse survival in patients. A recent study explains a promotional role of IL8 in the formation of NETs. In vitro adhesion system, CRC cells such as HT29 and MC38 cells are more prone to be entrapped into NETs than neutrophil monolayers, giving the chance for disseminated CRC cells to form micrometastases in the liver; this process can be enhanced by overexpression of IL8[91]. Another study focuses on exosomes secreted by CRC cells. The authors showed that exosomes derived from KRAS mutant cells could transfer mutant KRAS to receptors and upregulate IL8 Levels, eventually activating neutrophils to form NETs and leading to enhanced proliferation and invasion of cancer cells[92].

The aforementioned studies have highlighted an interaction between CRC cells and NETs, suggesting that NET formation is enhanced in tumours and that the increase in NETs can promote the progression and metastasis of CRC. Although several pathways have been described, the detailed mechanisms of how NETs interact with and boost the metastasis of CRC remain elusive. Most recently, a remarkable discovery not only precisely illustrates the underlying mechanism between CRC cells and NETs but also partially explains why CRC cells have a tendency to disseminate into the liver. Yang et al[93] investigated several metastatic lesions in the clinic and discovered the most abundant NET infiltration in liver metastases and the formation of NETs began before the appearance of the metastases. According to a pull-down assay, CCDC25, a potential cell-surface DNA receptor, was identified on the cytoplasmic membrane of cancer cells. Furthermore, amino acids 21-25 at the extracellular N-terminus of CCDC25 were found to be the binding site of NET-DNA. Further immunoprecipitation assays indicated that the intracellular C-terminus of CCDC25 binds integrin-linked kinase and that this interaction can be stimulated by NET-DNA, eventually inducing liver metastases of CRC cells via initiating the β-parvin-RAC1-CDC42 cascade.

NETS AS POTENTIAL BIOMARKERS AND THERAPEUTIC TARGETS

An increasing number of investigations have suggested a protumourigenic role of NETs in providing a microenvironment favouring interactions and promoting cancer proliferation, thrombosis and metastasis in GI cancers. This has led to the question of whether NETs can act as potential biomarkers and therapeutic targets.

Zhang et al[94] compared the levels of NETs in different patient populations, suggesting that NETs, as a diagnostic biomarker, have better value than carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 in GC. Additionally, NET formation is associated with tumour stage, poor overall survival and poor recurrence-free survival. Moreover, tumour-infiltrating NETs has been demonstrated to be an independent prognostic biomarker according to the assessment of clinical outcomes of cancer patients[95-97]. Considering the potential diagnostic significance of NETs, conventional histological staining is ineffective for their detection in tissue. To solve
this problem, a protocol has been created to detect NETs in paraffin-embedded tissue [98]. Additionally, machine learning is described as a useful tool to quantify NETosis [99]. Citrullinated histone H3, a central marker of NETs, is elevated in blood and regarded as a potential diagnostic and prognostic serum marker to predict the risk of venous thromboembolism and mortality in patients with advanced cancer [100-102]. A CitH3DNA binding assay has also been developed to quantify NET formation [103].

Taking into account the confirmed role of NETs in promoting the progression and metastasis of GI cancer cells, disruption of NETosis can be a possible new therapeutic strategy and the identification of NETosis inhibitors is of high interest. DNase I can degrade the DNA backbone of NETs and abrogate the protumourigenic ability of NETs in several aforementioned animal models. Moreover, DNase I has been safely used in cystic fibrosis and SLE patients [104], implying that DNase I can act as a promising candidate for the treatment of GI cancer patients. In a mouse model of CRC liver metastasis, an adenovirus-associated virus gene therapy vector was created to specifically express DNase I in the liver, inducing an inhibition of liver metastasis [105]. However, it is worth noting that everything is a double-edged sword. Inappropriate use of DNase I may lead to a systemic inflammatory response in patients [106]. Owing to the critical role of NE and PAD4 in the process of NETosis, NE inhibitors or PAD4-inhibitors are also commonly used to prevent the release of NETs. Nanoparticle-mediated delivery of NE inhibitors has been suggested as a feasible approach to decrease NETosis [107]. In addition, NOX inhibitors [108], MPO inhibitors [109] and RAF inhibitors [110] have been reported to arrest the inception of NETosis. Recently, a group of tetrahydroisoquinolines was found to be a novel class of NET formation inhibitors, but their underlying mechanism remains to be determined [111]. A drug screening of 126 compounds shows that appropriate use of anthracyclines (drugs used for cancer treatment) together with dexrazoxane could be a promising therapeutic candidate for suppressing NETosis without cytotoxicity against healthy neutrophils [112]. NET-associated CEA cell adhesion molecule 1 (CEACAM1) was identified by Rayes et al [113]. Knockdown of CEACAM1 on NETs abrogates the adhesion between NETs and colon cancer cells, thus indicating a potential therapeutic therapy or preventing liver metastases. A novel study revealed that PKCα, a lamin kinase that mediates the phosphorylation of lamin B, contributes to the formation of NETs; hence, blocking PKCα provides a new perspective towards treating NET-associated cancer progression [114]. Since the application of 5-fluorouracil (5FU) has been shown to trigger the formation of NETs in the blood of cancer patients, Amph-PVP self-assembled nanoparticles are proposed as an efficient delivery system for 5FU to avoid the generation of NETs, partially improving the anticancer effect and reducing the risk of long-term metastasis [115]. The previously mentioned studies mainly focus on the inhibition of NETosis or the proteins assembled in NETs. Unusually, Cao and King [116] proposed that NETs per se can be utilized as an anti-tumour drug delivery vehicle. By re-engineering neutrophils, supercharged eGFP-TRAIL, an apoptosis-inducing chimeric protein, is expressed on NETs to entrap and kill tumour cells.

**CONCLUSION**

NETs, initially identified as a host defense system designed to trap and kill bacteria, have now been suggested to play an important tumorigenic role in many cancers. Accumulating evidence has shown that NETs are involved in GI cancers, including GC and CRC. As mentioned above, NET infiltration in primary tumour tissues implies a poor outcome in GI cancer patients, indicating that a rapid intraoperative histopathological examination of NETs in the resected tissue margins may help determine the range of surgical resection. Moreover, several cytokines and genetic mutations trigger the formation of NETs and NETs per se can promote the progression of cancer partially by initiating downstream signalling pathways. On the other hand, the fact that NETs can trap disseminated cancer cells, combined with the situation that an abundance of NET infiltration in the liver is formed before metastases can be detected, increases the likelihood of liver metastasis and explains the high incidence of liver metastasis in patients with GI cancer.

Further studies are needed to elucidate the detailed underlying mechanism of the interaction between NETs and GI cancer. Clarifying the roles of NETs in cancer could open a new door in the design and development of therapeutic approaches. Transforming inhibitors of NETs into drugs that can be safely used in GI cancer patients or utilizing NETosis as a drug delivery system may evolve into promising anti-tumour therapies.
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