Cell-free DNA derived from cancer cells facilitates tumor malignancy through Toll-like receptor 9 signaling-triggered interleukin-8 secretion in colorectal cancer

Zhengchuan Niu†, Wentao Tang†, Tianyu Liu†, Pingping Xu†, Dexiang Zhu, Meiling Ji, Wenbai Huang, Li Ren, Ye Wei, and Jianmin Xu*

Department of General Surgery, Zhongshan Hospital of Fudan University, Shanghai 200032, China

†These authors contributed equally to this work.

*Correspondence address. Tel: +86-13501984869; Fax: +86-21-64041990-296; E-mail: xujmin@aliyun.com

Received 14 April 2018; Editorial Decision 24 May 2018

Abstract

Circulating cell-free DNA (cfDNA) has become a potential diagnostic and prognostic biomarker for colorectal cancer (CRC). In non-cancerous diseases, it has been confirmed that cfDNA can be recognized by Toll-like receptor 9 (TLR9), leading to a significant biological change. Nevertheless, the biological significance of cfDNA and its relationship with TLR9 in tumor malignancy is still unclear. Therefore, the purpose of this study is to explore the biological role of cfDNA in colorectal cancer (CRC). The expression of TLR9 was measured in different CRC cell lines and cancerous samples by RT-PCR or immunohistochemistry, which showed that high expression of TLR9 was significantly correlated with the tumor metastasis, advanced TNM stage and poor prognosis of patients. Then, cfDNA was obtained from fluorouracil (5FU)-induced apoptotic cancer cells in vitro and transfection techniques were used to transfect siRNA and cDNA plasmid for TLR9. Cancer cells were stimulated using isolated cfDNA fragments, and results showed that cfDNA could promote colorectal cancer cell proliferation via TLR9. Meanwhile, we demonstrated that the cfDNA binding to TLR9 could facilitate cell migration and invasion. Finally, we demonstrated that cfDNA initiated downstream TLR9-MyD88 signaling and induced robust release of chemokine interleukin 8 (IL-8), which helped to elucidate the mechanisms underlying these phenomena. Our data suggest that cancer cell-derived cfDNA contributes to cancer progression through activation of TLR9-MyD88 signaling and IL-8 secretion in CRC. These findings provide a novel perspective for understanding of tumor progression and provoke a potential therapeutic target for CRC treatment.

Key words: cell-free DNA, Toll-like receptor 9, interleukin 8, colorectal cancer

Introduction

Over the recent years, much attention has been focused on utilizing circulating cell-free DNA (cfDNA) as biomarkers in the liquid biopsy of cancer [1,2]. Nevertheless, very little is known about the biological function of cfDNA in cancerous diseases, despite its wide clinical exploration. According to previous studies [3–3], cfDNA fragments mainly originate from apoptotic or necrotic tumor cells, with 150–200 base pairs in length for apoptosis origin. DNA fragments can physiologically exist in human blood plasma, though significantly increased concentration of cfDNA is detected in patients with autoimmune and inflammatory diseases, as well as in patients with various cancers [6–9]. It has been reported that cfDNA
possesses the capability to activate the inflammatory signaling, thus contributing to the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE), and psoriasis [10,11]. In human cancers, cfDNA levels are significantly increased as disease stages advance [12–16]. A recent meta-analysis including 1076 patients has demonstrated that cfDNA measurement possesses the prognostic value for patients with metastatic colorectal cancer (mCRC), as patients with higher levels of cfDNA have a significantly shorter survival than those with lower levels [17]. These findings suggest that cfDNA may have significant clinical and potential biological implications. Accordingly, it remains an indeterminate but interesting question whether elevated cfDNA levels could contribute to malignancy.

Toll-like receptor 9 (TLR9) is a DNA receptor that can recognize microbial [18] and mammalian DNA containing unmethylated cytosine-guanosine (CpG) dinucleotides [11]. TLR9 is usually found on the intracellular vesicle membranes of conventional dendritic cells (cDCs), macrophages [19,20], as well as in different cancer cells. High TLR9 expression has been associated with poor survival in patients with glioma, prostate cancer, esophageal adenocarcinoma, and other types of cancers [21–25]. In addition, several studies have revealed a link between cfDNA and TLR9 in human-term labor and autoimmunity diseases, which can provoke inflammatory processes [26–30]. However, the exact relationship between cfDNA and TLR9 in human cancers remains unclear and needs to be further investigated. Strictly, only one study has superficially reported that cfDNA through active cellular secretion could stimulate proliferation of breast cancer cells via activation of the TLR9/NF-kB/cyclinD1 pathway [31].

Colorectal cancerous tissue exhibits global DNA hypomethylation and has higher amount of unmethylated CpG DNA sequences compared with the normal tissue [32–35]. It could be that these cfDNAs, containing unmethylated CpG sequences from cancer cells, are recognized by TLR9, exerting subsequent effect on colorectal cancer cells.

In the present study, we obtained cfDNAs from the supernatant of CRC cells induced by 5FU, and explored its effect on cell proliferation, invasion, and migration through activation of TLR9 signaling. In addition, we examined whether cfDNA-induced secretion of cytokines or chemokines could contribute to tumor malignancy.

**Materials and Methods**

**Clinical samples and immunohistochemistry**

A total of 217 patients were included in our study, who were pathologically diagnosed as colorectal cancer and received surgery between 2008 and 2010 at Zhongshan Hospital of Fudan University. Detailed patients’ characteristics are shown in Table 1. All patients gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Zhongshan Hospital of Fudan University. For the immunochemistry staining of TLR9 (Antibody from Sino Biological Inc., Beijing, China), cancerous and paracancerous sections from these patients were used in this study. Immunohistochemical staining of tissue sections for TLR9 was performed as follows. Sections were incubated for 60 min at 65°C, and then deparaffinized and rehydrated using xylene and ethanol series. Then tissue slides were immersed in EDTA antigen retrieval solution (pH 8.0; Solarbio, Beijing, China) for microwaving, with high temperature for 5 min and 40°C for 15 min. Incubation with anti-TLR9 primary antibody (1:150 in PBS solution) was completed overnight for 12 h at 4°C. Next day, after incubation with the HRP-conjugated secondary antibody and DAB substrate solution, the color of the antibody staining in the tissue sections was observed under a microscope. Staining scores were evaluated by three individuals as described in Konno’s study [36].

**Cell lines and culture conditions**

LoVo, SW480, HT29, SW620, HCT116, Colo320, and CCD-112CoN cell lines were all obtained from the American Type Culture Collection (ATCC, Manasas, USA). CCD-112CoN cells are from human normal colon tissue, while other cell lines are from human tissues of colorectal cancer. They were maintained as monolayers in DMEM (Gibco, Gaithersburg, USA) or Eagle's MEM (Merck, Darmstadt, Germany) containing 12% heat inactivated FBS (Sigma, St Louis, USA) and supplemented with 20 mM HEPES, 100 IU/ml penicillin and 100 μg/ml streptomycin (Merck). All cells were cultured at 37°C in humidified atmosphere containing 5% CO₂.

**Induction, extraction and addition of cfDNA fragments**

It is widely accepted that cfDNA originates from apoptotic processes or necrotic cells. Therefore, we used 5FU, known as a medication for cancer treatment, to induce the apoptosis and necrosis of cancer cells. HT29 and CDD-112CoN cells were cultured in 100-mm cell culture dishes (Corning Inc, Corning, USA) and treated with 50 μg/ml 5FU (Millipore, Darmstadt, Germany). Cell apoptosis was detected between 48 and 72 h after 5FU exposure, which was confirmed by typical morphological changes of nucleus and by apoptosis detection assay.

Then, the growth medium was collected at 72 h after 5FU treatment and DNA fragment was purified using phenol/chloroform extraction protocols [37]. The isolated protein-free DNA was subject to RNase A/T1 Mix at 37°C for 1 h (Thermo Scientific, Wilmington, USA). The concentration and the quality of the isolated DNA was measured using NanoDrop spectrophotometer (Thermo Scientific). Finally, the acquired cell-free DNA was added into culture plates for subsequent assays. Meanwhile, the genomic DNA isolated from cancer cells without 5FU treatment. Cytosine phosphate guanine oligonucleotides (CpG-ODN) ODN 2395 were synthesized by IDT Company (Integrated DNA Technologies, Inc., Skokie, USA). Cancer cells were treated with cfDNA at 5 μg/ml every 12 h in the subsequent assays.

**Propidium iodide and hoechst double staining assay**

Propidium iodide (PI) is a common red-fluorescent nuclear and chromosome counterstain, which is not permeant to live cells; PI can bind with DNA to detect dead or late apoptotic cells in a population. Hoechst dyes are cell-permeable and can bind to DNA in live or fixed cells. Cells were treated with PI (Thermo Fisher Inc., Waltham, USA) and Hoechst 33258 (Thermo Fisher) for the detection of cell death or late apoptosis induced by 5FU [38]. Images were acquired under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

**Flow cytometry analysis of apoptosis**

According to the manufacturer’s instructions, cells were washed twice with PBS and resuspended in binding buffer at a concentration of 1 × 10⁶ cells/ml. Then, AnnexinV-fluorescein isothiocyanate
Table 1. TLR9 expression and patients’ clinicopathological characteristics

<table>
<thead>
<tr>
<th>Clinicopathological factors</th>
<th>n</th>
<th>TLR9 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High (n = 94)</td>
<td>Low (n = 123)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>Male 132</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female 85</td>
<td>38</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td>&gt;65 70</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≤65 147</td>
<td>62</td>
</tr>
<tr>
<td>Diameter</td>
<td></td>
<td>&gt;5 137</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≤5 90</td>
<td>34</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td>Right 48</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left 52</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rectum 117</td>
<td>50</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td>T1 6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2 31</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3 48</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T4 132</td>
<td>58</td>
</tr>
<tr>
<td>Pathological type</td>
<td></td>
<td>Adenocarcinoma 184</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mucinous aden. 33</td>
<td>16</td>
</tr>
<tr>
<td>N stage</td>
<td></td>
<td>N0 105</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N1 68</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N2 44</td>
<td>19</td>
</tr>
<tr>
<td>M stage</td>
<td></td>
<td>M0 160</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1 57</td>
<td>34</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td>I 28</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II 65</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III 67</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV 57</td>
<td>34</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td>Well 34</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate 102</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poor/undifferentiated 81</td>
<td>38</td>
</tr>
<tr>
<td>Survival (60 months)</td>
<td></td>
<td>Censor 157</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Death 60</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PFS (36 months) No 146</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes 71</td>
<td>38</td>
</tr>
</tbody>
</table>

(FITC; BD, Franklin Lakes, USA) and PI solution were added into the cell suspension. After 20 min of incubation in the dark at room temperature, cells were measured using a flow cytometer (BD) and analyzed using Flowjo 7.6 software.

Synthesis and transfection of siRNA and plasmid DNA

To inhibit the expression of TLR9, cells were transfected with specific small interfering RNA (siRNA). The siRNA against TLR9, as well as the negative control was synthesized by GenePharma (Biosune, Shanghai China). The modified plasmid pcDNA3-TLR9-YFP containing human TLR9 ORF cDNA together with empty vector plasmid was purchased from Bofeng Biological Company (Shanghai, China). Cells were transfected with plasmids or siRNA using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA). The subsequent assays were performed 48 h after transfection. The siRNA sequences against TLR9 were as follows: sense 1 strand: 5′-AAACCUUGAGCUACACAAACAUCATG-3′/antisense 1 strand: 5′-CAUGAUGUUGUGUUGACUCAGGUUAG-3′ and sense 2 strand: 5′-AAACUGAGrUCACACAAACAUCAGUA-3′/antisense 2 strand: 5′-UCAUUGAUGUUGUGUAGCUAGGUUUAA-3′.

Western blot analysis

The proteins were extracted from cells using lysis buffer. Then western blot analysis was performed using antibodies against TLR9, MyD88, NF-κB, β-actin (Abcam, Cambridge, UK), c-fos, c-jun (Santa Cruz Biotechnology, Santa Cruz, USA), MAPK p42/44, p-p42/44, MAPK p38, p-p38, JNK, and p-JNK p-p65 (Cell Signaling Technology Inc., Danvers, USA) as described previously [39]. β-actin was used as the internal control.

RNA preparation and real-time PCR

RNA was extracted from cells using Trizol reagent (Invitrogen) and then the reverse transcription reaction was performed using the PrimeScript™ RT-PCR kit (TaKaRa, Dalian, China) according to the manufacture’s instruction. Afterwards, SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA) was used to perform quantitative PCR on the ABI Prism 7900HT. Data were calculated by the 2−ΔΔCt method normalized by GAPDH. The primer sequences used in this study were listed as following: TLR9 forward 5′-AGACCCCTTCTGGAGAAGCC-3′/reverse 5′-AGCTCACAGGTCGAAAGG-3′; and GAPDH forward 5′-ACATCGCTCAGACACCATG-3′/reverse 5′-TGATTGGAGCTCAATGAGGG-3′.

Cell proliferation assay

Cell proliferation was measured using Vybrant® MTT Cell Proliferation Assay kit (Invitrogen). Briefly, harvested cells were seeded in a 96-well plate, and then treated with DNA fragments from different origins. Subsequently, MTT solution was added into each well for measurement. Absorbance at 540 nm was obtained using a Microplate Reader (Bio-Tech Instruments, Winooski, USA).

Migration and invasion assays

Migration and invasion assays were performed using modified Transwell chambers (8 μm pore size; Corning) with the membrane pre-coated with (invasion) or without (migration) Matrigel. Transfected cells were pretreated with cfDNA or PBS for 72 h. Then, 1 × 105 cells per well in 150 μl serum-free medium were seeded into the upper chamber. In the lower chamber, 600 μl medium containing 20% serum was added as a chemo attractant. After 36 h of incubation at 37°C, migrated cells were staining with 0.1% crystal violet and examined under an inverted microscope [40]. For the wound healing assay, transfected cells were incubated in 6-well plates until 90% confluence. Then, the cell monolayer was scratched with a fine pipette tip, and cultured in serum-free medium with cfDNA or PBS for 36 h. Photographs were taken along the scrape line under a microscope.

Cytokine measurement by ELISA

Transfected cells were pretreated with cfDNA or PBS for 12 h. Then, cytokine levels in the culture supernatants were measured...
using ELISA kit developed by Bofeng Biological Company (Shanghai, China) according to the manufacturer’s instructions.

Statistical analysis
Statistical analyses were performed with SPSS 13.0 software. The histogram shown was plotted as the mean and standard deviation of three independent experiments. Survival analyses were carried out using the Kaplan–Meier method and the log-rank test. Statistical differences were compared using One-Way ANOVA, Chi-square test or t-test in our study. P < 0.05 was considered statistically significant.

Results

TLR9 is expressed in colorectal cancer tissues and cell lines
Recent studies have demonstrated that cfDNA can serve as an endogenous ligand for TLR9 in non-cancerous diseases [27,41]. Yet, the expression of TLR9 in cancers, especially in colorectal tumor, remains unclear. Therefore, we explored the expression of TLR9 and its clinical significance in colorectal cancer using tissue microarray via immunohistochemistry staining. Results showed that TLR9 was mainly expressed on the intracellular vesicle membranes, and less on the cell membranes of tumor cells. Among 217 cases, 43.3% (94/217) exhibited high TLR9 expression, while others were classified into low expression group (Fig. 1A and Table 1). In addition, the TLR9 expression in cancerous tissue showed a much higher staining score than that in paracancerous normal tissue (Fig. 1B). Furthermore, we analyzed the association between TLR9 and clinicopathologic variables (Table 1). There was a significant relationship between high TLR9 expression and M stage or TNM stage (P = 0.004 and 0.017 respectively). Kaplan–Meier survival analysis showed that patients with high TLR9 expression had significantly shorter 5-year overall survival and 3-year progression-free survival (PFS) compared with patients with low TLR9 expression (P = 0.032 and 0.012, the log-rank test, respectively) (Fig. 1C,D). We also examined TLR9 mRNA expression in colorectal cancer cell lines. Among them, SW480 cells expressed higher TLR9 mRNA, while LoVo cells expressed lower TLR9 mRNA (Fig. 1E).

Concentration of cfDNA is associated with cell apoptosis and necrosis in vitro
It has been suggested that circulating cfDNA in human serum mainly originates from apoptotic or necrotic cells. To clarify this idea, we investigated the relationship between cfDNA and cell apoptosis or necrosis in vitro. Firstly, we induced cell apoptosis and necrosis in colorectal cell line HT29 using a common chemotherapeutic drug 5FU. As shown in Fig. 2A, obvious morphological nuclear changes characterized by nucleus pyknosis, condensation and fragmentation were observed in dying cells treated with 5FU compared with cells treated with PBS. Meanwhile, the proportion of PI positive cells was significantly higher in cells treated with 5FU compared with those treated with PBS. Furthermore, flow cytometry data showed that cell apoptosis and necrosis rate were increased with prolonged 5FU treatment (Fig. 2B,C). Correspondingly, the concentration of cfDNA circulating in supernatant were increased in parallel with changes of cell apoptosis and necrosis rate (Fig. 2D). The above data suggested that cfDNA could be obtained from dying cells, and its concentration is correlated with the cell apoptosis or necrosis rates.

cfDNA promotes cell migration and invasion via TLR9 receptor in colorectal cancer
It has been reported that synthetic CpG-ODN could induce TLR9-mediated invasion in cancer cells [42,43]. However, whether cfDNA from cancer cells could also affect cell migration and invasion is still unknown. By using transwell migration assay, we showed that cfDNA could induce cell migration. When the expression of TLR9 was knocked down by siRNA in SW480 cells, cfDNA-induced cell migration ability was inhibited and decreased to a similar level to blank control (Fig 4A–G). Meanwhile, higher invasion ability was observed after cells were treated with both TLR9 cDNA plasmid and cfDNA, when compared with cells treated with cfDNA-only (Fig. 4H–N). Similarly, cfDNA could lead to a higher invasion ability, compared with the blank control group. However, changes of TLR9 expression alone did not result in obvious changes in cell migration and invasion ability. These results indicated that cfDNA could play an important role in promoting cell proliferation in vitro via TLR9 receptor in colorectal cancer.

cfDNA promotes cell proliferation via TLR9 receptor in colorectal cancer
To investigate whether cfDNA can affect CRC cell proliferation and tumorigenesis through TLR9 receptors, we successfully downregulated and overexpressed TLR9 in SW480 and LoVo cells, respectively. The protein expressions of TLR9 in these cells are shown in Fig. 3A–D. Consequently, we treated SW480 cells with DNA from different origins, and observed cell proliferation using MTT method. As shown in Fig. 3E, cfDNA from cancer cells significantly promoted the proliferation of SW480 cells similar to CpG ODN (5 μM), while cancer cells treated with cfDNA from normal colonic epithelial cells and intact DNA from non-apoptotic cancer cells showed a much lower proliferative ability. Next, cfDNA derived from cancer cells were used to stimulate SW480 and LoVo cells transfected with siRNA or plasmid. Our results indicated that when treated with cfDNA, cells with higher TLR9 expression, including wild SW480 or TLR9-plasmid-transfected LoVo cells, had a higher proliferative ability (Fig. 3F,G). In addition, our results also showed that the changes of TLR9 expression alone did not result in obvious changes in cell proliferation ability. These results indicated that cfDNA could play an important role in promoting cell proliferation in vitro via TLR9 receptor in colorectal cancer.

cfDNA stimulates the TLR9-MyD88 pathway and triggers the secretion of IL-8
Recently, accumulating evidence has indicated that MyD88 is essential for the activation of TLR signaling and the TLR-MyD88 pathway may lead to activation of NF-κB and activator protein (AP)-1, thus promoting the secretion of cytokines and chemokines [44]. Nonetheless, whether cfDNA could stimulate this pathway in colorectal cancer remains unclear. As shown in Fig. 5A, when SW480 cells were exposed to cfDNA for 48 h, both NF-κB and AP-1 pathways were provoked as predicted, with obvious increase in activated p65 and AP-1 levels. AP-1 activation is mediated by mitogen-activated protein kinases (MAPks) including c-Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK), which leads to higher phosphorylation of c-fos and c-jun, while p38 MAPK was not found to be activated in our study. Interestingly, cfDNA did not show these effects when it was added into cells treated with TLR9 siRNA.
Furthermore, we examined the production of an array of cytokines (IL-1β, IL-6, IL-8, TGF-β, and TNF-α) after binding cfDNA to TLR-9. Interestingly, ELISA assay showed that only IL-8 level in medium was significantly increased after 12 h of exposure to cfDNA (Fig. 5B), while IL-8 secretion could be abrogated when TLR9 was downregulated by siRNA or blocked by TLR9 inhibitor chloroquine (CQ, 10 mM; NBP2-29386, Novus, Littleton, USA) (Fig. 5C). Since IL-8 plays a vital role in malignant activities, we further explored whether cfDNA-induced IL-8 secretion could participate in tumor malignancy. It was found that cfDNA alone could promote cell proliferation (Fig. 5D); while this effect was inhibited by CQ, and also by reparixin (100 nM), an inhibitor of IL-8 receptor CXCR1/2.

Consistent with our hypothesis, when recombinant human IL-8 (50 ng/ml) was added into cells treated with both cfDNA and CQ, cell proliferation could be recovered, and again IL-8-induced proliferation was abrogated by reparixin. Similarly, cfDNA-induced cell migration could

Figure 1. TLR9 expression in colorectal cancer tissues and cell lines  (A) TLR9 expression was evaluated in paracancerous and cancerous colorectal tissues. Negative, Positive +, Positive ++ and Positive +++ were observed on normal, Tumor 1, Tumor 2 and Tumor 3 tissue, respectively. Upper row: scale bar = 200 μm; lower row: scale bar = 50 μm. (B) TLR9 expression in 217 tumor tissues was significantly higher compared with those in paracancerous normal tissue. (C,D) Patients’ 3-year Progression-free survival (PFS) and 5-year Overall survival (OS) according to TLR9 expression. (E) TLR9 mRNA expression was detected in different colorectal cancer cell lines. *P < 0.05. Cum Survival: Cumulative survival.
also be inhibited by both CQ and reparixin (Fig. 5E,F). Moreover, additional IL-8 could recover CQ-caused inhibition of cell migration. These data suggested that cfDNA-induced IL-8 secretion could contribute to tumor malignancy via the TLR9-MyD88 pathway.

Discussion
Clinical use of ‘liquid biopsy’ has received considerable attention over the past years. As a novel biomarker, circulating cfDNA exhibits increased sensitivity for early detection and selection of therapy in colorectal cancer [16]. However, the biological significance of cfDNA in cancers has not yet been elucidated. In this study, we obtained cfDNA from the medium of CRC cells induced by 5FU in the in vitro model, and found that cfDNA could promote cell proliferation, invasion and migration via activation of TLR9 signaling in colorectal cancer. In addition, we demonstrated that cfDNA-induced IL-8 secretion could contribute to tumor malignancy.

TLR family plays a critical role in innate immune response, as well as subsequent initiation of adaptive immune response [45]. Among ~13 TLR family members [46], TLR9 was originally thought to specifically recognize microbial or viral DNA; whereas now it is evident that mammalian DNA containing unmethylated CpG-rich DNA sequence can also be an effective TLR9 ligand [11]. Recent studies have demonstrated that cfDNA can also serve as an endogenous ligand for TLR9 [27,41]. When interacting with TLR9 receptor, cfDNA can biologically activate the inflammatory cascade, which can promote the development and progression of autoimmune diseases [8,26], chronic adipose tissue inflammation [41], and even trigger the onset of parturition during pregnancy [47]. TLR9 was also found to be expressed in different cancerous cells including breast, ovarian, esophageal, prostate, pancreas cancer, all of which predicted poorer prognosis and were related to tumor malignant behaviors [21,23,24,48,49]. Consistent with findings in our study, TLR9 was expressed in clinical samples and CRC cell lines, while higher
expression of TLR9 was associated with patients’ earlier disease progression and shorter survival. In vitro and in vivo studies have suggested that the treatment of cancer cells with TLR9 agonist, CpG motif containing CpG-ODN, significantly increased tumor cell proliferation as well as inflammatory host response [48]. As is known, CpG-ODN is short (only 20–30 bases) single-stranded synthetic mimics of viral or bacterial DNA, functioning as TLR9 agonist and immune modulator in basic research and even clinical trials [50].

However, unlike synthetic CpG-ODN, cfDNA released from apoptotic and necrotic cancer cells has not been well investigated regarding its relationship with TLR9 in human cancers. Therefore, we utilized cfDNA from our in vitro model to treat cancer cells. It was found that cfDNA could participate in cell proliferation, colony formation and in vivo tumor formation. Moreover, cfDNA showed the ability to promote cell migration and invasion through TLR9 signaling. Interestingly, in our study, cfDNA showed a stronger stimulatory effect on cell proliferation and viability, compared with intact genomic DNA from cancer cells and cfDNA from normal cells. It is known that cancerous tissue exhibits global DNA hypomethylation with greater unmethylated CpG DNA sequences [33], which can be more likely recognized by TLR9 receptor, compared with normal tissues. This may provide a reasonable interpretation on different stimulatory abilities between different DNAs. In human plasma of patients with colorectal cancer, the cfDNA level is higher than that in healthy ones, thus cfDNA could bind to TLR9 on tumor cells of colorectal cancer, while there was no effect on the biological activity of cells when TLR9 alone was up- or downregulated in vitro in absence of ligand cfDNA. Taken together, cfDNA released from cancer cells is involved in tumor malignancy through TLR9 receptor in colorectal cancer.
Previous studies have demonstrated that in innate immune response, upon ligand binding, the stimulation of TLR9 can recruit MyD88 that forms a supramolecular complex with other intracellular components, leading to subsequent inflammation characterized by increased expressions of various interleukins and cytokines [44, 45]. In this study, we found that cfDNA from colorectal cancer cells could not only activate the NF-κB pathway, but also trigger the activation of AP-1 mediated by the MAPKs pathway. Meanwhile, we further measured changes of cytokines and chemokines in the supernatant of cancer cells exposed to cfDNA. Among them, IL-8...
showed a remarkable increase following cfDNA addition. IL-8, also known as CXCL-8, is a member of the CXC chemokine family, which has been proved to play a critical role in tumor metastasis, growth and angiogenesis through interactions with CXCR1 and CXCR2 in colorectal cancer [51–53]. Furthermore, we demonstrated that cfDNA-induced IL-8 secretion could participate in tumor malignancy via the TLR9-MyD88 pathway, which could be inhibited by CXCR1/2 inhibitor reparixin. It could, at least partially, account for the role of cfDNA-TLR9 axis in cancer progression.

In summary, our data suggested that cfDNA released from damaged cancer cells could promote cell proliferation, migration, and invasion through activation of TLR9-MyD88 signaling and IL-8 secretion in colorectal cancer. These findings provide a novel perspective on our understanding of tumor progression and may provide a potential therapeutic target for CRC treatment. Although the in vitro model could not completely represent the actual condition of patients, we provided useful information concerning the biological implication of cfDNA through TLR9-mediated recognition in colorectal cancer. In the nearer future, we will further explore the functions of cfDNA in in vivo models and investigate the synergistic effect of immune system and cancer cells underlying cfDNA-TLR9 axis-induced cancer progression in patients.

**Funding**

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 81402341 and 81602036) and Shanghai Engineering Research Center of Colorectal Cancer Minimally Invasive (No. 17DZ2252600).

**References**