NUCLEAR TRANSLOCATION OF GASDERMIN D (GSDMD) SENSITIZES CHEMOTHERAPY THROUGH INHIBITING PARP-1 IN COLORECTAL CANCER

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ABSTRACT

Gasdermin D (GSDMD) has recently been identified to play a central role in pyroptosis of monocytes, through its N-terminal effector domain cleaved by activated inflammatory caspases to form pores on cell membrane. However, as the universally expressed protein in cancer cells, its function beyond pyroptosis, the pyroptotic function and pyroptosis-independent function of GSDMD in cancer cells have been poorly characterized. In my dissertation, I found GSDMD nuclear translocation and the apoptotic role of GSDMD for the first time. My first objective was to explore the correlation between the nuclear localization of GSDMD and overall survival rate of colorectal cancer patients. To address my first objective, I demonstrated upregulation of GSDMD mRNA in adenocarcinoma compared with GSDMD expression in adenoma and normal mucosal epithelia. Further studies using anti-GSDMD antibody in clinical specimens validated the upregulation of GSDMD protein in adenocarcinoma tissue. However, statistical analysis revealed no significant correlation of GSDMD expression levels with the overall survival rate of colorectal cancer patients. Very interestingly, I discovered the existence of GSDMD nuclear location in colorectal cancer cells of patients, and such nuclear translocation of GSDMD induces apoptosis and has significantly positive correlation with a better overall survival of patients with colorectal cancer. For the first time, I reported that non-pyroptotic GSDMD plays a beneficial role in promoting chemotherapy-induced apoptosis of tumor

cells via its nuclear localization, and insufficient or absent GSDMD nuclear translocation predicts worse prognosis of colorectal cancer patients. My second objective was to define the non-pyroptotic function of GSDMD in colorectal cancer cells with chemotherapy and investigate how full-length GSDMD translocates into nucleus to induce apoptosis. My findings indicated that non-pyroptotic full-length GSDMD promotes chemotherapy-induced apoptosis of tumor cells via its nuclear translocation and subsequent regulation of poly (ADP-ribose) polymerase-1 (PARP-1) mediated DNA repair. After chemotherapeutic drug treatment induces full-length GSDMD nuclear translocation, GSDMD interacts with nuclear protein PARP-1 to dramatically inhibit its DNA Damage Repair-related function, to act similarly like the PARP inhibitor Olaparib. My findings confirmed that GSDMD related apoptosis is nuclear translocation dependent. My final objective was to dissect the potential clinical value of GSDMD nuclear translocation for chemosensitization by manipulating GSDMD expression and nuclear localization signal (NLS) and ataxia-telangiectasia mutated (ATM) signaling activation. My results indicated that NLS and ATM dependent mechanism of GSDMD nuclear translocation sensitize DNA damage related chemotherapeutic drug treatment. Together, these studies redefined the pyroptosis-independent function of GSDMD in its full-length form. My studies demonstrate that full-length GSDMD nuclear translocation facilitates chemotherapy-induced apoptosis through inhibiting PARP-1-mediated DNA Repair. Overall, our data strongly suggest that the combination of platinum-based chemotherapy and Olaparib in GSDMD low-expressed

clinical cases, and subcellular localization of GSDMD may serve as a promising target for therapy of colorectal cancer and a good indicator for clinical outcomes of colorectal cancer patients. These studies are innovative in the context of GSDMD nuclear localization, cleavage-independent non-pyroptotic action, chemosensitization and better prognosis.

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CRC	Colorectal cancer
CEA	Carcinoembryonic antigen
GSDMD	Gasdermin D
PAMP	Pathogen-associated molecular pattern
DAMP	Damage associated molecular patterns
NLS	Nuclear localization signal
IHC	Immunohistochemistry
CCK8	Cell counting kit-8
IF	Immunofluorescence
5-Fu	5-Fluorouracil
FCM	Flow cytometry
FACS	Fluorescence activated cell sorting
Co-IP	Co-Immunoprecipitation
PI	Propidium Iodide
DAPI	4, 6-diamidino-2-phenylindole
PBS	Phosphate Buffered Saline
IL-1β	Interleukin 1β
IL-18	Interleukin 18
LPS	lipopolysaccharide
PMA	Phorbol myristate acetate
PBS	Phosphate buffer saline
TBST	Tris Buffered Saline Tween
FBS	Fetal Bovine Serum
MS	Mass spectrometry
PARP	Poly(ADP-ribose) polymerase
PAR	poly(ADP-ribosyl)ation

SSBs	Single strand breaks
DSBs	Double strand breaks
BER	Base excision repair
FDA	Food and Drug Administration
TCGA	The Cancer Genome Atlas
GEO	Gene Expression Omnibus
SC-seq	Single-cell sequencing
scATAC-seq	Single-cell sequencing assay for transposase-accessible chromatin
Ran	Ras-related nuclear protein
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
KRT	Keratin
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
TGF-β	Transforming growth factor β
BRCA	Breast cancer gene
γ-H2AX	γ-H2A histone family member X
HIV-1	Human immunodeficiency virus type 1
Vpr	Viral protein R
PIC	Pre-integration protein complex
GFP	Green fluorescent protein
GST	Glutathione S-transferase

CHAPTER 1: INTRODUCTION

1.1 The Epidemiology and Biology of Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer in the world, ranking the second in mortality, behind lung cancer [1]. The latest global cancer statistics report shows that there are 1.8 million new cases of colorectal cancer worldwide each year and 896,000 cases of death [2]. For early-stage adenoma, endoscopic surgery is recommended [3]. For patients with progressive and advanced colorectal cancer, comprehensive treatments include preoperative chemoradiotherapy, surgery, neoadjuvant chemoradiotherapy and radiotherapy are required [4]. The 5-year survival rate of patients with early colorectal cancer is about 90% after surgery, while the regional and distant-stages are reduced to 70.4% and 14%, respectively [5]. Therefore, to explore the tumorigenesis of colorectal cancer and strengthen the prevention and early diagnosis of colorectal adenocarcinoma will allow high-risk people to benefit from early screening [2].

As the most common malignant tumor of the digestive system, colorectal cancer is affected by genetic factors, susceptibility factors and environmental factors [6, 7]. About 10% -20% of patients with CRC have a family cancer history, the rest are sporadic. Patients with sporadic colorectal cancer have no family history [7]. The tumorigenesis is closely related to genetic mutation of specific genes and epigenetics changes [8]. About 85% of patients with sporadic colorectal cancers have undergone the histological change process of the

normal mucosal epithelial-adenoma-adenocarcinoma sequence [9]. Over time, the proliferative polyps gradually develop into adenoma polyps, progressing into a typical precancerous lesion, the colorectal adenomas. Finally, adenomas develop into colorectal adenocarcinomas [10, 11].

The occurrence and development of sporadic colorectal cancer is not limited to genetic factors [10]. The carcinogenesis of sporadic colorectal cancer is also considered to be influenced by environmental factors, such as, diet, lifestyle, etc. Sporadic colorectal cancer is reported to be a multi-gene accumulative mutation-related disease [11, 12]. Currently recognized mechanisms of CRC carcinogenesis include chromosomal instability, microsatellite instability and increased methylation level in CpG island [13].

The inactivation of anti-oncogenes, such as APC, is usually considered to be an important initiating event for the development of abnormal adenomas into early adenomas, subsequent activation of the proto-oncogene PIK3CA can promote the progression from early adenomas to the mid-stage adenomas [14, 15]. Finally, the loss of function mutation of tumor suppressor genes, P53, DCC and SMAD2 / SMAD4, promote the progression of mid-stage adenomas into advanced adenomas and colorectal adenocarcinoma [11].

Almost all colorectal cancers have abnormal methylation of DNA, of which, about 10 -20% are characterized by high abnormal methylation of CpG island [16]. Studies show that abnormal changes in DNA methylation in the early stages of CRC evolution exceed the incidence of gene mutations and are the most valuable diagnostic molecular markers for

the identification of colon polyps and adenocarcinoma [17, 18]. High specificity of abnormal DNA methylation levels can be used as an important indicator for early diagnosis and prognostic risk assessment of colorectal cancer [19, 20].

As mentioned above, the pathogenesis of colorectal cancer is a multi-step, multi-stage, and multi-gene involved process [21, 22]. Therefore, understanding the characteristics of multi-omics and their interactions during the transformation of normal mucosa to adenoma and eventually to adenocarcinoma in each patient with sporadic colorectal cancer is of great significance to elucidate the mechanism of most common colorectal cancer. However, due to the difficulty in obtaining adenoma samples, most studies lack analysis of intermediate state-adenomas from the same patient's lesions. In the transition of colorectal tissues from normal mucosa to adenoma, and then from adenoma to adenocarcinoma, benign adenomas accumulate a series of genetic and epigenetic driver mutations that cause changes in gene expression profiles. This change leads to biological changes such as abnormal proliferation, differentiation, and migration of cells, which is the basis for adenoma to progress to malignant adenocarcinoma and gradually acquire invasive characteristics. Existing multi-omics sequencing of colorectal cancer does not elucidate the molecular evolution mechanism between normal mucosa-adenoma and adenoma-adenocarcinoma, ignoring important intermediate states.

Tumor markers can reflect tumor progression and have values for early screening, auxiliary diagnosis, and prognosis of CRC. At present, the tumor marker which is commonly used

to clinically monitor the recurrence and metastasis of colorectal cancer is Carcinoembryonic antigen (CEA) [23, 24], but CEA is a non-specific tumor-associated antigen, with low sensitivity of 56%. Other markers, such as DNA methylation and gene mutation are still in clinical trials [25, 26]. Multi-gene molecular marker testing has been developed [27]. Due to the lack of sensitivity and specificity of current tumor markers in CRC, there is an urgent need to understand the underlying mechanisms of CRC progression and find more specific tumor markers in CRC [28, 29].

1.2 Current Treatment Strategies for Colorectal Cancer and Chemotherapy Resistance

Comprehensive treatment strategies for colorectal cancer depend on the clinical and pathological stages of the disease [30]. Over the past decade, the treatment mode of colorectal cancer has changed from simple surgical resection to a multidisciplinary comprehensive treatment mode, such as, preoperative chemoradiotherapy, neoadjuvant chemoradiotherapy, radiotherapy, monotherapy, and immunotherapy [31, 32]. However, current targeted monotherapy with specific colorectal tumor somatic mutations such as EGFR and ERBB2, such as Panitumumab, Trastuzumab and Lapatinib, are facing the dilemma of unsatisfactory therapeutic effects and drug resistance. And immunotherapies have off-target effect [32, 33].

Despite recent advances in novel therapies, such as targeted therapy [34, 35] and immunotherapy [36, 37], chemotherapy remains the first line of treatment for the metastatic

colorectal cancer. The mainstay of treatment for colorectal cancer, ranking the 3rd incidence of malignancy worldwide with a high mortality rate of 9.2% [2], remains to be chemotherapy with cytotoxic agents in clinic [38], most of which could induce DNA Damage Response. DNA damage induced by chemotherapeutic drugs, exceeding the DNA repair capacity, leads to cancer cell cycle arrest and cell death [39]. Resistance to the fundamental chemotherapeutic drugs, including 5-fluorouracil (5-Fu) and Oxaliplatin, still remains a significant obstacle to effective treatment of colorectal cancer, leading to relapse and further poor outcomes [40]. A better understanding of the accurate mechanisms contributing to drug resistance may help develop more effective strategies in the management of colorectal cancer.

1.3 The Role of GSDMD in Pyroptosis

The dynamic balance between cell proliferation and death is crucial for homeostasis in life processes [41]. Programmed cell death refers to the process of cell death under certain conditions, through the activation of specific genes in the cell with a specific death pattern [42]. The entire apoptotic process is finely regulated. Apoptosis is the most extensively studied programmed cell death, which plays an extremely important role in various physiological and pathological processes [43]. Cell apoptosis remove damaged or redundant cells and help maintain homeostasis [44]. The morphological characteristics of apoptosis include karyopyknosis, chromosome breakage with specific DNA

electrophoresis band and the formation of apoptotic bodies. Apoptotic bodies are formed during plasma membrane blebbing. Then, apoptotic bodies are phagocytosed by macrophages and fuse with lysosomes within macrophages to prevent damage to the surrounding cell or tissue. The entire apoptotic process is not accompanied by inflammation [44]. Pyroptosis is a more recently identified type of programmed cell death [45, 46]. Pyroptosis is a highly inflammatory form of lytic programmed cell death associated with inflammation.

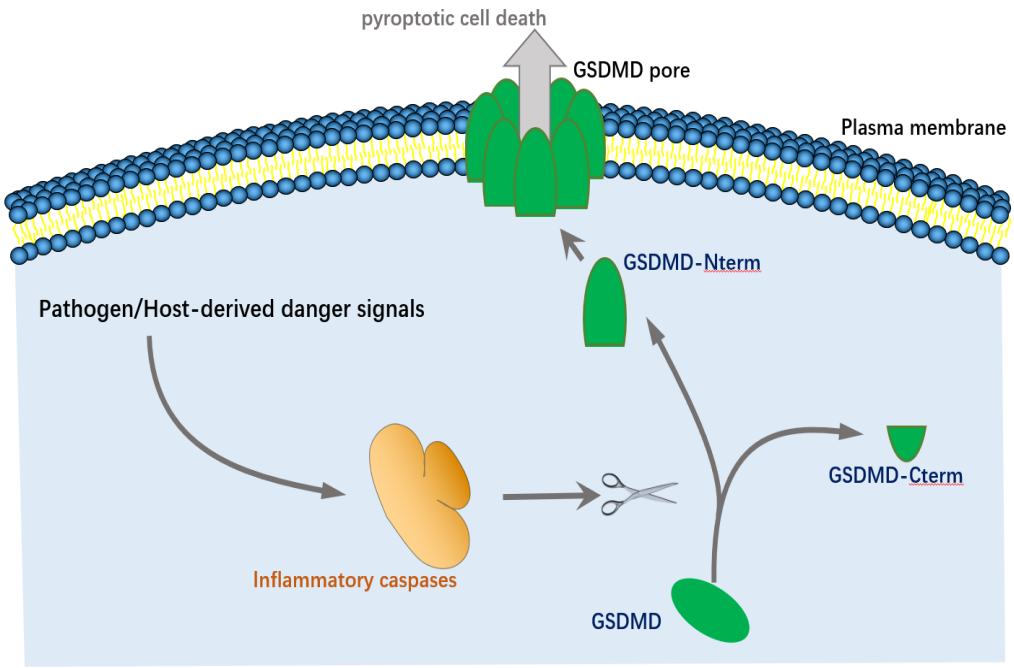

Figure 1.1 - The classical role of GSDMD in pyroptosis.
GSDMD is regarded as a predominantly cytoplasm-localized protein playing a key role in pyroptosis. The N-terminal effector domain of GSDMD, cleaved by the activated inflammatory caspase, incorporates into membrane to form pores and perforates cell membrane, resulting in a lytic form of programmed cell death (pyroptosis) with release of pro-inflammatory cytokine IL-1β and IL-18. This cartoon was made with BioRender.

GSDMD plays a central role in monocyte death, known as pyroptosis, a form of programmed necrosis, mostly investigated in the field of innate immunity. The N-terminal effector domain of GSDMD, cleaved by activated inflammatory caspase 1, 4, 5, 11, incorporates into membrane to form pores and perforates cell membrane, resulting in a lytic form of cell death (pyroptosis) with release of pro-inflammatory cytokine IL-1β and IL-18 [47-49] (**Figure 1.1**). GSDMD belongs to Gasdermin superfamily, consisting of GSDMA, GSDMB, GSDMC, GSDMD, GSDMDE (DFNA5) and DFNB59, which have been reported to be exclusively expressed in a highly tissue-specific manner in epithelium of skin and gastrointestinal tract [50]. In this study, I focus on GSDMD, since GSDMD has been reported to be widely expressed in a variety of cancer cells compared to other Gasdermin superfamily members [52, 64]. GSDMD-mediated pyroptosis also plays a key role in non-alcoholic steatohepatitis associated with lobular inflammation and hepatocellular swelling [51], and in alcoholic hepatitis with worsened hepatocellular lytic death and polymorphonuclear leukocyte-related inflammation [52].

The linear amino acid structure of GSDMD consists of 487 amino acid residues with a molecular weight of 53KDa [53]. Activated inflammatory caspase recognizes Asp276 locus in GSDMD and cleaves the protein into the N-terminal domain and C-terminal domain [54]. The N-terminal domain is the main functional domain of pyroptosis, producing the pore and the lytic form of cell death, while the C-terminal domain has the

function of self-inhibition of GSDMD [55]. When GSDMD is not cleaved and activated, the N-terminal domain and C-terminal domain of GSDMD are connected by a loop, the C-terminal domain blocks the pore formation effect of the N-terminal domain and makes the N-terminal domain lose its affinity with the plasma membrane lipids [56, 57]. The binding of the N-terminal domain of GSDMD to lipids was found to function only in the inner surface of the plasma membrane, which is highly consistent with the distribution of phosphatidylserine and phosphoinositide in the plasma membrane [57]. The cytotoxic N-terminal domain of GSDMD can only bind phosphatidylserine and phosphoinositide in the inner surface of cell membrane, so the cytotoxic N-terminal domains are only oligomerized in the intracellular surface of cell membrane to form pores and induce cell lysis [58]. Therefore, the N-terminal domains of GSDMD released by cell lysis cannot bind to the outer surface of cell membrane of adjacent cells, and do not induce cell lysis of adjacent cells [59]. According to the lipid distribution of bacteria, cardiolipin with affinity to the N-terminal domain of GSDMD is distributed on the outer side of bacterial plasma membrane, previous reports showed that the cytotoxic N-terminal domain of GSDMD can directly kill bacteria [60].

Among Gasdermin superfamily members, GSDMA, GSDMC and GSDMD were reported to function as tumor suppressor gene [61-63], but the mechanism underlying the tumor-suppressing function of Gasdermin superfamily and their downstream pathways remain to be clarified. Currently, it is controversial in terms of the role of GSDMD in cancer after the

redefinition of GSDMD function in pyroptosis. Previously, one study has shown that downregulated GSDMD promotes proliferation of gastric cancer, while another study has shown that downregulation of GSDMD suppresses proliferation of non-small cell lung cancer. The contrary functions of GSDMD above were both associated with pyroptosis [52, 64]. Conventionally, GSDMD is regarded as a predominantly cytoplasm-localized protein playing a key role in pyroptosis, particularly in immune cells and recently cancer cells [65, 66]. The role and mechanism of Gasdermin superfamily, including GSDMD, and their downstream pathways in the tumorigenesis and progression remain to be determined. Our preliminary finding on GSDMD nuclear translocation in chemotherapeutic sensitization will highlight a new direction for cancer biology and anti-cancer therapy.

1.4 DNA Repair Enzyme PARP-1

Poly(ADP-ribose) polymerase-1 (PARP-1) is a DNA repair enzyme widely existing in eukaryotic cells, belonging to the PARP superfamily, which has 17 members, among which PARP-1 is the most widely studied. The primary protein structure of PARP-1 is composed of 1014 amino acids with molecular weight of 116 KDa [67, 68]. PARP-1 is mainly located in the promoter region of the nucleus [69]. DNA binding domain of PARP-1 recognize and bind to the damaged DNA structures [70]. DNA binding domain of PARP-1 is involved in mediating the binding of PARP-1 to the damaged DNA and regulating its catalytic activity [71]. The major function of PARP-1 is to repair damaged DNA. PARP-1 recognizes DNA

single and double strand damage and plays an important role in the stability of genome. PARP-1 mainly uses base excision repair (BER) to repair single strand breaks (SSB) of DNA. When single strand breaks occur in genomic DNA, replication fork stops, or DNA cross-links, these situations activate the PARP-1. Poly(ADP-ribosyl)ation (PAR) is posttranslational modification of proteins by linear or branched chains of ADP-ribose units [72]. PAR has been reported to regulate protein stability, protein subcellular localization, protein-protein interactions, and transcriptional regulation. PARP-1 has been reported to play important roles in DNA damage repair, cell proliferation, cell differentiation, and apoptosis [73, 74]. PARP-1 has been studied in synthetic lethality, which refers to the phenomenon in which mutations in two genes together result in cell death, but a mutation in either gene alone does not [75, 76]. It has been reported that tumor cells with BRCA1/BRCA2 (Breast cancer gene) gene mutations resulting in homologous recombination repair malfunctions are more sensitive to PARP-1 inhibitors than cells with functional BRCA1 and BRCA2, PARP-1 and mutant BRCA1/BRCA2 gene work together to induce synthetic lethality of the tumor cells [77, 78]. Previous reports showed that homologous recombination repair deficient colorectal cancer cells are sensitive to PARP-1 inhibitor treatment [79].

Olaparib is a potent PARP inhibitor. Nowadays, the combination of platinum-based chemotherapeutic drugs and the PARP inhibitor Olaparib has been approved by the FDA for breast and ovarian cancer [80, 81]. Olaparib sensitizes the apoptotic action of the DNA

damage-related chemotherapeutic drugs in breast and ovarian cancer cells, likely due to its inhibition on DNA repair function [81]. Here in the preliminary study, we found that down-regulation of GSDMD contributes to Oxaliplatin chemotherapeutic resistance, which can be eliminated by the combined application with Olaparib, implying that GSDMD functions in the same way as PARP inhibitor Olaparib to some extent. The nuclear localization pattern of GSDMD in CRC cancer cells may give guidance to the combinational application of PARP inhibitor Olaparib and basic chemotherapeutic drugs in colorectal cancer.

1.5 ATM Regulated DNA Damage Response Signaling Pathway

DNA Damage Response signaling pathway is orchestrated by ATM and ATR kinases, the central regulators of this network. ATM/ATR kinase can be activated by DNA damage, with non-redundant function and distinct downstream signaling pathway [82]. ATM is a central element of DNA damage response signaling pathway. The ATM protein belongs to a protein kinases family containing the phosphatidylinositol-3 catalytic domain, including ATM, ATR and PI3K [83]. ATM is located at the core of DNA damage pathway. ATM is activated after DNA double-strand break (DSB). Through a variety of complex cellular reaction processes, ATM triggers its autophosphorylation, and subsequent activation to promote DNA repair [84]. The study has shown that when ATM is mutated or insufficient, the cell is unable to recognize the DNA damage that has occurred and to respond

appropriately to the damage, which destroys the integrity of the intracellular genome and in turn causes cell death [85].

Researchers have proposed anticancer therapeutic approaches by inhibiting ATM. By inhibiting ATM, DNA damage response mediated cell cycle arrest, DNA repair and apoptosis pathways are blocked, thereby inducing apoptosis of cancer cells [86]. Studies have shown that the application of ATM kinase inhibitor KU55933 or other ATM/ATR inhibitor CGK733 lead to increased apoptosis of cells. The interesting finding of this study is that this type of ATM-specific inhibitor only let the cells that are affected by cancer and prematurely senescence lose the ability to resist apoptosis, thereby destroying cancer cells, whereas there has no intervention on normally senescent cells [87, 88].

It is reported a high proportion of ATM aberrations of B-cell chronic lymphocytic leukemia, which is associated with relatively short progression-free survival following chemotherapy and adverse prognoses. Interestingly, some studies found that ATM mutation events may increase the chemotherapy sensitivity of cancer patients [89, 90]. For example, ATM mutations may cause a higher sensitivity to platinum drugs among the cancer patients, particularly breast and ovarian cancer [91].

One study used systems biology methods to build mathematical and bioinformatics models that can better predict the therapeutic function and effect of ATM inhibitors as potential anticancer drugs. These models improved clinical drug intervention strategies targeting ATM signaling pathways [92]. Preclinical data also provided a strong rationale for clinical

testing of ATM inhibitor both in combination with chemotherapy, and in synthetic lethal approaches to treat tumors. One study has reported that highly selective small molecule inhibitors of ATM are under the preclinical and clinical development [92].

1.6 Nuclear Localization Signal

The nuclear membrane that isolates DNA is one of the characteristics of eukaryotic cells. Thus, the nuclear membrane separates the nuclear processes of DNA replication and RNA transcription from the cytoplasmic processes of protein production [93]. The proteins needed in the nucleus must be delivered there by some mechanism. Nuclear localization signal (NLS) are amino acid sequences that mark proteins for nuclear translocation [93]. Different nuclear localization proteins may share the same NLS sequence [93]. The function of NLS is the opposite of that of nuclear export signals [94]. The transport into and out of nucleus of cargo molecules with molecular weight > 40-60 kDa is largely mediated by members of the Importin Superfamily of transporters, consisting of α- and β-types [94]. The movement of proteins between the cytoplasm and nucleus usually involves recognition of nuclear localization signals by members of the Importin superfamily and then translocation though nuclear pore complexes embedded in the nuclear membrane [94]. The NLS labeled protein bind to importin and the complex pass through the nuclear pore complex together [94]. At this point, RAN-GTP binds to the Importin-protein complex, which causes Importin to lose its affinity for the protein. After that, the protein is released

and the RAN-GTP/Importin complex move out of the nucleus through the nuclear pore complex [95]. Next, the GTP-activated protein in the cytoplasm hydrolyzes Ran-GTP to GDP, which results in a conformational change of Ran that ultimately reduces its affinity for the Importin protein[95]. Finally, Importin is released, and Ran-GDP is recycled back to the nucleus, where guanine nucleotide exchange factor converts its GDP back into GTP [95].

Targeted NLS sequence recognition represents a critical control point for nuclear transport regulation. The affinity of the targeted nuclear localized protein and its specific NLS sequence interaction is a key parameter to determine transport efficiency [94]. Different nuclear localized proteins have different NLS binding specificities, which allows the system to be regulated by the differential expression of NLS protein itself as well as by competition between different NLSs for the same protein and between different proteins for the same NLS [94]. Previous studies have found that targeted nuclear localized protein and its specific NLS sequence interaction can be affected directly by phosphorylation increasing the affinity of the interaction with importins, or by targeting sequence masking through phosphorylation or specific protein binding [96].

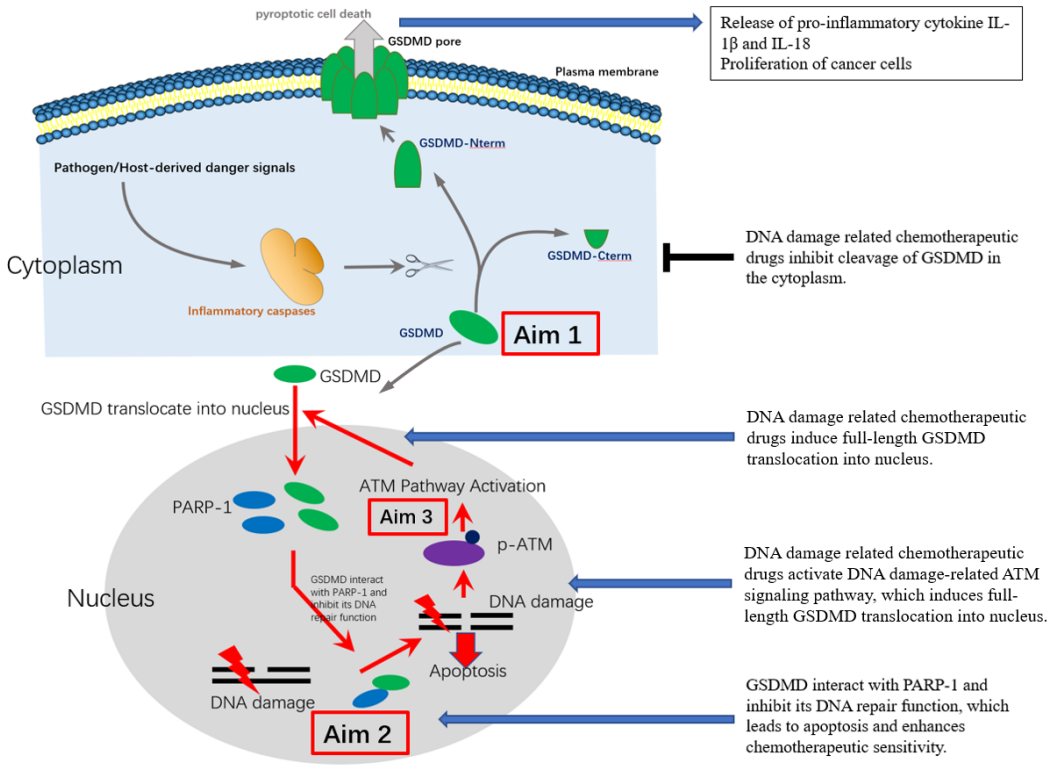

Figure 1.2 - Schematic overflow of GSDMD cytosolic pyroptosis and potential mechanisms for GSDMD nuclear translocation, and nuclear apoptosis in colorectal cancer cells.

We found GSDMD nuclear translocation and the apoptotic role of GSDMD for the first time. GSDMD interacts with PARP-1 and inhibits its DNA repair function, which leads to apoptosis and enhances chemotherapeutic sensitivity. This cartoon was made with BioRender.

Specific Aim 1: To validate the preliminary finding that GSDMD nuclear localization in colorectal cancer cells has better clinical outcomes in colorectal cancer patients;

Specific Aim 2: To define the non-pyroptotic function of GSDMD in colorectal cancer cell with chemotherapy and investigate how GSDMD translocate into nucleus to interact with PARP-1 and inhibit the DNA repair response of PARP-1;

Specific Aim 3: To determine ATM and NLS dependent mechanisms for GSDMD nuclear translocation that sensitize chemotherapy of DNA damage-related agents.

1.7 Hypothesis and Specific Aims

In our preliminary studies using multi-omics strategies, we found that Gasdermin D

(GSDMD) mRNA expression is significantly higher in adenocarcinoma than the normal mucosa and adenoma. However, statistical analysis revealed no significant correlation of GSDMD expression levels with the overall survival of colorectal cancer patients. Further studies showed chemotherapy induced robust nuclear localization of non-cleaved GSDMD in cancer cells, which correlated with better overall survival rate. Therefore, we hypothesize that **non-pyroptotic GSDMD plays a beneficial role in promoting chemotherapy-induced apoptosis of tumor cells via its nuclear localization and insufficient GSDMD nuclear translocation predicts worse prognosis of colorectal cancer patients. Non-pyroptotic GSDMD promotes chemotherapy-induced apoptosis of tumor cells via its nuclear translocation and subsequent regulation of PARP-1-mediated DNA repair.**

We will test our central hypothesis via the following specific aims as shown in **Figure 1.2**:

Specific Aim 1: To validate the preliminary finding that GSDMD nuclear localization in colorectal cancer cells has better clinical outcomes in colorectal cancer patients;

Specific Aim 2: To define the non-pyroptotic function of GSDMD in colorectal cancer cell with chemotherapy and investigate how GSDMD translocate into nucleus to interact with PARP-1 and inhibit the DNA repair response of PARP-1;

Specific Aim 3: To determine ATM and NLS dependent mechanisms for GSDMD nuclear translocation that sensitize chemotherapy of DNA damage-related agents.

CHAPTER 2: GSDMD NUCLEAR LOCALIZATION IN COLORECTAL CANCER HAS BETTER CLINICAL OUTCOMES IN COLORECTAL CANCER PATIENTS

2.1 Introduction

The pathogenesis of colorectal cancer is a multi-step, multi-stage, and multi-gene involved process [21, 22]. Therefore, understanding the characteristics of multi-omics and their interactions during the transformation of normal mucosa to adenoma and eventually to adenocarcinoma in each patient with sporadic colorectal cancer is of great significance to elucidate the mechanism of most common colorectal cancer. However, due to the difficulty in obtaining adenoma samples, most studies lack analysis of intermediate state-adenomas from the same patient's lesions.

To explore the dynamic regulatory mechanism, paired samples of normal mucosa-adenoma-adenocarcinoma in the same area, and multi-omics study of gene expression by genomic variation and epigenetic modification in three important pathological stages of polyp formation-adenoma progression-adenocarcinoma malignancy at individual sporadic colorectal cancer in the Chinese people were studied. Any potential variations in ethnicity, environmental factors and dietary habits were excluded as much as possible, in order to provide a more rigorous and reliable research model for delineating the transformation mechanism during CRC tumorigenesis.

Previous reports on the role of GSDMD in cancer were contradictory. On one hand, downregulated GSDMD promotes gastric cancer proliferation[64], while on the other hand, downregulated GSDMD suppresses tumor proliferation in non-small cell lung cancer [97]. Both of the contrary functions of GSDMD above involve cleavage-dependent pyroptosis. However, another study on the function of GSDMD in gastric cancer revealed no evidence of GSDMD cleavage in cancer cells upon treatment with chemotherapeutic drug 5-Fu [98]. The role of non-cleaved GSDMD in tumorigenesis and chemotherapeutic resistance remains elusive. Apoptosis and pyroptosis use different caspase family members. Pyroptosis use pro-inflammatory caspase 1,4,5,11 [45, 46]. Apoptosis use caspase 3,7 [43]. In cancer cells, there are protein expression of pro-inflammatory caspase. But there is no activated pro-inflammatory caspase in cancer cells. So, there should be no pyroptosis occurring in cancer cells [45, 46]. However, some previous research showed that innate immune cell derived activated pro-inflammatory caspase can travel though the cell membrane of cancer cell by exosomes and then trigger pyroptosis of cancer cells [47-49]. Our preliminary multi-omics studies in Trio samples identified that GSDMD mRNA expression was significantly higher in adenocarcinoma than the normal mucosa and adenoma but its correlation with prognosis was unclear. Conventionally, GSDMD is regarded as a predominantly cytoplasm-localized protein playing a key role in pyroptosis [65, 66], which leads to cell proliferation via release of proinflammatory cytokines (**Figure 1.1**). Unexpectedly, our preliminary studies found that most cancer tissue samples show

positive nuclear staining of GSDMD. By Kaplan-Meier survival analysis of GSDMD nuclear staining intensity and their correlations with prognosis, we found that GSDMD nuclear localization in colorectal cancer cells has better clinical outcomes in CRC patients. The objective of this specific aim and chapter is to explore the correlation between the nuclear localization of GSDMD and overall survival rate of colorectal cancer patients. The central hypothesis is that higher level of GSDMD expression in colorectal tumor tissues may promote pyroptotic inflammation and tumorigenesis, while chemotherapy may induce nuclear translocation of non-cleaved GSDMD and subsequent apoptosis in cancer cells, leading to better survival rate in CRC patients. We performed the immunohistochemical staining of GSDMD in the tumor masses of CRC patient samples as well as Western blot analysis of cytosolic and nuclear fractions. We evaluated whether the GSDMD nuclear localization exists in cancer cells of CRC patients. We found that GSDMD nuclear localization plays a crucial role in apoptosis (**Figure 1.2**).

2.2 Materials and Methods

Reagents, Antibodies and Plasmids.

Etoposide (#E1383), Cisplatin (#C2210000), propidium iodide (#P4170) and 5-Fu (#F6627) were purchased from Sigma-Aldrich. Olaparib (HY-10162), was obtained from MedChem Express. Mitomycin C (#S8146) was purchased from Selleck. Puromycin (#A610593-0025) was purchased from Sangon Biotech. Oxaliplatin was treated at a dose

of 0.5μM and 5-Fu was treated at a dose of 25μg/ml for 2/12/24 hours in CRC cell lines. The antibodies were used as below: anti-Flag (#F3165) for western blotting and IHC was from Sigma-Aldrich; anti-Myc (#05-724) was purchased from Millipore; anti-PARP-1 (#9532) and anti-γ-H2AX (#9718) were from Cell Signaling Technology; anti-PAR (#4335-AMC-050) was from Trevigen; anti-human GSDMD (#sc-393656) for IF at a dilution of 1:100 was purchased from Santa Cruz Biotechnology; anti-Ki67 (#ab15580) for IHC and anti-human GSDMD(#ab215203) for detection of N-terminal domain cleavage at a dilution of 1:1000 for western blotting were from abcam; anti-Human GSDMD(#20770-1-AP) for IHC at a dilution of 1:100 and western blots at a dilution of 1:2000 was obtained from Proteintech; anti- Cleaved Caspase-3 (#9664) was from CST. The PCS2-Flag tagged human GSDMD plasmid was kindly gifted from Prof. Feng Shao. Human GSDMD-3×Flag-pLVX-IRES-puro plasmid was constructed by Cyagen Co., Ltd and verified by sequencing. The pIP-Myc tagged human PARP-1 and PARP-1 domain truncation plasmids were from Prof. Bin Li. The shRNA sequences targeting human GSDMD were as follows: GSDMD-sh1 CAGCACCTCAATGAATGTGTA; GSDMD-sh2 GTGTGTCAACCTGTCTATCAA.

Patients and tissue specimens

A total of 80-case colorectal cancer tissue microarray specimens collected from the patients, who had undergone the operation at the Department of General Surgery of the First

People's Hospital of Shanghai in China, were used in this study. Informed consent had been obtained from all patients and the project had been approved by the local Ethics Committee.

Joint analysis of single-cell and multi-omics sequencing data

For the combined data of mutation, methylation, and expression, we constructed the differential co-expression network of between N and AD, and the differential co-expression network of methylation and expression between AD and TD using R package DiffCorr (Version 0.4.1) [99]. It calculated the correlation matrices for each dataset, identified the first principal component-based "eigen-molecules" in the correlation networks, then tested differential correlation between the two conditions by using Fisher's z-test. Fisher's z-test was based on stringency test as well as its conservative estimates. We used the Seurat package (Version 4.0.6) to filter single-cell sequencing data and drew cluster analysis maps. Furthermore, FindClusters in the Seurat package was used to determine the number of clusters obtained by downstream clustering analysis, and RunUMAP was used to perform dimensionality reduction and visual display. To decipher the regulations among mutation, methylation and GSDMD expression patterns of each cell cluster in different stages, we constructed the Bayesian network using R package bnlearn (Version 4.7.1) [100].

Sequencing data processing and quality control

The raw sequencing reads from whole-exome sequencing was filtered by NGSQC Tool kit (version 2.3.3) with default parameters. All the samples produced enough high-quality sequencing data with averagely 13.26 Gb raw data after Illumina pass filtering (B160-folds genome coverage). Clean reads were aligned to human reference genome (hg38) using Burrows–Wheeler Aligner (version 0.7.15) with default parameters. SAM tools (version 1.3.1) was used to convert files in SAM format to BAM format. Then, polymerase chain reaction (PCR) duplicates were removed by using Mark Duplicates of Picard tools (version 2.5.0). Deduplicated reads were realigned at known indel positions with GATK Indel Realigner and base quality recalibration was performed by GATK Base Recalibrator (version 4.2.6.1).

Mutation Detection and annotation

Germline variants calling were carried by GATK Haplotype Caller with default parameters and filtered out with common single nucleotide polymorphism. Somatic variants calling were performed for matched samples (carcinoma adenoma sample to normal sample) of each patient by Varscan2 (version 2.4.2). To avoid false-positive, several filtering criteria were applied. We required the minimum coverage of both normal tissue and matched tumor tissue should be no less than 10 reads. Moreover, the mutation should be supported by at least 5% of the local mapping reads in the tumor tissue, and each variant should be

supported by at least three reads in the tissue. For each candidate somatic mutation site, one-tailed Fisher's exact test was used for the allele depth and frequency of the case and germline samples to eliminate the mutations found in the normal and tumor tissues (p-value< 0.01). The variants in the intergenic region and intronic region were also excluded in the following analysis. The potential effects of the somatic SNV and indels were annotated using ANNOVAR (hg38).

Immunohistochemistry (IHC)

IHC was performed on 3μm thick paraffin-embedded section of tumor tissues. Specimens were gradually de-paraffinized in xylene and rehydrated in gradient alcohols to water. Blocking endogenous peroxidase activity was performed with 3% H2O2 in PBS for 15 minutes. For antigen retrieval, the sections were microwaved with 10mM citrate buffer with pH=6.0. Then sections were blocked with 10% goat serum to exclude unspecific staining, incubated with the indicated primary antibodies at appropriate dilution overnight at 4°C and then washed with PBST for three times and incubated with biotinylated secondary antibodies for 40 minutes at room temperature. GSDMD antibody, anti-human GSDMD (#96458), for IHC was purchased from Sigma-Aldrich. After washing with PBST, slides were incubated with streptavidin-peroxidase complex in PBS for 20 minutes at 37°C. The sections were rinsed with PBST, and staining was then revealed using 3, 3-diaminobenzadine substrate and counterstained with hematoxylin for 15 minutes. The IHC

staining intensity scoring of Tissue Microarray were strictly evaluated by 3 senior pathologists manually, not blindly, with the immunoreactive score system (IRS) that has been described as follows: negative, 0-1 point; mild (+), 2-3 points; moderate (++), 4-8 points; strongly positive (+++), 9-12 points. The IHC staining showing IRS scores ≥ 4 points was considered as high, and IRS scores ≥ 2 points was considered as positive.

Kaplan-Meier Plotter Database Analysis

Kaplan-Meier plotter can assess the effect of all the specific key genes on survival using 80 colorectal cancer samples. The correlation between GSDMD expression and survival in colorectal cancers was analyzed by Kaplan-Meier plotter (http://kmplot.com/analysis/) [101]. The hazard ratio (HR) with 95% confidence intervals and log-rank P-value was also computed.

Cell Culture and Transfection.

The PCS2-Flag tagged human GSDMD plasmid was kindly gifted from Prof. Feng Shao. Human GSDMD-3×Flag-pLVX-IRES-puro plasmid was constructed by Cyagen Co., Ltd and verified by sequencing. The shRNA sequences targeting human GSDMD were as follows: GSDMD-sh1 CAGCACCTCAATGAATGTGTA; GSDMD-sh2 GTGTGTCAACCTGTCTATCAA. HEK293T, SW1116 and RKO cell lines were purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM,

Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin-streptomycin. Caco-2, HCT1116, COLO 205, SW620 and HT-29 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin-streptomycin. THP-1 cell line was grown in Gibco Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin. All cells were maintained at 37 °C in a 5% CO2 incubator. HEK293T cell line was transfected with the indicated plasmids using polyethyleneimine (Polyscience, 23966) reagent by following the manufacturers' instructions. Polyethylenimine Linear was used as the transfection reagent. For construction of stably expressing cell lines, the lentivirus infection technology was used for overexpression and knockdown of human GSDMD gene. In brief, lentivirus plasmids were co-transfected with the packing plasmids Δ8.9 and VSVG into HEK293T cells. The viral supernatants were harvested 48h and 72h after transfection, 5ml viral supernatant was added to 5ml fresh cell culture media and used to infect SW1116 and RKO cell lines along with polybrene (Sigma-Aldrich, H9268), and then subjected to positive selection with puromycin. Puromycin (#A610593-0025) was purchased from Sangon Biotech.

Western blot analysis

Equivalent amounts of protein were loaded and separated by SDS/PAGE, and protein expression was quantified by western blotting with primary antibody against human

GSDMD (#96458) from Sigma-Aldrich, primary antibody against Flag (#F3165) from Sigma-Aldrich, and primary antibody against PARP-1 (#9532) from Cell Signaling Technology. anti-Human GSDMD for western blots at a dilution of 1:2000; anti-Flag for western blots at a dilution of 1:2000; anti-PARP-1 for western blots at a dilution of 1:1000.

Colony Formation Assay

Cells were seeded at a density of 400 cells/well in 6-well plate with complete medium. After culture for 15 days, cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After three times of washes with PBS, cells were dyed with reagent crystal violet, followed by three times of washes with distilled water. Images were acquired with microscope.

Cell Growth Assay and Cytotoxicity Test

Cells were seeded at a density of 1500 cells/well to detect cell growth in 96-well plate with complete medium; cells were seeded at a density of 10000 cells/well to detect cytotoxicity of Oxaliplatin at a dose of 0.5μM for 24 hours in 96-well plate with complete medium. The results were measured by CCK-8 cell proliferation kit (CK04, Dojindo) according to the manufacturer's instructions.

Immunofluorescence (IF) assay

Cells were seeded on coverslips in 24-well plate and then treated with corresponding reagents/conditions for an indicated time. Stimulated cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After three times of being washed with PBS, cells were permeabilized in 0.2% (v/v) TritonX-100 for 20 minutes at room temperature. Coverslips were then washed three times with PBS and incubated with blocking buffer containing 5% (wt/v) BSA and 5% (v/v) FBS. Afterwards, cells were incubated with primary antibodies for 16 hours at 4°C diluted in PBS with 1% BSA and 1% FBS. anti-Flag (#F3165) for IF was purchased from Sigma-Aldrich; anti-human GSDMD (#sc-393656) for IF was purchased from Santa Cruz Biotechnology. After three times washes with PBST (0.1% tween-20 in PBS), cells were incubated with Alexa Fluor ® secondary antibodies (Invitrogen) and nuclei stain DAPI (Invitrogen, H3569) for 1 hour at room temperature. Coverslips were washed four times with PBST and then images were acquired using ZEISS LSM 710 Metal confocal microscope.

Nuclear/Cytoplasmic Fractionation

1×10^7 cells were washed with cold PBS and harvested with 200µl cold buffer A containing 10mM Hepes buffer (pH7.4), 10mM KCl, 1.5mM MgCl2, 0.5mM EDTA, 0.5mM EGTA with 1mM PMSF, 1mM Na3VO4, 1mM NaF and protease inhibitor. After placing on ice for 15 minutes, cells were rinsed 2 times with a 1mL syringe with 25G

needle and fraction were separated by spinning at 1000 rpm for 5 minutes at 4°C. The cytoplasmic supernatants were further clarified by spinning at 14000 rpm for 10 minutes and the supernatants were collected as the cytoplasmic fraction. The nuclear pellets were washed three times with buffer A and then were lysed by rotating in cold buffer B, comprising of 10mM Hepes buffer (pH7.4), 0.42mM NaCl, 2.5%(v/v) glycerol, 1.5mM MgCl2, 0.5mM EDTA, 0.5mM EGTA, 1mM DTT supplemented with 1mM PMSF, 1mM Na3VO4, 1mM NaF and protease inhibitor. After incubation on ice for 30 minutes, the lysates were centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatants were then collected as the nuclear fraction.

Statistical analysis

All experiments were repeated at least 3 times with comparable results. Statistical analysis of data was carried out with GraphPad Prism 7 software, and results were presented as mean ± SD with indicated statistical methods. Statistical significance was distinguished with p value: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.3 Results

Multi-omics profiling identified upregulation of GSDMD mRNA in adenocarcinoma compared to normal mucosa and adenoma in Trio samples.

To explore the role of GSDMD in the pathogenesis and prognosis of colorectal cancer, we

collected paired Trio colorectal samples of normal mucosa-adenoma-adenocarcinoma in the same area and same sporadic colorectal cancer in 110 patients and performed extensive multi-omics data analysis. We found that GSDMD mRNA expression is significantly higher in adenocarcinoma than the normal mucosa and adenoma (**Figure 2.1A**). To explore the role of GSDMD in the pathogenesis and prognosis of colorectal cancer, we hope to gain in-depth understanding of normal mucosa (N) - adenoma (AD) - adenocarcinoma (TD) processes through multi-omics comprehensive analysis, to verify the relationship and correlation between GSDMD nuclear translocation and chemotherapy sensitization and cancer progression/prognosis.

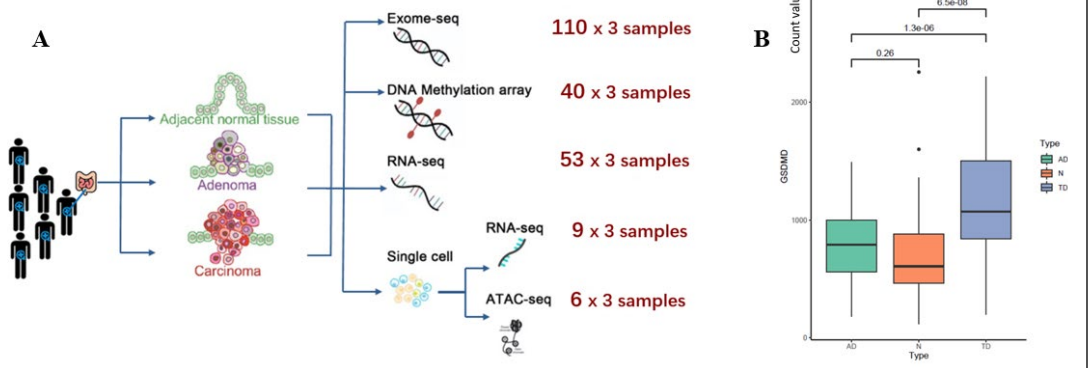

Figure 2.1 - Multi-omics profiling identified upregulation of GSDMD mRNA in adenocarcinoma.

(**A**). Diagram for trio sample collection and multi-omics methods. Exome-seq: whole exome sequencing; DNA Methylation array: 850K DNA methylation chips and Pyrosequencing; RNA-seq: RNA sequencing; scRNA-seq: single-cell RNA sequencing; scATAC-seq: single-cell Assay for Transposase-Accessible Chromatin sequencing. (**B**). ANOVA analysis of GSDMD mRNA expression among three groups. N: normal tissue; AD: adenoma; TD: adenocarcinoma.

We collected paired Trio colorectal samples of normal mucosa-adenoma-adenocarcinoma in the same area and same sporadic colorectal cancer of 110 Chinese patients to perform extensive multi-omics data analysis. Trio samples (Normal tissue- adenoma-adenocarcinoma) were collected from the same CRC patient, who had undergone the operation at the Department of General Surgery of the West China Hospital, Sichuan University. Trio sample from 110 CRC patents were collected. High-quality DNA and RNA from matched Trio samples of these patients were extracted to perform multi-omics studies and single cell studies. We obtained the multi-omics data from all these CRC patients, including 346 whole exome sequencing samples of 110 patients, 189 RNA sequencing samples of 68 patients and 126 methylation samples of 42 patients (**Figure 2.1A**). We cooperate with a Bioinformatics team from the Chinese Academy of Sciences to analyze the data. Initial analysis revealed that GSDMD mRNA expression is significantly higher in adenocarcinoma than the normal mucosa and adenoma (**Figure 2.1B**). With the in-depth development of research, we have found that more signals from cell populations are obtained through traditional multi-omics sequencing. While single-cell (sc) transcriptome technology can further explore the tumor heterogeneity of colorectal cancer, the immune microenvironment, and the different stages of intestinal adenoma carcinogenesis. From this, we have added single-cell sequencing analysis technology to explore more in-depth molecular regulation mechanisms at the individual level. Thus, we obtained another 27 scRNA sequencing samples of 9 patients and 18 scATAC (Single-cell sequencing assay for

transposase-accessible chromatin) sequencing samples of 6 patients, which are also paired Trio colorectal sequencing samples of normal mucosa-adenoma-adenocarcinoma (**Figure 2.1A**).

Through whole-exome sequencing analysis of Trio samples, we found three single nucleotide polymorphism sites of GSDMD in CRC cancer cells, all of which are located on the long arm of chromosome 8, region 2, band 4, sub-band 3. There are three single nucleotide variant mutations that occur in the exon region. The first single nucleotide polymorphism site is in rs143559394. This nonsynonymous mutation occurs on exon 2 of NM_024736, resulting in a nucleobase mutation at position 59 from Guanine to Thymine, and the mutation of the amino acid at position 20 from Glycine to Valine. The second single nucleotide polymorphism site is in rs143561373. This nonsynonymous mutation occurs on exon 6 of NM_024736, resulting in the nucleobase mutation at position 686 from Thymine to Cytosine, and the mutation of the amino acid at position 229 from Valine to Alanine. The third single nucleotide polymorphism site is rs143562859. There is a synonymous mutation.

Positive nuclear expression of GSDMD is associated with favorable clinical outcomes, while negative nuclear expression of GSDMD is associated with worse clinical outcomes.

GSDMD is mainly expressed in cytoplasm of colorectal cancer cells. We unexpectedly observed robust nuclear localization of GSDMD in the CRC patients, which is

significantly correlated with better prognosis. Further studies using anti-GSDMD antibody in clinical specimens validated the expression of GSDMD protein in adenocarcinoma tissue. Representative IHC images of tissue chip of colorectal cancer paired with paratumor tissue show lower expression of GSDMD in cancer tissue with significant statistical difference by paired student's t test, ***, $p < 0.001$. Very interestingly, we observed the existence of GSDMD nuclear location in cancer cells of CRC patients (**Figure 2.2A**), and such nuclear translocation of GSDMD has significantly positive correlation with a better overall survival of patients with colorectal cancer (**Figure 2.2B**), although there was no significant correlation between the total GSDMD expression and the prognosis (**Figure 2.2C**). Survival plot with Kaplan-Meier Analysis of the above 80-case cancer tissue microarray observes no significant difference when grouped by high and low the expression level of GSDMD in the whole cells. While Kaplan-Meier analysis confirmed the statistically significant correlation between the expression level of nuclear GSDMD in the colorectal cancer cells and the survival rate of patients. These results indicated that high nuclear GSDMD expression indicates favorable clinical outcomes, all these above give hints that GSDMD location in the nuclei but not cytoplasm of CRC cells may have effects on malignant biological properties of cancer (**Figure 2.2B-C**). These preliminary observations strongly support our central hypothesis (**Figure 1.2**). Inferring from the above clinical data, GSDMD may act as a predictive

marker of clinical outcomes in advanced colorectal cancer, and it may serve as a promising target for therapy of colorectal cancer.

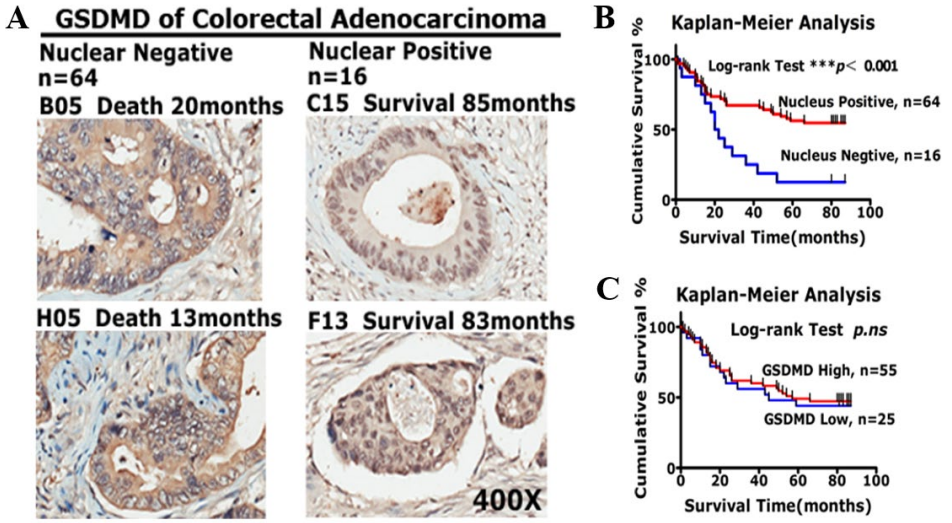

Figure 2.2 - GSDMD nuclear localization correlates with better clinical outcomes. (**A**). Representative IHC images of tissue chip show 2 positive nuclear staining loci with better clinical outcomes and 2 negative nuclear staining loci with worse clinical outcomes. (**B**) Survival plot for GSDMD nuclear positive expression (n=64) and negative expression (n=16) in colorectal cancer show that positive nuclear expression favors clinical outcomes, while negative nuclear expression indicates worse clinical outcomes with significant statistical difference by log-rank test, ***, $p < 0.001$. (**C**) Survival plot for GSDMD low expression (IHC staining intensity – and +, n=25) and high expression (IHC staining intensity ++ and +++, n=55) in colorectal cancer is of no significant statistical difference with log-rank test to show GSDMD expression is not a good indicator for clinical outcomes.

Establishment of GSDMD overexpression and knockdown stably expressing colorectal cancer cell lines

To investigate the role of GSDMD in malignant progression of colorectal cancer, we first established GSDMD overexpression with 3×Flag tag and knockdown by short hairpin

RNA in stably expressing RKO and SW1116 cell lines. RKO and SW1116 cell lines are commonly used CRC cell lines. The targeting sequence of shRNA knockdown plasmid is as follows, GSDMD-sh1: CAGCACCTCAATGAATGTGTA; GSDMD-sh2: GTGTGTCAACCTGTCTATCAA. After that, Establishment of GSDMD overexpression and knockdown stably expressing RKO and SW1116 CRC cell lines were verified by western blot **(Figure 2.3A-B)**.

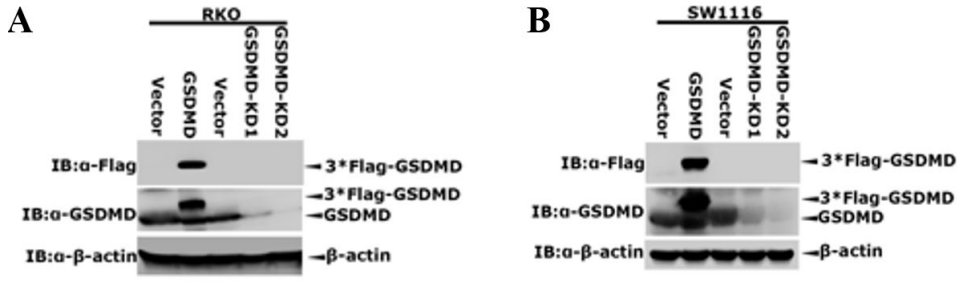

Figure 2.3 - Western blot verify the establishment of GSDMD overexpression and knockdown stably expressing cell lines of colorectal cancer in RKO and SW1116.
(**A-B**). Establishment of GSDMD overexpression with 3×Flag tag and knockdown by short hair RNA stably expressing cell lines in RKO and SW1116 are shown by Western Blot.

GSDMD had little effect on colony formation ability and cell growth ability in colorectal cancer cell lines

To determine whether GSDMD overexpression is involved in CRC cell colony formation ability, we performed colony formation assay in GSDMD vector and GSDMD overexpression cell lines **(Figure 2.4A)**. The colony formation assay results showed that GSDMD had little effect on colony formation ability on colorectal cancer cell lines under

cell culture conditions in vitro **(Figure 2.4B-C)**.

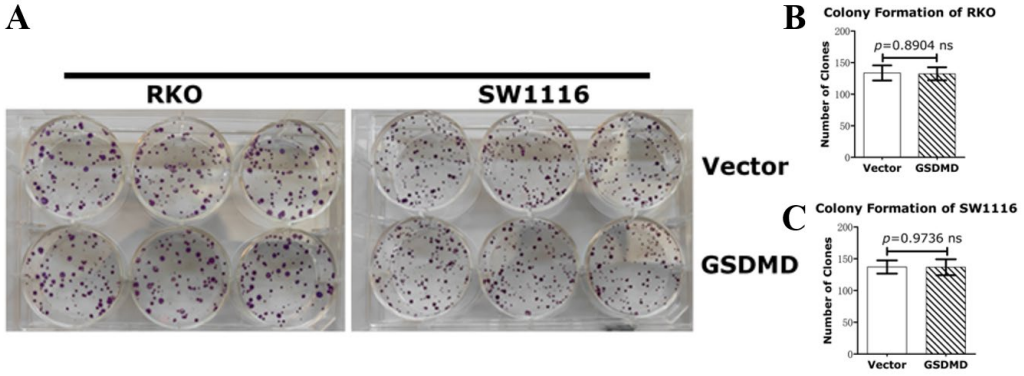

Figure 2.4 - GSDMD has little effect on colony formation ability on colorectal cancer cell lines.
(**A-C**). Colony formation assay stained by crystal violet indicates GSDMD has little effect on colony formation ability measured by number of colonies in RKO and SW1116 in vitro. Data were shown as mean ± SD with p ns.

To determine whether GSDMD overexpression is involved in CRC cell growth, we performed Cell Counting Kit-8 (CCK8) assay to examine the cell proliferation and viability ability in GSDMD vector and GSDMD overexpression CRC cell lines. Cancer cell growth were measured by absorbance value in the GSDMD stably expressing cell lines. The results showed that GSDMD had little effect on colorectal cancer cell growth under cell culture conditions in vitro **(Figure 2.5A-D)**.

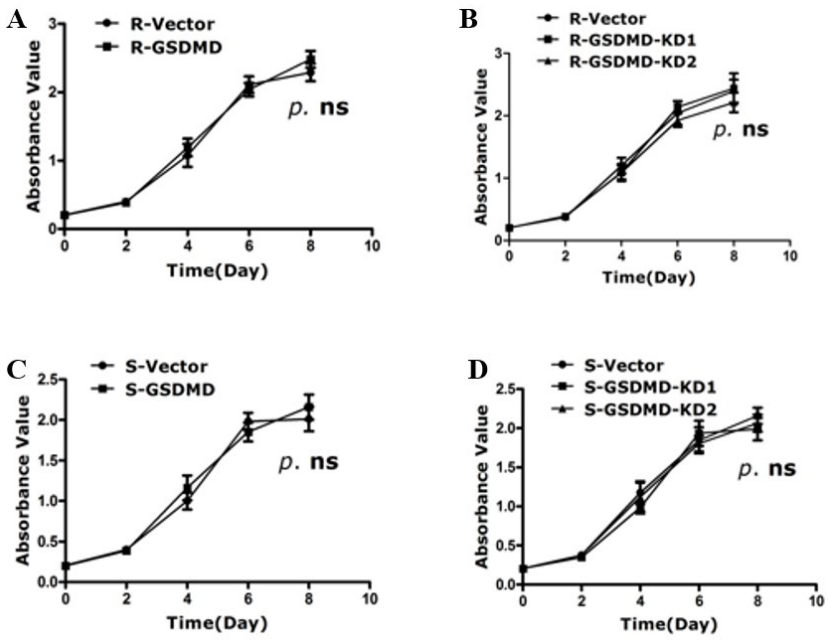

Figure 2.5 - GSDMD has little effect on colorectal cancer cell growth.
(**A-D**). CCK8 assay indicates that GSDMD overexpression and knockdown have little effect on cell growth in RKO and SW1116 under cell culture conditions in vitro. Data were shown as mean ± SD (standard deviation) with p ns (no statistical significance).

GSDMD had potent promoting effect on chemotherapeutic drug Oxaliplatin induced cytotoxicity

To further investigate the effect of GSDMD on the biological behavior of colorectal cancer, we examined the effect of this gene on first-line chemotherapeutic drug, Oxaliplatin, induced apoptosis in colorectal cancer. After chemotherapeutic drug Oxaliplatin treatment, we performed CCK8 assay to detect the cell viability ability in GSDMD overexpression and knockdown stable cell lines **(Figure 2.6A-B)**. The CCK8 assay results show that when treated with Oxaliplatin, first-line chemotherapy drug of colorectal cancer, GSDMD

overexpression showed potent promoting effect on Oxaliplatin induced cytotoxicity. However, GSDMD knockdown inhibited Oxaliplatin induced cytotoxicity. These results suggest that GSDMD may sensitize CRC cancer cells' response to chemotherapy.

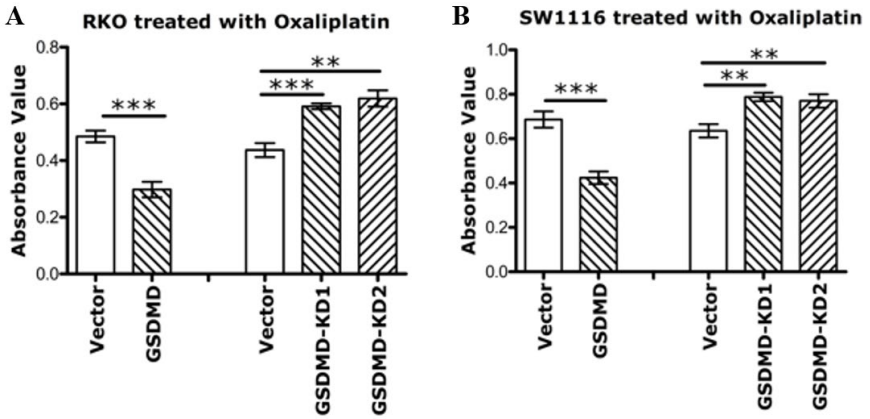

Figure 2.6 - GSDMD has promoting effect on chemotherapeutic drug Oxaliplatin induced cytotoxicity.
(**A-B**). Cytotoxicity assay measured by CCK8 reagent shows that GSDMD overexpression promotes cytotoxicity, while GSDMD knockdown inhibits cytotoxicity in vitro when treated with Oxaliplatin at a dose of 0.5μM for 24 hours. Data were shown as mean ± SD with ** $p \leqslant 0.01$ and *** $p \leqslant 0.001$.

The universality of the phenomenon of GSDMD nuclear localization in different CRC cell lines with different chemotherapeutic drugs treatment

We observed the existence of GSDMD nuclear location in cancer cells of patients with chemotherapy, and such nuclear translocation of GSDMD caused by chemotherapy has significantly positive correlation with a better overall survival of therapeutic patients with colorectal cancer (**Figure 2.2A-B**). GSDMD has been regarded as the protein localized in

cytoplasm under in vitro cell culture conditions in a variety of cells [53]. To test the universality of the phenomenon of GSDMD nuclear localization in CRC cells with chemotherapeutic drug treatment, we examined the expression pattern of GSDMD in a colorectal cell line panel, consisting of Caco-2, HCT1116, COLO 205, RKO, SW1116, SW620 and HT-29. According to results of immunofluorescence (IF) assay obtained by Laser Scanning Confocal Microscopy, GSDMD is predominantly localized in cytoplasm under in vitro cell culture conditions, which is in accordance with the reported results **(Figure 2.7)**.

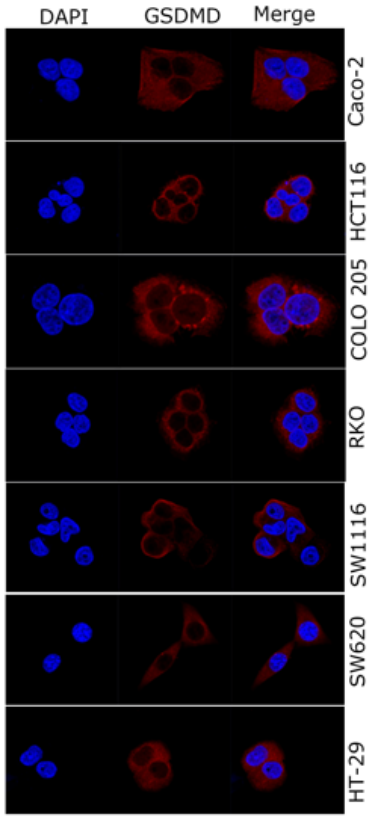

Figure 2.7 - GSDMD is predominantly localized in cytoplasm.
IF of colorectal cancer cell lines panel shows endogenous GSDMD is predominantly localized in cytoplasm under in vitro culture conditions.

We found GSDMD nuclear translocation takes place when treated with Oxaliplatin and 5-Fu. To explore the universality of the phenomenon of GSDMD nuclear localization in CRC cells with different chemotherapeutic drugs treatment, we treated the GSDMD-3×Flag stably expressing SW1116 cell line with other chemotherapeutic drugs, Cisplatin, Etoposide and Mitomycin C **(Figure 2.8A-B)**. All these drugs are of genotoxicity via

similar DNA damage response-induced apoptotic pathway. According to results of immunofluorescence (IF) assay obtained by Laser Scanning Confocal Microscopy, we found that all these drugs could induce GSDMD nuclear translocation and DNA damage. **(Figure 2.8)**.

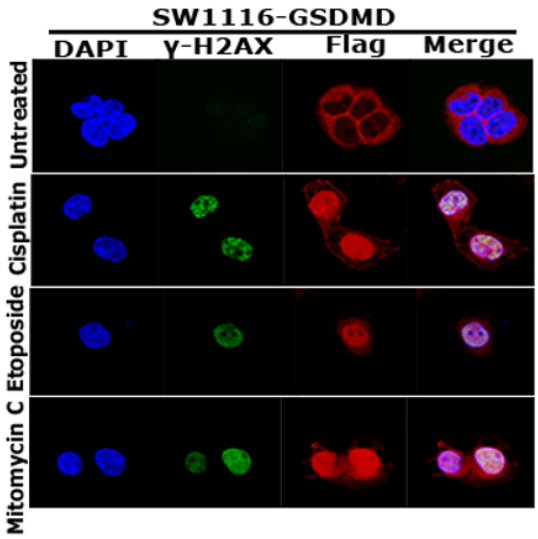

Figure 2.8 - Chemotherapy drugs, Cisplatin, Etoposide and Mitomycin C, induce GSDMD nuclear translocation.
IF images of SW116-GSDMD show remarkable nuclear translocation of GSDMD and DNA damage when treated with chemotherapy drugs, Cisplatin, Etoposide and Mitomycin C for 2 hours in vitro cell culture conditions.

GSDMD nuclear localization in CRC cells were verified by western blot of nuclear-cytosolic fractionation when treated with or without Oxaliplatin

To further verify the phenomenon of GSDMD nuclear localization in CRC cells after chemotherapeutic drug treatment, we performed western blot of nuclear-cytosolic

fractionation of GSDMD in RKO and SW1116 CRC cell lines when treated with or without chemotherapeutic drug Oxaliplatin, at a dose of 0.5μM for 24 hours. The western blot of nuclear-cytosolic fractionation results showed that Oxaliplatin significantly induced nuclear translocation of GSDMD in RKO and SW1116 CRC cell lines, compared to the groups without chemotherapeutic drug treatment **(Figure 2.9A-B)**.

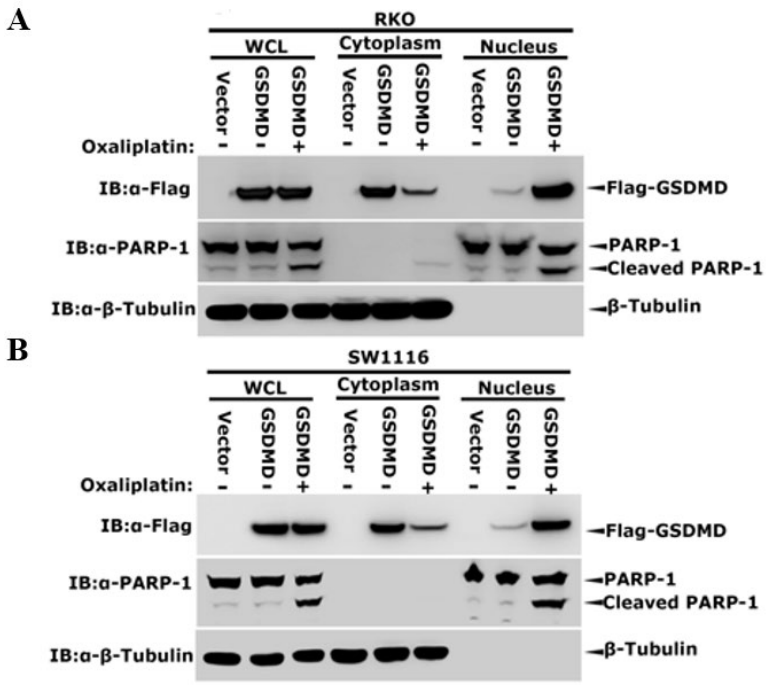

Figure 2.9 - The western blot of nuclear-cytosolic fractionation show that Oxaliplatin significantly induced nuclear translocation of GSDMD in RKO and SW1116 CRC cell lines.

(**A-B**). Western blotting of nuclear-cytosolic fractionation verifies translocation of GSDMD when treated with cytotoxic drug Oxaliplatin, at a dose of 0.5μM for 24 hours.

2.4 Discussion

Our preliminary studies found that most cancer tissue samples show positive nuclear staining of GSDMD. GSDMD nuclear localization in colorectal cancer cells represent better clinical outcomes in colorectal cancer patients. However, we also found that 20-25 % CRC patients have GSDMD nuclear translocation failure. This group of patients have poor prognosis. I hypothesize that this group of CRC patients have GSDMD or GSDMD related accessory protein mutation. In aim 1, we found the correlation between the nuclear localization of GSDMD and overall survival rate of colorectal cancer patients.

For our multi-omics study, we do not anticipate any problems with the techniques proposed because these are well established in the lab and tested in the preliminary studies. All paired Trio colorectal samples have been sequenced on a mature-scale biotechnology platform to avoid most potential errors and problems. All sequencing data have performed rigorous sorting and quality control to ensure the accuracy of the follow-up bioinformatics analysis, which provides reliable technical support for the in-depth study.

I collected 25 patients with full sets of matched Trio samples for full exome, transcriptome and DNA methylation sequencing to explore how GSDMD is regulated by genomic variation and epigenetic modification dynamically in three important pathological stages of polyp formation - adenoma progression - adenocarcinoma malignancy. We also performed the single-cell sequencing to further determine the heterogeneity of colorectal tumors at the individual cellular level. Consequently, we may distinguish the GSDMD

expression patterns of individual cells in different stages of disease development and hopefully dissect the potential mechanism (i.e., methylation, mutation, signal pathways) underlying GSDMD nuclear translocation.

We proposed research to analyze the intermediate state adenomas of the same patient, but due to the difficulty of obtaining adenomas, we may not have a sufficient amount of paired Trio colorectal samples for our study. Furthermore, the bioinformatics analysis of clinical patients from a single region or country may not give strong evidence to illustrate the mechanism of colorectal tumorigenesis. Alternatively, we may use numbers of large-scale pan-cancer public datasets, The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO), to carry out further analysis of verification and comparison, from which we could evaluate the expected results of our own research.

The combined analysis of single-cell and multi-omics sequencing data will reveal in detail the genome-driven mutation events, epigenetic abnormal changes, and dynamic changes in GSDMD expression during the occurrence and metastasis of colorectal cancer from multiple dimensions. These data may help to dissect the potential mechanism underlying GSDMD nuclear translocation during chemotherapy. Accomplishment of these studies will provide a comprehensive understanding of the early accumulation of abnormal molecular events in the adenoma state of colorectal cancer patients and provide a more rigorous and reliable proof of concept for pursuing the molecular mechanism for the transformation of adenoma to adenocarcinoma. Additionally, it also provides more precise and rigorous guidance for the next in-depth exploration of GSDMD as a novel therapeutic target.

Through whole-exome sequencing analysis, we found two nonsynonymous mutations of GSDMD in CRC cancer cells. I hypothesize that these GSDMD mutations are corelated to

the malignant progression/prognosis of colorectal cancer. In our further research, we plan to establish colorectal cancer cell lines transfected with wild type or mutants of GSDMD. Further in vitro CCK 8 assay, colony formation assay and in vivo tumor xenograft mouse model will be performed to detect the specific correlation between GSDMD mutation and some malignant phenotypes of colorectal cancer.

In conclusion, we found that DNA damage-related chemotherapeutic drugs induce the nuclear translocation of full-length non-cleaved GSDMD (**Figure 1.2**). GSDMD nuclear translocation is correlated with chemotherapy sensitization and cancer progression/prognosis.

CHAPTER 3: THE NON-PYROPTOTIC FUNCTION OF GSDMD IN COLORECTAL CANCER WITH CHEMOTHERAPY: GSDMD TRANSLOCATE INTO NUCLEUS TO INTERACT WITH PARP-1 AND INHIBIT THE DNA REPAIR RESPONSE OF PARP-1

3.1 Introduction

PARP-1 is well known to play a primary role in DNA repair response of DNA single strand breaks, through the modification of poly(ADP-ribosyl)ation, to maintain integrity of the genome [102, 103]. Olaparib, a potent PARP inhibitor, induces synthetic lethality in BRCA1/2 deficient tumor cells by formation of double strand breaks (DSBs) of DNA that leads to cell death [104]. Olaparib monotherapy represents a new therapeutic measure for patients with advanced, heavily pretreated germline BRCA mutation-associated ovarian cancer [105-107]. The combination of Platinum-based chemotherapeutic drugs and PARP inhibitor Olaparib has been in clinical application approved by FDA for breast and ovarian cancer, especially triple negative metastatic breast cancer and advanced ovarian cancer with "BRCAness", i.e. cancer cells with BRCA1 or BRCA2 mutations that result in Homologous Recombination Repair defects [80, 81]. However, monotherapy of Olaparib after failure of standard systemic therapy, was proven ineffective for colorectal cancer regardless of microsatellite status, which is also another main cause for genomic instability in colorectal cancer [108]. Nevertheless, combined application of Oxaliplatin and Olaparib

has been reported to increase the sensitivity of colorectal cancer to Oxaliplatin to induce more G2/M cell cycle arrest and DNA damaged related γ-H2AX (γ-H2A histone family member X) foci formation and hence more cell death [109]. However, the mechanism for GSDMD induced chemosensitization remains elusive. In this study, we found that GSDMD functions the same way as Olaparib. Both GSDMD and Olaparib can interact with PARP-1 in the nucleus, suppress DNA damage repair function of PARP-1, and finally lead to apoptosis of CRC cells.

The preliminary studies unexpectedly identified the presence of GSDMD nuclear location in chemotherapeutic colorectal cancer tissues. The objective of this specific aim and chapter is to explore the apoptosis promoting role of GSDMD and mechanism of GSDMD nuclear translocation triggered apoptosis of CRC cells induced by chemotherapy both in vitro and in vivo. The central hypothesis is that the non-cleaved full-length GSDMD would induce apoptosis independent of the conventional pyroptosis in colorectal cancer cells via its nuclear translocation and the chemotherapy would induce/facilitate nuclear translocation of GSDMD that subsequently interacts with PARP-1 and suppresses its DNA damage repair function, leading to apoptosis.

3.2 Materials and Methods

FACS Analysis of Cell Apoptosis

Cells were plated in 12-well plate one day before indicated treatment. 12-well plate with

radius about 1cm. Then cells were treated with given reagents or stimulations for an indicated time. The cells were harvested at the density 60-70% and processed for cell apoptosis analysis with the APC Annexin V Apoptosis Detection Kit (BD Bioscience, 640930) and propidium iodide, according to the instructions. 10000-15000 events were collected and analyzed. Briefly, cells were washed with PBS, then digested with trypsin with no EDTA, resuspended in binding buffer containing 5µl of APC-Annexin V antibody and incubated in dark for 15 minutes. Subsequently, 5µl propidium iodide was added and incubated in dark for 5 minutes. Finally, cell apoptosis data was acquired by FACS analysis with BD LSR Fortessa X-20 cell analyzer and FlowJo 7.6.

Western blot analysis

Equivalent amounts of protein were separated by SDS/PAGE, and protein expression was quantified by western blotting with primary antibody against human GSDMD (#96458) from Sigma-Aldrich, primary antibody against Flag (#F3165) from Sigma-Aldrich, primary antibody against PARP-1 (#9532) from Cell Signaling Technology, primary antibody against PAR (#4335-AMC-050) from Trevigen, and primary antibody against human GSDMD(#96458) for detection of C-terminal domain cleavage from Cell Signaling Technology.

Co-Immunoprecipitation (Co-IP)

Cells were lysed in IP RIPA buffer containing 50mM TrisHCl (pH=7.5), 150mM NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 1mM EDTA with 1mM PMSF, 1mM Na3VO4, 1mM NaF and protease inhibitor (Sigma, P8340), and the lysate was centrifuged at 10000 rpm for 15 minutes. Then, the supernatants were pre-cleared with protein A/G agarose beads (Santa Cruz Biotechnology, sc-2003) for half an hour and incubated with 1μg of the indicated antibodies for 16 hours at 4°C. Primary antibody against human GSDMD (#96458) was purchased from Sigma-Aldrich, primary antibody against Flag (#F3165) was purchased from Sigma-Aldrich, primary antibody against PARP-1 (#9532) was purchased from Cell Signaling Technology, and primary antibody against Myc (#05-724) was purchased from Millipore. Thereafter, 15μl of protein A/G agarose beads was incubated with the protein complex for 2 hours at 4°C. Then the pellets were washed 5 times with above IP RIPA buffer containing 1mM PMSF and resuspended in sample loading buffer and finally examined by western blot and mass spectrometry.

Immunofluorescence (IF) assay

Cells were seeded on coverslips in 24-well plate and then treated with corresponding reagents/conditions for an indicated time. Stimulated cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After three times washes with PBS, cells were permeabilized in 0.2% (v/v) TritonX-100 for 20 minutes at room temperature.

Coverslips were then washed three times with PBS and incubated with blocking buffer containing 5% (wt/v) BSA and 5% (v/v) FBS. Afterwards, cells were incubated with primary antibodies for 16 hours at 4°C diluted in PBS with 1% BSA and 1% FBS. anti-Flag (#F3165) for IF was purchased from Sigma-Aldrich; anti-human GSDMD (#sc-393656) for IF was purchased from Santa Cruz Biotechnology; anti-PARP-1 (#9532) and anti-γ-H2AX (#9718) for IF were purchased from Cell Signaling Technology. After three times washes with PBST (0.1% tween-20 in PBS), cells were incubated with Alexa Fluor ® secondary antibodies (Invitrogen) and nuclei stain DAPI (Invitrogen, H3569) for 1 hour at room temperature. Coverslips were washed four times with PBST and then images were acquired using ZEISS LSM 710 Metal confocal microscope.

Statistical analysis

All experiments were repeated at least 3 times with comparable results. Statistical analysis of data was carried out with GraphPad Prism 7 software, and results were presented as mean ± SD with indicated statistical methods. Statistical significance was distinguished with p value: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.3 Results

GSDMD promotes apoptosis induced by chemotherapeutic drugs in a non-pyroptotic manner.

As the most studied type of cell death, apoptosis is an ordered and orchestrated cellular

process occurring in physiological and pathological conditions[110]. Most of anti-cancer therapies induce apoptosis and related cell death network to eliminate malignant cells[111]. To better clarify the function of GSDMD in cancer cell growth and death, we analyzed the effects of GSDMD on apoptosis induced by first-line chemotherapeutic drugs of colorectal cancer, Oxaliplatin and 5-Fu. The effects of GSDMD on apoptosis was detected by flow cytometry (FCM) when treated with first-line chemotherapy drugs of colorectal cancer Oxaliplatin and 5-Fu **(Figure 3.1A-H)**. We found that GSDMD enhanced apoptosis in RKO and SW1116 induced by chemotherapeutic drugs.

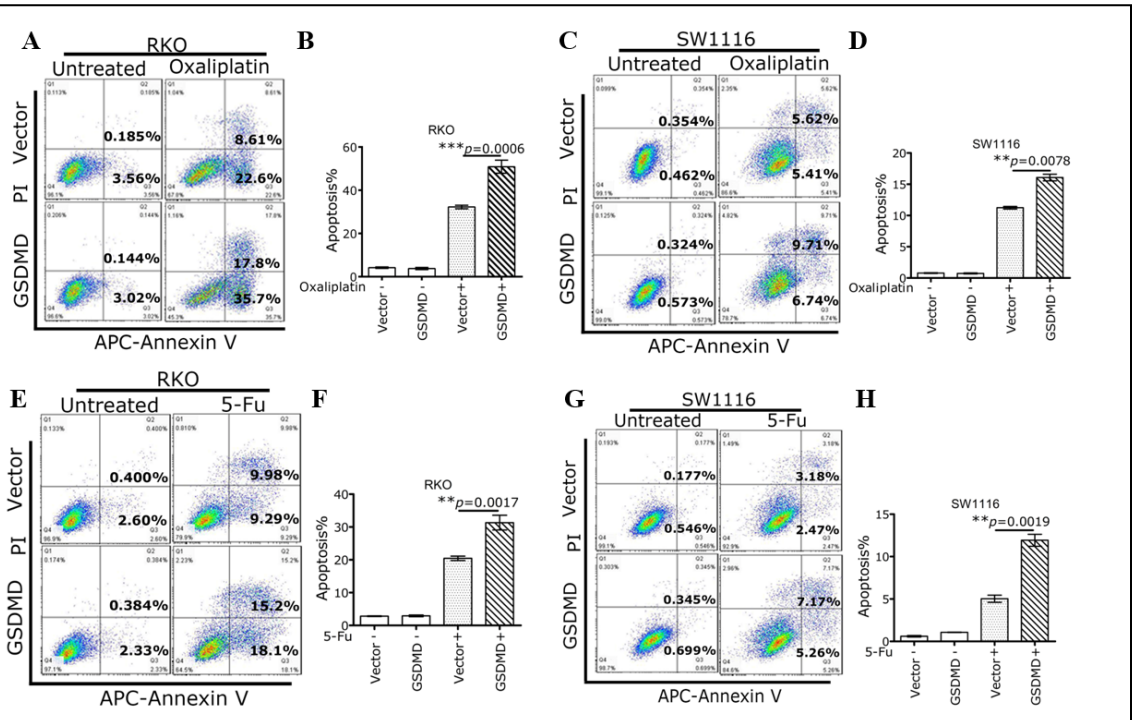

Figure 3.1 - GSDMD promotes apoptosis induced by Oxaliplatin and 5-Fu in RKO and SW1116, independent of GSDMD-related pyroptosis.

(**A-H**). FCM shows GSDMD promotes colorectal cancer cell apoptosis when treated with 5-Fu at a dose of 25μg/ml for 24 hours or Oxaliplatin at a dose of 0.5μM for 24 hours in RKO (**, p=0.0017; ***, p=0.0006) and SW1116 (**, p=0.0019; **, p=0.0078).

It's well established that GSDMD functions as the executor of pyroptosis through assembling N-terminal effector domains to form pores on cell membranes after cleavage by inflammatory caspases [47, 49, 112]. To determine whether the above apoptotic effects of GSDMD were pyroptosis related, we performed Western blot analysis on the cleavage of GSDMD in colorectal cancer cell lines treated with chemotherapeutic drug Oxaliplatin using human monocyte cell line THP-1 treated with PMA (phorbol myristate acetate, 50ng/ml 24h), LPS (lipopolysaccharide, 5μg/ml, 4h) and nigericin(1μM, 1h) sequentially to induce pyroptosis as a positive control. No cleavage of GSDMD was observed in cancer cell lines of RKO and SW1116 treated with Oxaliplatin **(Figure 3.2)**. From the above, we draw conclusions that GSDMD promotes apoptosis induced by chemotherapy drugs in colorectal cancer, in a pyroptosis-independent manner. The apoptosis promoting effects of GSDMD is pyroptosis independent.

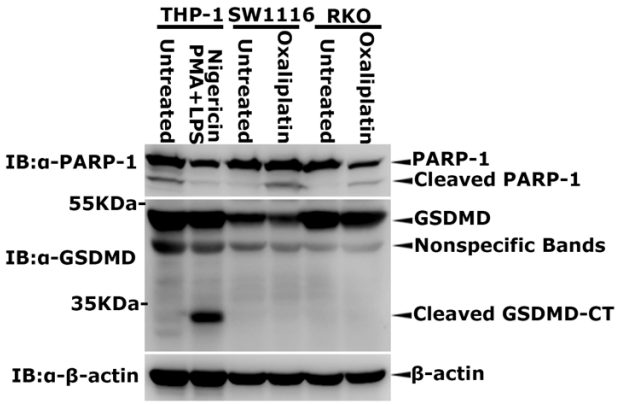

Figure 3.2 - No cleavage of GSDMD in RKO and SW1116 treated with Oxaliplatin. Western blot results of RKO and SW1116 treated with Oxaliplatin detected by specific antibody to identify both full length and C-terminal domain of GSDMD show that no cleavage of GSDMD was observed, compared with positive control of human monocyte cell line THP-1 treated with pyroptotic induction conditions.

Nuclear localized GSDMD interacts with PARP-1 to inhibit its DNA Repair-related function.

In order to elucidate the mechanism underlying the effects of GSDMD on apoptosis, mass spectrometry (MS) was carried out to identify the proteins interacting with GSDMD. Immunoprecipitation (IP) was performed before MS with Flag-tag antibody in GSDMD-3×Flag stably expressing SW1116 cell line. As is shown, Poly(ADP-ribose) polymerase-1 (PARP-1) involved in DNA repair response, is a promising candidate to interact with GSDMD **(Table 1).**

	Gene Name	PepCount	UniquePepCount	CoverPercent
Vector	GSDMD	0	0	0%
	PARP-1	0	0	0%
GSDMD	GSDMD	11	9	15.50%
	PARP-1	4	4	3.94%

Table 1. PARP-1 is a promising candidate protein to interact with GSDMD. Identification of interacting proteins of GSDMD by Mass Spectrometry was carried out with immunoprecipitated proteins by Flag tag antibody in 3×Flag-GSDMD stably expressing SW1116 cell line to show that PARP-1 is a promising interacting candidate protein.

This interaction was verified by co-IP analysis of the semi-endogenous and endogenous proteins **(Figure 3.3A-D)**, as well as immunofluorescent colocalization studies **(Figure 3.4A-D)**. PARP-1 is a nuclear protein, and GSDMD is localized predominantly in cytoplasm at steady state [67]. Surprisingly, GSDMD translocated into nucleus after 2 hours treatment with chemotherapeutic drug Oxaliplatin, and complementarily colocalized with PARP-1 in the nucleus **(Figure 3.4A-D)**. In this study, chemotherapeutic drug oxaliplatin treatment time is different in different experiments. A significant difference of cell death can be observed 24 hours after chemotherapeutic drug Oxaliplatin treatment, so, I chose the time point of 24 hours post Oxaliplatin treatment to detect its apoptosis-related phenomenon and mechanism. Besides, blockage of PARP-1 function is the mechanism accounting for nuclear GSDMD translocation-related apoptosis promoting effects, so I

took the midpoint 12h post Oxaliplatin treatment to detect the smear band of PAR, representing PARP-1 related DNA repair function.

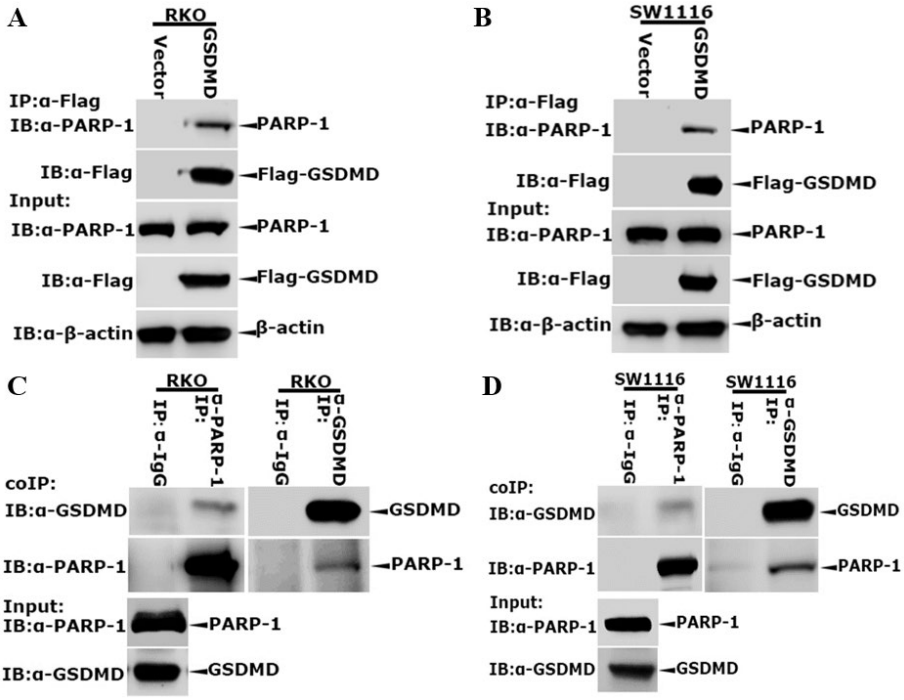

Figure 3.3 - Semi-endogenous co-IP and endogenous co-IP verify the interaction of GSDMD and PARP-1.

(**A-D**). The interaction of GSDMD with PARP-1 was verified by semi-endogenous and endogenous co-IP.

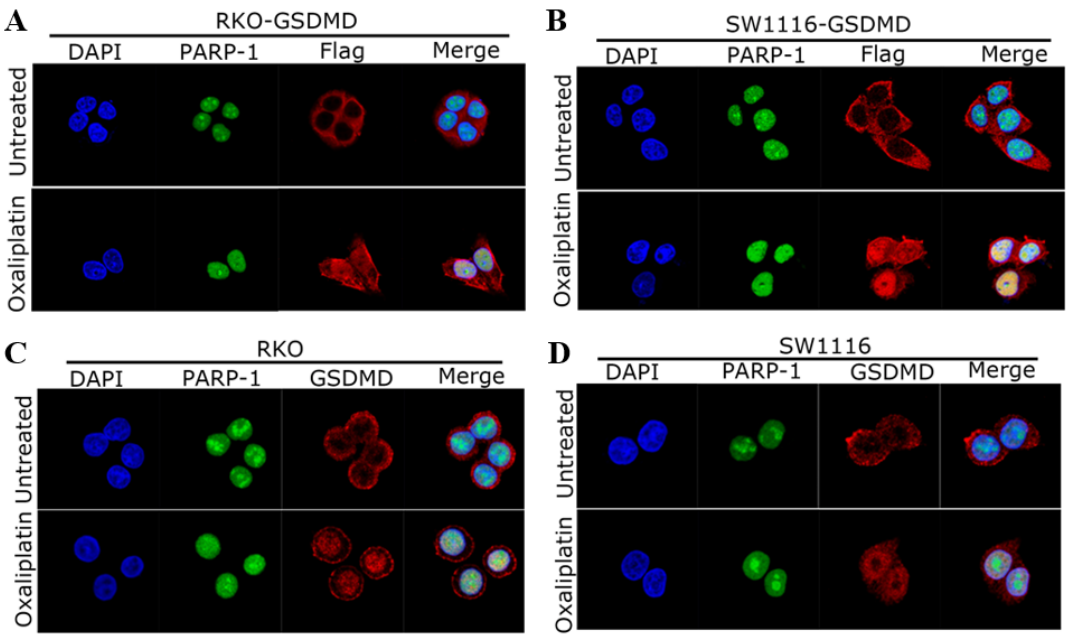

Figure 3.4 - GSDMD is translocated into nucleus upon treatment with chemotherapeutic drug Oxaliplatin, and colocalized with PARP-1 in the nucleus.
(**A-D**). Representative IF images of exogenous and endogenous GSDMD in RKO and SW1116 cell lines treated with Oxaliplatin for 2 hours show a remarkable translocation of GSDMD into nucleus to form complementary co-distribution with PARP-1.

PARP-1 plays a primary role in DNA Repair Response of DNA single strand breaks (SSBs), through the modification of poly(ADP-ribosyl)ation, to maintain integrity of genome [102, 103]. DNA damage, exceeding the DNA repair capacity, leads to cancer cell cycle arrest and cell death [39]. Thus, we checked whether the interaction of these two proteins had effects on DNA damage repair-related function of PARP-1. The poly(ADP-ribose)ation modification to its substrates was used to detect the PARP-1 function via Western blot

analysis in GSDMD stably expressing cell lines. PARP-1 function, evidenced by smear bands of poly(ADP-ribose)ation, was dramatically attenuated by GSDMD overexpression when treated with Oxaliplatin in both cell lines **(Figure 3.5A-B)**.

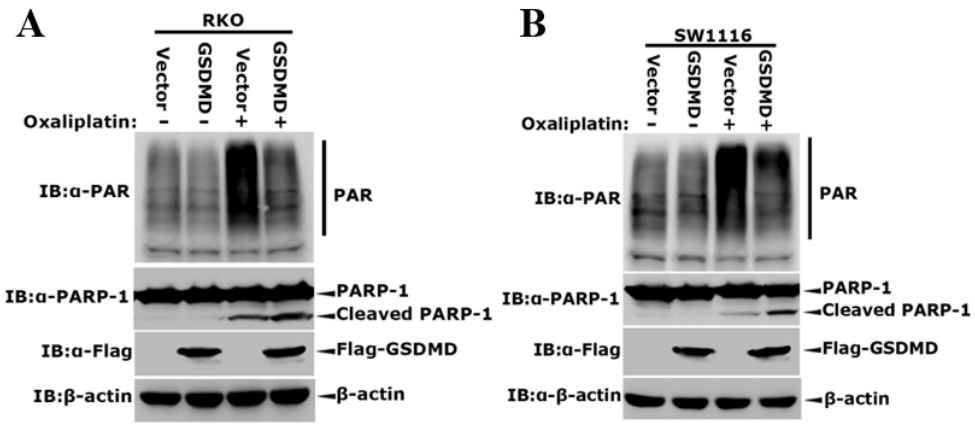

Figure 3.5 - GSDMD translocate into nucleus when treated with Oxaliplatin to block PARP-1 associated DNA Repair function.
(**A-B**). Effects of GSDMD on PARP-1 function in RKO and SW1116 cell lines treated with Oxaliplatin were demonstrated with smear poly(ADP-ribosyl)ation bands to show that overexpression of GSDMD inhibits PARP-1 associated DNA Repair function.

GSDMD interacts with CA Domain of PARP-1, like the way Olaparib (PARP-1 inhibitor) functions.

To elucidate the underlying mechanism of the inhibitory effects of GSDMD on PARP-1, we first identified the interacting domain of PARP-1 with GSDMD. By domain mapping co-IP, we identified the Catalytic Domain (CA Domain) at the C terminal of PARP-1 as the interacting domain of GSDMD, GSDMD interacts with CA Domain of PARP-1. **(Figure 3.6)**.

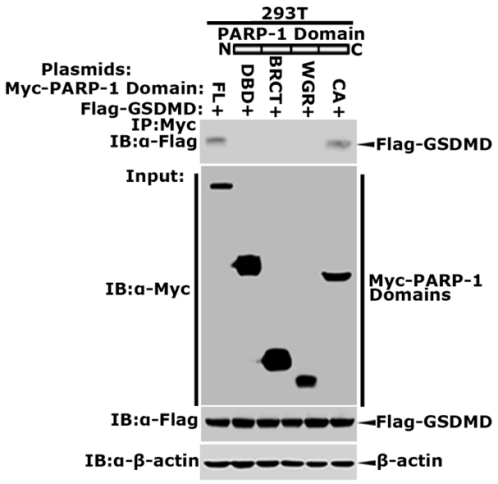

Figure 3.6 - GSDMD interacts with CA Domain of PARP-1.
Domain mapping of PARP-1 with GSDMD identified by co-IP in ectopic transfected HEK293T shows that GSDMD interacts with CA Domain of PARP-1.

Olaparib is a small molecule PARP inhibitor that induces synthetic lethality in homozygous BRCA-deficient cancer cells[104] and it functions as a competitive inhibitor of NAD+ at the CA Domain of PARP-1 and PARP-2. PARP-1 plays a central role in the repair of SSBs mediated via the base excision repair (BER) pathway and inhibition of the BER pathway by Olaparib leads to formation of deleterious DSBs with accumulation of unrepaired SSBs[113]. Given the same binding domain, we speculated that GSDMD may function the same way like PARP-1 inhibitor, Olaparib. Then we observed that Olaparib competitively inhibits the interaction of GSDMD with PARP-1 by co-IP **(Figure 3.7A-B)**.

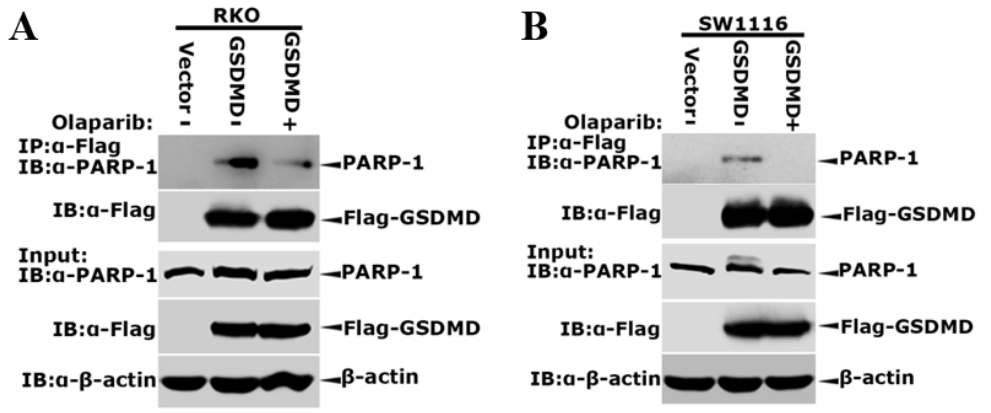

Figure 3.7 - Olaparib competitively inhibits the interaction of GSDMD with PARP-1. (**A-B**). PARP inhibitor Olaparib impairs the interaction of GSDMD and PARP-1 shown by co-IP in GSDMD stably expressing RKO and SW1116 cell lines to further verify the interplay manner of PARP-1 and GSDMD.

To exclude the possibility of Olaparib induced nuclear translocation of GSDMD, we performed IF to detect the effects of Olaparib on Oxaliplatin induced GSDMD nuclear translocation in GSDMD overexpression cells. IF results showed that there are no differences of GSDMD nuclear translocation between Olaparib treatment group and combination of Oxaliplatin and Olaparib treatment group. Olaparib has no effect on the nuclear translocation of GSDMD **(Figure 3.8A-B)**.

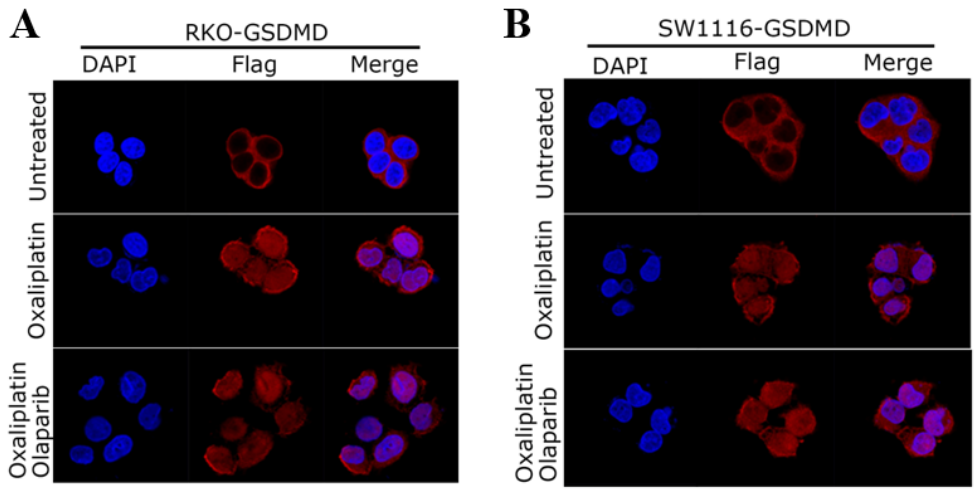

Figure 3.8 - Olaparib has no effect on the nuclear translocation of GSDMD.
(**A-B**). IF shows that Olaparib has no effect on the nuclear translocation of GSDMD when treated with Oxaliplatin at a dose of 0.5μM for 2 hours and combination of Oxaliplatin and Olaparib at a dose of 0.5/0.4μM for 2 hours.

GSDMD induces γ-H2AX foci formation and severe DNA damage in CRC cells after Oxaliplatin treatment.

There is a strong and quantitative correlation between γ-H2AX foci formation and induction of DNA double strand breaks (DSBs) [101]. Immunofluorescence (IF) assays of the DNA damage degree characterized by number of γ-H2AX foci were performed by IF staining of γ-H2AX in GSDMD stably expressing RKO and SW1116 cell lines and GSDMD Vector control cell line. The IF assay results showed that GSDMD overexpression induces more γ-H2AX foci formation and more severe DNA damage in comparison with the Vector control with significant statistical difference, when treated with Oxaliplatin

(Figure 3.9A-D). We verified the novel apoptosis role of GSDMD. GSDMD interacts with PARP-1 and inhibit the DNA repair function of PARP-1, which leads to apoptosis and enhances chemotherapeutic sensitivity.

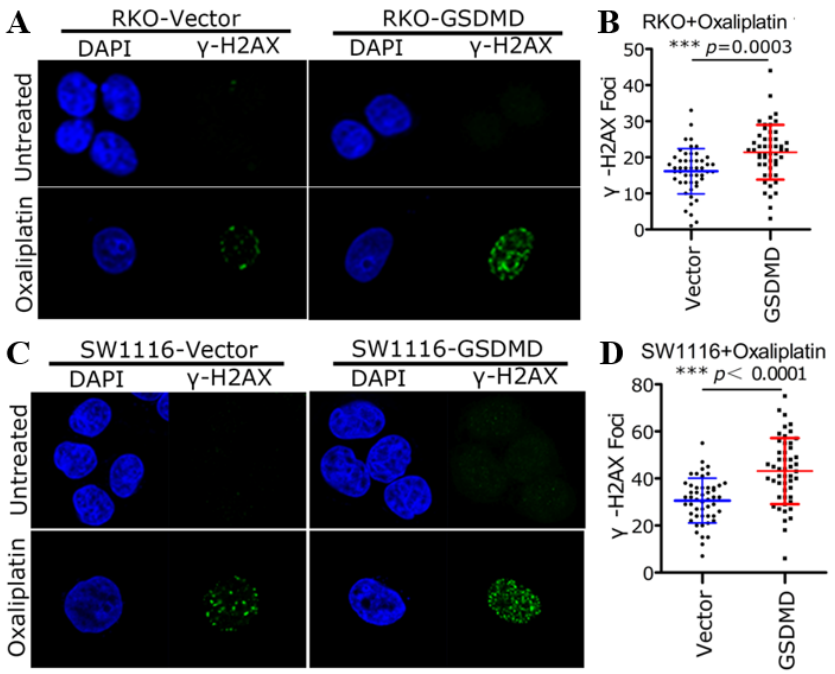

Figure 3.9 - GSDMD overexpression induce severe DNA damage in CRC cells after Oxaliplatin treatment.
(**A-D**). Representative IF images show a significant increment of γ-H2AX foci when treated with Oxaliplatin at a dose of 0.5μM for 24 hours in RKO-GSDMD (***, p=0.0003) and SW1116-GSDMD (***, p < 0.0001) in comparison with vector control.

GSDMD knockdown results in apoptosis resistance when treated with Oxaliplatin. PARP inhibitor, Olaparib, eliminates apoptosis resisting effects of GSDMD knockdown.

The role of Olaparib on Oxaliplatin induced apoptosis were detected in RKO-Vector and RKO-GSDMD-KD1/2. From the results of FCM, GSDMD knockdown results in apoptosis

resistance when treated with Oxaliplatin by FACS analysis of cell apoptosis **(Figure 3.10A-B)**. This result is in accordance with the above results of GSDMD overexpression **(Figure 3.9A-D)**. However, PARP inhibitor, Olaparib, could eliminate apoptosis resisting effects of GSDMD knockdown **(Figure 3.10A-B)**, which suggests that GSDMD functions partly the same way as Olaparib. These results suggested that Olaparib may compensate the apoptosis promoting effects of nucleus-localized GSDMD in subgroup CRC patients with low GSDMD expression clinically.

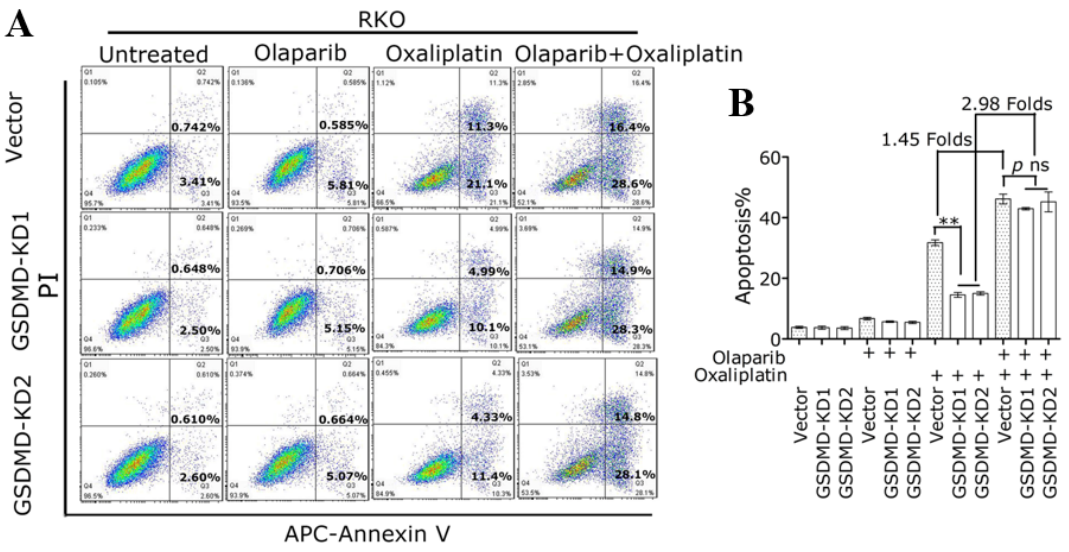

Figure 3.10 - GSDMD knockdown results in apoptosis resistance when treated with Oxaliplatin. PARP inhibitor, Olaparib, eliminates apoptosis resisting effects of GSDMD knockdown.

(**A-B**). FCM shows effects of Olaparib on GSDMD-related apoptosis induced by Oxaliplatin, which are detected in GSDMD knockdown stably expressing RKO cell line with western blotting, demonstrating that GSDMD-knockdown reduces Oxaliplatin-induced apoptosis, **, $p<0.01$. Meanwhile, Olaparib normalized the reduced Oxaliplatin-induced apoptosis caused by GSDMD-knockdown, ns, $p > 0.05$.

3.4 Discussion

For shRNA knockdown studies, the GSDMD loss of function may not last for a long time due to siRNA silencing or compensatory regulation. Alternatively, CRISPR/Cas9 genome editing will be used to knockout GSDMD in cancer cell line. Although the in vitro cancer cell lines could address the scientific question conceptually, novel xenograft models such as cell line-derived xenograft or patients-derived xenograft could be used to validate the GSDMD nuclear translocation induced by chemotherapy.

We verified the interaction of GSDMD and PARP-1 by Co-Immunoprecipitation and mass spectrometry analysis. Then, we observed a remarkable translocation of GSDMD into nucleus to form co-distribution with PARP-1 in the nucleus by Immunofluorescence staining of GSDMD and PARP-1. Further western blot analysis verified that overexpression of GSDMD inhibits PARP-1 associated DNA Damage Repair function by observing the poly(ADP-ribose)ation modification bands of PARP-1. Immunofluorescence images of GSDMD stably expressing CRC cell lines treated with Oxaliplatin showed a remarkable increment of γ-H2AX foci and induced more severe DNA damage. GSDMD knockdown resulted in apoptosis resistance when treated with chemotherapeutic drugs, Oxaliplatin, while GSDMD overexpression enhanced chemotherapeutic apoptosis determined by FACS analysis. Then, by Co-Immunoprecipitation in domain map of PARP-1 with GSDMD, we identified the Catalytic Domain (CA Domain) at the C terminal of PARP-1 as the interacting domain. As is known, Olaparib is a small molecule PARP

inhibitor. Olaparib is already approved for the treatment of patients with recurrent ovarian cancer and BRCA mutation. Olaparib functions as a competitive inhibitor of NAD+ at the CA Domain of PARP [114]. Given the same binding domain, we speculated that GSDMD may function the same way as PARP inhibitor Olaparib. After that, I observed that PARP inhibitor Olaparib competitively inhibits the interaction of GSDMD with PARP-1 in GSDMD stably expressing CRC cell lines by Co-Immunoprecipitation. Oxaliplatin chemotherapeutic resistance can be eliminated by the combined treatment of chemotherapy with PARP inhibitor Olaparib. This result is in accord with previous reports that combination of Platinum-based chemotherapeutic drugs and PARP inhibitor Olaparib has synergistic efficacy in breast and ovarian cancer [80, 81]. I demonstrate that GSDMD function in the same way as PARP inhibitor Olaparib. Nuclear localized GSDMD may serve as a marker for clinical outcomes of colorectal cancer and its subcellular localization pattern may give guidance to the combinational application of PARP inhibitor Olaparib and basic chemotherapeutic drugs in colorectal cancer.

CHAPTER 4: ATM AND NLS DEPENDENT MECHANISMS OF GSDMD NUCLEAR TRANSLOCATION SENSITIZE CHEMOTHERAPY OF DNA DAMAGE-RELATED AGENTS

4.1 Introduction

The molecular weight of GSDMD is 52,801 Da. The amino acid length of GSDMD is 484. The amino acids sequence of GSDMD is "MGSAFERVVRRVVQELDHGGEFIPVTS-LQSSTGFQPYCLVVRKPSSSWFWKPRYKCVNLSIKDILEPDAAEPDVQRGRSFHF YDAMDGQIQGSVELAAPGQAKIAGGAAVSDSSSTSMNVYSLSVDPNTWQTLLH ERHLRQPEHKVLQQLRSRGDNVYVVTEVLQTQKEVEVTRTHKREGSGRFSLPGA TCLQGEGQGHLSQKKTVTIPSGSTLAFRVAQLVIDSDLDVLLFPDKKQRTFQPPA TGHKRSTSEGAWPQLPSGLSMMRCLHNFLTDGVPAEGAFTEDFQGLRAEVETIS KELELLDRELCQLLLEGLEGVLRDQLALRALEEALEQGQSLGPVEPLDGPAGAV LECLVLSSGMLVPELAIPVVYLLGALTMLSETQHKLLAEALESQTLLGPLELVGS LLEQSAPWQERSTMSLPPGLLGNSWGEGAPAWVLLDECGLELGEDTPHVCWEP QAQGRMCALYASLALLSGLSQEPH"

DNA Damage Response signaling pathway is orchestrated by ATM and ATR kinases, the central regulators of this network. ATM/ATR kinase can be activated by DNA damage, with non-redundant function and distinct downstream signaling pathway [82]. ATM is a central element of DNA damage response signaling pathway. The ATM protein belongs to

a protein kinases family containing the phosphatidylinositol-3 catalytic domain, including ATM, ATR and PI3K [83]. ATM is located at the core of DNA damage pathway. ATM is activated after DNA double-strand break (DSB). Through a variety of complex cellular reaction processes, ATM triggers its autophosphorylation, and subsequent activation to promote DNA repair [84]. When ATM is mutated or insufficient, the cell is unable to recognize the DNA damage that has occurred and to respond appropriately to the damage, which destroys the integrity of the intracellular genome and in turn causes cell death [85]. Researchers have proposed anticancer therapeutic approaches by inhibiting ATM. By inhibiting ATM, DNA damage response mediated cell cycle arrest, DNA repair and apoptosis pathways are blocked, thereby inducing apoptosis of cancer cells [86]. Studies have shown that the application of ATM kinase inhibitor KU55933 or other ATM/ATR inhibitor CGK733 lead to increased apoptosis of cells. The interesting finding of this study is that this type of ATM-specific inhibitor only let the cells that are affected by cancer and prematurely senescence lose the ability to resist apoptosis, thereby destroying cancer cells, whereas there has no intervention on normally senescent cells [87, 88].

It is reported a high proportion of ATM aberrations of B-cell chronic lymphocytic leukemia, which is associated with relatively short progression-free survival following chemotherapy and adverse prognoses. Interestingly, some studies found that ATM mutation events may increase the chemotherapy sensitivity of cancer patients[89, 90]. For example, ATM mutations may cause a higher sensitivity to platinum drugs among the cancer patients,

particularly breast and ovarian cancer [91].

One study used systems biology methods to build mathematical and bioinformatics models that can better predict the therapeutic function and effect of ATM inhibitors as potential anticancer drugs. These models improved clinical drug intervention strategies targeting ATM signaling pathways [92]. Preclinical data also provided a strong rationale for clinical testing of ATM inhibitor both in combination with chemotherapy, and in synthetic lethal approaches to treat tumors. One study has reported that highly selective small molecule inhibitors of ATM are under the preclinical and clinical development [92].

The nuclear membrane that isolates DNA is one of the characteristics of eukaryotic cells. Thus, the nuclear membrane separates the nuclear processes of DNA replication and RNA transcription from the cytoplasmic processes of protein production [93]. The proteins needed in the nucleus must be delivered there by some mechanism. Nuclear localization signal (NLS) are amino acid sequences that mark proteins for nuclear translocation [93]. Different nuclear localization proteins may share the same NLS sequence [93]. The function of NLS is the opposite of that of nuclear output signals [94]. The transport into and out of nucleus of molecular weight > 45 kDa is largely mediated by members of the Importin Superfamily of transporters, consisting of α- and β- types [94]. The movement of proteins between the cytoplasm and nucleus usually involves recognition of nuclear localization signals by members of the Importin superfamily and then translocation though nuclear pore complexes embedded in the nuclear membrane [94].

The NLS labeled protein bind to importin and the protein complex pass through the nuclear pore complex together [94]. At this point, RAN-GTP binds to the Importin-protein complex, which causes Importin to lose its affinity for the protein. After that, the protein is released and the RAN-GTP/Importin complex move out of the nucleus through the nuclear pore complex [95]. Next, the GTP-activated protein in the cytoplasm hydrolyzes Ran-GTP to GDP, which results in a conformational change of Ran that ultimately reduces its affinity for the Importin protein [95]. Finally, Importin is released, and Ran-GDP is recycled back to the nucleus, where guanine nucleotide exchange factor converts its GDP back into GTP [95].

Based on our preliminary data that GSDMD cleavage was lacking in the chemotherapeutic samples, nuclear localized GSDMD enhances chemotherapeutic sensitivity by interacting with PARP-1 in the nucleus and inhibiting its DNA repair activity. The objective of this specific aim and chapter is to dissect the potential clinical value of GSDMD nuclear translocation for chemosensitization by manipulating GSDMD expression and ATM/ATR signaling activation. Since most chemotherapeutic reagents induce DNA damage, we expect that DNA damage-dependent ATM pathway activation could also contribute to GSDMD nuclear translocation. The central hypothesis is that translocation of GSDMD into nucleus in colorectal cancer cells relies on the uncleaved GSDMD and the activation of ATM or ATR activation (**Figure 1.2**). Chemotherapeutic drugs induced GSDMD nuclear translocation is not by passive diffusion. GSDMD could translocate into nucleus and

promote apoptosis of CRC cells with the guidance of a well-known NLS.

4.2 Materials and Methods

Mice Experiments

Male BALB/c Nude mice aged for 4 weeks were purchased from SLAC Laboratory Co., Ltd (Shanghai, China) and divided into subgroups randomly. RKO-Vector and RKO-GSDMD (5×10^6 resuspended in PBS, mixed with Matrigel at ratio 1:1) were injected subcutaneously into BALB/c Nude mice. In the second mice experiment, after the primary tumor volume amounting to about 100 mm^2, mice were randomly divided into Oxaliplatin and solvent control group (n=6 for each group). Intraperitoneal injection of Oxaliplatin was executed into mice at a dose of 7.5mg/kg every 4 days, with control mice receiving saline. Tumor size was assessed every two days by precision caliper, then tumor volume was calculated according to the formula Volume= ($0.5\times length\times width^2$). All mice were sacrificed at the indicated time point for further analysis. The above mice experiment was approved by Institutional Animal Care and Use Committee of Shanghai according to the National Research Council Guide for Care and Use of Laboratory Animals (SCXK [Shanghai 2007–0005]).

Immunohistochemistry (IHC)

IHC was performed on 3μm thick paraffin-embedded section of tumor tissues. Specimens were gradually de-paraffinized in xylene and rehydrated in gradient alcohols to water. Blocking endogenous peroxidase activity was performed with 3% H2O2 in PBS for 15 minutes. For antigen retrieval, the sections were microwaved with 10mM citrate buffer with pH=6.0. Then sections were blocked with 10% goat serum to exclude nonspecific antibody staining, incubated with the indicated primary antibodies at appropriate dilution overnight at 4°C and then washed with PBST for three times and incubated with biotinylated secondary antibodies for 40 minutes at room temperature. GSDMD antibody, anti-human GSDMD (#96458), was purchased from Sigma-Aldrich. γ-H2AX antibody, anti-γ-H2AX (#9718), was purchased from Cell Signaling Technology. After washing with PBST, slides were incubated with streptavidin-peroxidase complex in PBS for 20 minutes at 37°C. The sections were rinsed with PBST, and staining was then revealed using 3, 3-diaminobenzadine substrate and counterstained with haematoxylin. The immunoreactive score was evaluated by four pathologists independently with rules as follows: negative (-); mild (+); moderate (++); strongly positive (+++).

Western blot analysis

Equivalent amounts of protein were separated by SDS/PAGE, and protein expression was quantified by western blotting with primary antibody against human GSDMD (#96458)

from Sigma-Aldrich, primary antibody against Flag (#F3165) from Sigma-Aldrich, primary antibody against PARP-1 (#9532) from Cell Signaling Technology, primary antibody against PAR (#4335-AMC-050) from Trevigen, and primary antibody against Myc (#05-724) from Millipore

Co-Immunoprecipitation (Co-IP)

Cells were lysed in IP RIPA buffer containing 50mM TrisHCl (pH=7.5), 150mM NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 1mM EDTA with 1mM PMSF, 1mM Na3VO4, 1mM NaF and protease inhibitor (Sigma, P8340), and the lysate was centrifuged. Then, the supernatants were pre-cleared with protein A/G agarose beads (Santa Cruz Biotechnology, sc-2003) for half an hour and incubated with 1μg of the indicated antibodies for 16 hours at 4°C. Primary antibody against human GSDMD (#96458) was purchased from Sigma-Aldrich, primary antibody against Flag (#F3165) was purchased from Sigma-Aldrich, primary antibody against PARP-1 (#9532) was purchased from Cell Signaling Technology, and primary antibody against Myc (#05-724) was purchased from Millipore. Thereafter, 15μl of protein A/G agarose beads was added for 2 hours incubation at 4°C. Then the pellets were washed 5 times with above IP RIPA buffer containing 1mM PMSF and resuspended in sample loading buffer and finally examined by western blot and mass spectrometry.

Immunofluorescence (IF) assay

Cells were seeded on coverslips in 24-well plate and then treated with corresponding reagents/conditions for an indicated time. Stimulated cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After three times washes with PBS, cells were permeabilized in 0.2% (v/v) TritonX-100 for 20 minutes at room temperature. Coverslips were then washed three times with PBS and incubated with blocking buffer containing 5% (wt/v) BSA and 5% (v/v) FBS. Afterwards, cells were incubated with primary antibodies for 16 hours at 4°C diluted in PBS with 1% BSA and 1% FBS. anti-Flag (#F3165) for IF was purchased from Sigma-Aldrich; anti-human GSDMD (#sc-393656) for IF was purchased from Santa Cruz Biotechnology; anti-Myc (#05-724) for IF was purchased from Millipore; anti-PARP-1 (#9532) and anti-γ-H2AX (#9718) for IF were purchased from Cell Signaling Technology. After three times wishes with PBST (0.1% tween-20 in PBS), cells were incubated with Alexa Fluor ® secondary antibodies (Invitrogen) and nuclei stain DAPI (Invitrogen, H3569) for 1 hour at room temperature. Coverslips were washed four times with PBST and then images were acquired using ZEISS LSM 710 Metal confocal microscope.

FACS Analysis of Cell Apoptosis

Cells were plated in 12-well plate one day before indicated treatment. Then cells were treated with given reagents or stimulations for an indicated time. The cells were harvested

and processed for cell apoptosis analysis with the APC Annexin V Apoptosis Detection Kit (BD Bioscience, 640930) and propidium iodide, according to the instructions. Briefly, cells were washed with PBS, then digested with trypsin with no EDTA, resuspended in binding buffer containing 5μl of APC-Annexin V antibody and incubated in dark for 15 minutes. Subsequently, 5μl propidium iodide was added and incubated in dark for 5 minutes. Finally, cell apoptosis rate was acquired by FACS with BD LSR Fortessa X-20 cell analyzer.

Statistical analysis

All experiments were repeated at least 3 times with comparable results. Statistical analysis of data was carried out with GraphPad Prism 7 software, and results were presented as mean ± SD with indicated statistical methods. Statistical significance was distinguished with p value: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.3 Results

GSDMD robustly enhances chemotherapeutic sensitivity in vivo.

The above results in vitro showed that GSDMD translocates into nucleus upon stimulation of chemotherapeutic drugs. More GSDMD expression in cancer cells, more GSDMD translocation upon treatment of chemotherapeutic drugs, and more DNA Damage related apoptosis, thus suggest that GSDMD sensitizes CRC cancer cells' response to

chemotherapy. We then investigated the effects of GSDMD on chemotherapeutic sensitivity in vivo with methods of subcutaneous injection of RKO-GSDMD overexpression and RKO-Vector control cell lines into nude mice, with or without treatment of Oxaliplatin. Exponentially growing RKO-Vector and RKO-GSDMD cells were harvested and subcutaneously injected into the back of 8-week-old male nude mice, 10 days after inoculation of the RKO cells, mice were treated every 4 days with Oxaliplatin or saline by intraperitoneal injection (i.p.) at the dose of 7.5mg/kg **(Figure 4.1A-B)**. By analyzing results of measurement, tumor masses of GSDMD overexpression at sacrifice point nearly disappeared upon treatment of Oxaliplatin in comparison with Vector control, revealing that GSDMD robustly enhances chemotherapeutic sensitivity in vivo.

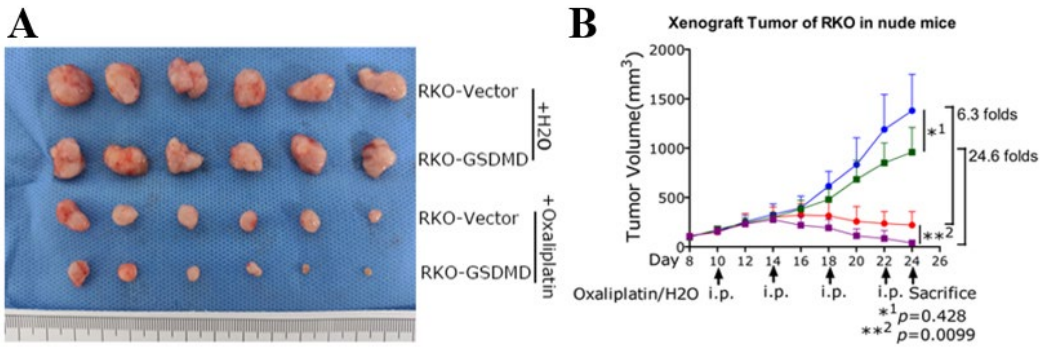

Figure 4.1 - GSDMD overexpression robustly enhance the chemotherapeutic sensitivity to Oxaliplatin in vivo.

(**A-B**). GSDMD sensitizes chemotherapy effect of Oxaliplatin in vivo. Exponentially growing RKO-Vector and GSDMD cells were harvested and subcutaneously injected into the back of 8-week-old male nude mice, followed by treatment of intraperitoneal injection of Oxaliplatin and solvent control saline from indicated timepoint Day 10, every 4 days. The left panel shows the harvested tumor masses at Day 24 of the 4 groups, to show GSDMD promote chemotherapy effect of Oxaliplatin, **, p=0.0099.

We then investigated the effects of chemotherapeutic drug Oxaliplatin induced GSDMD nuclear translocation in vivo in xenograft tumor masses of mice. We performed IHC staining of GSDMD in the tumor xenografts of RKO-Vector and RKO-GSDMD groups treated with or without Oxaliplatin **(Figure 4.2A-F)**. As is known, systematic chemotherapy delivery to the tumor masses relies on the simple diffusion gradients from the intravascular to the interstitial space. While various ischemic factors within the tumor mass, such as chaotic vascular architecture, heterogeneous fluid flux and increased solid tumor pressure, impedes the permeation of chemotherapy drugs[115]. External zones of the harvested tumor masses have higher chemotherapeutic drug Oxaliplatin concentration,

internal zones of the harvested tumor masses have lower chemotherapeutic drug Oxaliplatin concentration. Significant nuclear translocation of GSDMD was observed in the external zone of the harvested tumor masses with relatively higher Oxaliplatin concentration.

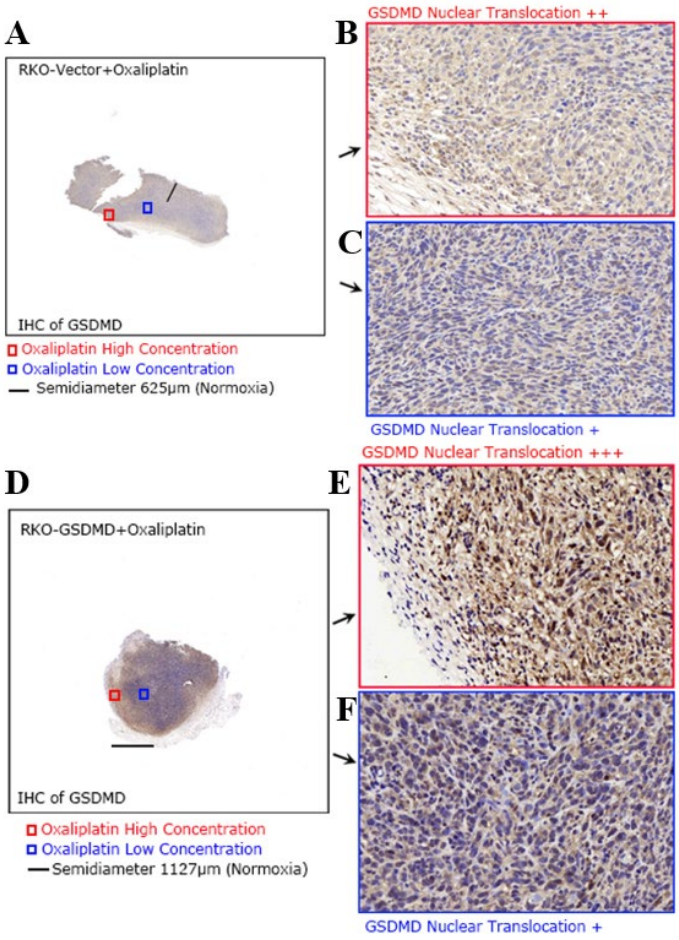

Figure 4.2 - Chemotherapeutic drug Oxaliplatin induce GSDMD nuclear translocation in vivo.

(**A-F**). Representative IHC images of tumor xenografts of RKO-Vector and RKO-GSDMD treated with Oxaliplatin showing nuclear translocation of GSDMD in the external zone exposing to a relatively higher concentration of Oxaliplatin.

Finally, we determined the correlation of DNA damage in tumor tissues with Oxaliplatin treatment. We examined the DNA damage degree in our collected tumor masses by immunohistochemical staining with γ-H2AX. According to the chemotherapeutic drug diffusion gradients from the external zones to the internal zones of the tumor masses, DNA damage induced by Oxaliplatin was demonstrated by using γ-H2AX staining where a narrow annular zone of positive staining at the external area was observed. We defined this narrow annulus as "DNA Damage Ring" **(Figure 4.3F and H)**. By analyzing the Oxaliplatin treated groups, a significantly thicker DNA Damage Ring was observed in intact tumors of RKO-GSDMD (178μm) than in RKO-Vector (121μm) **(Figure 4.3E-I)**, with no positive γ-H2AX staining observed in groups treated with saline **(Figure 4.3A-D)**, suggesting that GSDMD overexpression and translocation facilitates DNA damage induced by chemotherapy drugs in vivo.

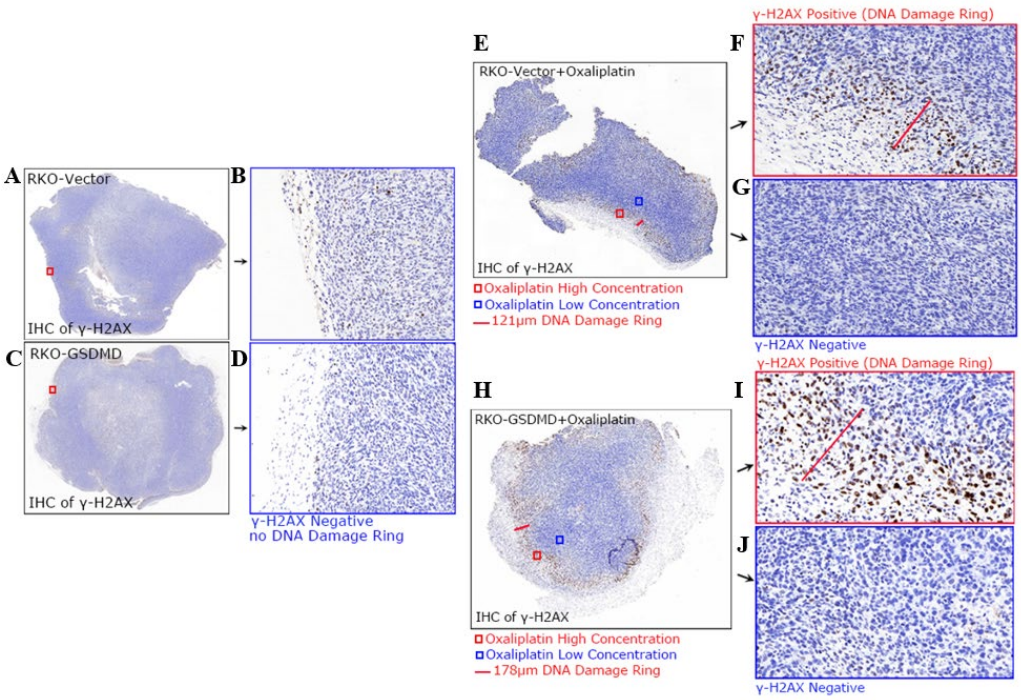

Figure 4.3 - GSDMD overexpression and translocation facilitates Oxaliplatin induced γ-H2AX staining DNA Damage Ring in tumor xenografts.
(**A-I**). Representative IHC images of tumor xenografts of RKO-Vector and RKO-GSDMD treated with Oxaliplatin showing a thicker γ-H2AX positive ring indicating double-strand DNA damages in the external zone.

Chemotherapy drugs induced GSDMD nuclear translocation is ATM kinase signaling pathway dependent

DNA damage response signaling pathway is integrated by ATM/ATR kinase, which is the regulatory hub of the entire DNA damage repair network. Both ATM and ATR kinases can be activated during DNA damage and have different functions and downstream signaling pathways [82]. After that, I checked whether the activation of ATM or ATR played a role

in the translocation of GSDMD. ATM and ATR inhibitors were used to block the activation of the related pathways. By performing IF staining of GSDMD, we found that it is the ATM inhibitor Ku-55933, rather than ATR inhibitor VE-882, that inhibited the translocation of GSDMD in stably expressing RKO and SW1116 cell lines **(Figure 4.4A-B)**.

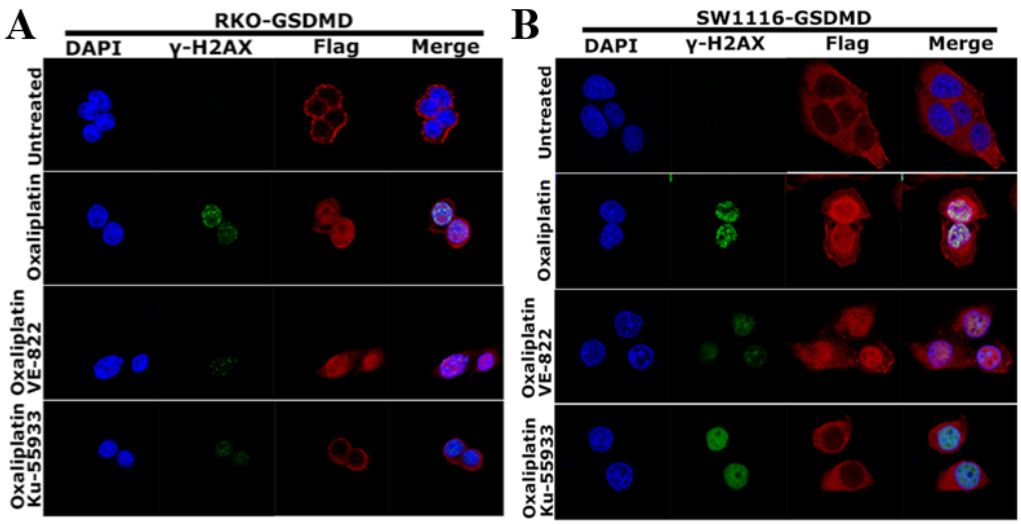

Figure 4.4 - GSDMD translocation into nucleus induced by various genotoxic chemotherapeutic drugs is ATM phosphorylation activation dependent.
(**A-B**). Representative IF images of GSDMD stably expressing SW1116 and RKO cell lines treated with Oxaliplatin with or without ATR inhibitor VE-822 or ATM inhibitor Ku-55933 for 24h show that Ku-55933 inhibits the translocation of GSDMD into nucleus induced by Oxaliplatin.

Next, we checked whether ATM inhibitor Ku-55933 could reduce GSDMD translocation into nucleus from cytoplasm and result in retention of GSDMD in cytoplasm. We performed nuclear-cytosolic fractionation in GSDMD stably expressing cell line treated with Oxaliplatin and ATM inhibitors **(Figure 4.5)**. The Western blot of nuclear-cytosolic

fractionation in GSDMD results showed that the ATM kinase inhibitor KU-55933 can significantly inhibit oxaliplatin induced GSDMD nuclear translocation, and correspondingly increase the retention of GSDMD protein in the cytoplasm.

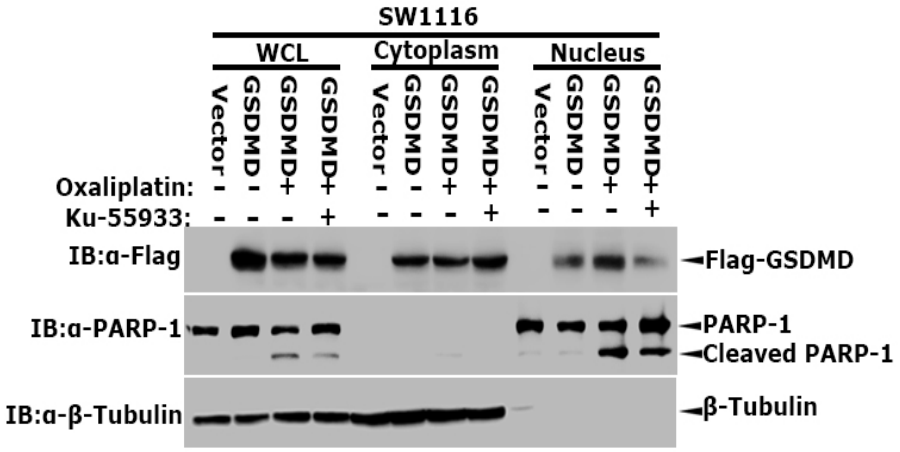

Figure 4.5 - ATM kinase inhibitor KU-55933 inhibits oxaliplatin induced GSDMD nuclear translocation.
Nuclear-cytosolic fractionation performed in GSDMD stably expressing SW1116 treated with Oxaliplatin with or without Ku-55933 shows that Ku-55933 inhibits the translocation of GSDMD into nucleus resulting in retention of GSDMD in cytoplasm.

Finally, we determined whether ATM inhibitor Ku-55933 could block the promoting effects of GSDMD on apoptosis induced by Oxaliplatin. We performed FACS analysis of cell apoptosis to detect the effects of ATM inhibitors on apoptosis in GSDMD stably expressing cell lines induced by Oxaliplatin **(Figure 4.6A-B)**. The FACS analysis results showed that GSDMD overexpression promoted oxaliplatin induced apoptosis. However, ATM inhibitor Ku-55933 weakened the promoting effect of GSDMD overexpression on

oxaliplatin induced apoptosis. This result further demonstrated that chemotherapy drugs induced GSDMD nuclear translocation is ATM kinase signaling pathway dependent.

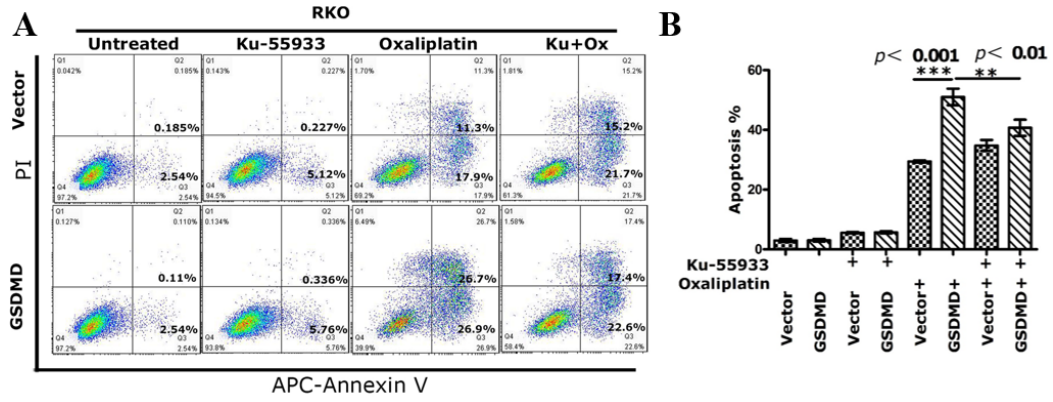

Figure 4.6 - GSDMD overexpression promote oxaliplatin induced apoptosis. ATM inhibitor Ku-55933 weaken the promoting effect of GSDMD overexpression on oxaliplatin induced apoptosis.

(**A-B**). Effects of Ku-55933 on apoptosis induced by Oxaliplatin is detected in RKO GSDMD knockdown stably expressing cell lines with double staining of Annexin-V-APC/PI, and the statistical results with significant statistic difference marked on the figure are shown to the right to demonstrate that GSDMD promotes apoptosis and ATM inhibitor Ku-55933 impairs apoptotic promoting effects of GSDMD induced by Oxaliplatin.

GSDMD nuclear translocation is nuclear localization signal (NLS) dependent

The transport into and out of nucleus of molecular weight >45kDa is largely mediated by members of the Importin Superfamily of transporters, consisting of α- and β- types[116, 117]. The molecular weight of GSDMD is 53 kDa. To determine the nuclear transport pattern of GSDMD and exclude the possibility of passive diffusion, co-IP in RKO and SW1116 CRC cell lines were performed **(Figure 4.7)**. The result of co-IP showed that GSDMD interact with Importin β, rather than Importin α. Thus, we verified that the

transport of GSDMD is Importin β dependent. We indirectly demonstrated that chemotherapeutic drugs induced GSDMD nuclear translocation is not by passive diffusion.

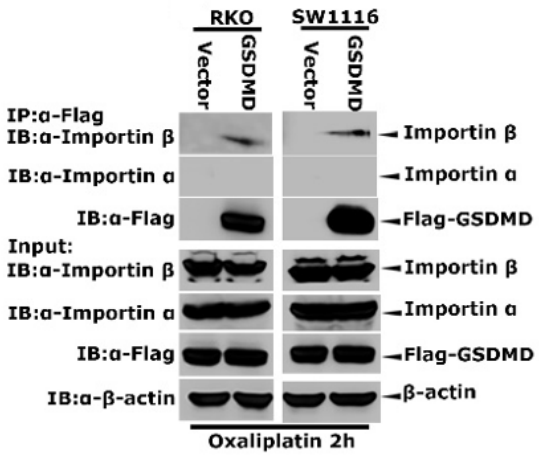

Figure 4.7 - GSDMD interacts with Importin β, rather than Importin α, to guide GSDMD nuclear translocation.

Western blot shows the verification of interaction of GSDMD with importin β, rather than importin α by semi-endogenous co-IP pretreated with Oxaliplatin at a dose of 0.5μM for 2 hours to suggest the nucleocytoplasmic transport manner of GSDMD.

However, the NLS (Nuclear Localization Signal) of GSDMD remains unknown. A short amino acids sequence of "Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val" termed as a well-known NLS (Nuclear Localization Signal) is responsible for the nuclear location of some certain proteins[118, 119]. And then, Myc & NLS-tagged GSDMD stably expressing cells were established in RKO and SW1116 CRC cell lines **(Figure 4.8A-B)**. The expression of Myc-GSDMD-NLS (GSDMD with Myc tag and well known NLS tag) was verified by western blot.

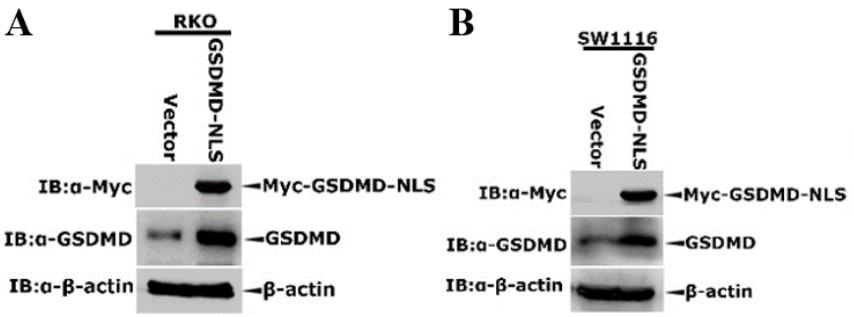

Figure 4.8 - The establishment of Myc & NLS-tagged GSDMD stably expressing cell lines in RKO and SW1116.
(**A-B**). Western blot shows the verification of stably expressing cell lines of RKO and SW1116 with Myc-GSDMD-NLS.

Then, ectopic expression and subcellular localization of Myc-GSDMD-NLS were further verified by IF **(Figure 4.9A-B)**. The IF images showed that, without chemotherapeutic drug treatment, GSDMD cannot translocate in the nucleus in 3×Flag-GSDMD overexpression cell lines. However, GSDMD can translocate in the nucleus in Myc-GSDMD-NLS overexpression cell lines with the well-known NLS tag.

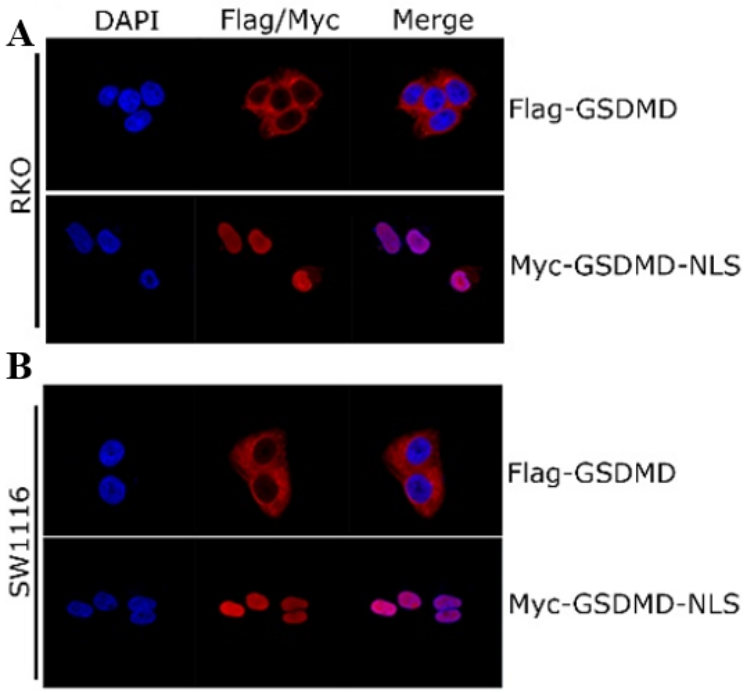

Figure 4.9 - Ectopic expression and subcellular localization of Myc-GSDMD-NLS. (**A-B**). IF images show the subcellular localization of stably expressing cell lines of RKO and SW1116 with 3×Flag GSDMD and Myc-GSDMD-NLS.

To further determine whether GSDMD could translocate into nucleus and promote apoptosis of CRC cells with the guidance of a well-known NLS, in the context of above established stably expressing cell lines of RKO and SW1116 with and without the NLS tag, apoptosis was detected by FCM and FACS analysis of apoptosis **(Figure 4.10A-D)**. FACS analysis results showed that, without chemotherapeutic drug treatment, GSDMD cannot translocate into the nucleus and GSDMD cannot promote apoptosis in GSDMD overexpression and Vector control groups. However, GSDMD can translocate into the

nucleus and promote apoptosis in GSDMD-NLS overexpression group with the well-known NLS tag. The above results indirectly demonstrated that GSDMD can translocate into nucleus and promote apoptosis of CRC cells with the guidance of a well-known NLS.

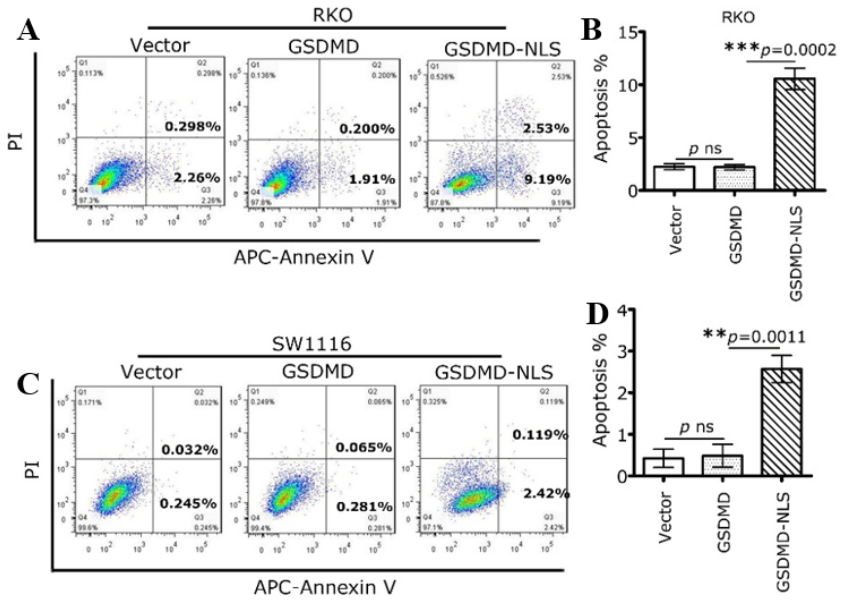

Figure 4.10 - GSDMD translocate into nucleus and promote apoptosis of CRC cells with the guidance of a well-known NLS.
(**A-D**). FCM shows nucleus-localized GSDMD, rather than cytoplasm-localized GSDMD, promotes colorectal cancer cell apoptosis in RKO (***, p=0.0002) and SW1116 (***, p=0.0011).

4.4 Discussion

Targeted NLS sequence recognition represents a critical control point for nuclear transport regulation. The affinity of the targeted nuclear localized protein and its specific NLS sequence interaction is a key parameter to determine transport efficiency [94]. Different nuclear localized proteins have different NLS binding specificities, which allows the

system to be regulated by the differential expression of NLS protein itself as well as by competition between different NLSs for the same protein and between different proteins for the same NLS [94].

The molecular weight of GSDMD is 53 kDa. The transport into and out of nucleus of molecular weight > 40-60 kDa is largely mediated by members of the Importin Superfamily of transporters, consisting of α- and β- types [95]. We verified that the transport of GSDMD is Importin β dependent. We indirectly demonstrated that chemotherapeutic drugs induced GSDMD nuclear translocation is not by passive diffusion. However, no NLS (Nuclear Localization Signal) sequence in human GSDMD was predicted by online Nuclear Localization Signal Prediction softwares. The NLS of GSDMD remains unknown. This is a deficiency in my research. We found that, GSDMD can translocate in the nucleus in GSDMD-NLS overexpression cell lines with the well-known NLS tag. These results indirectly demonstrated that GSDMD can translocate into nucleus with the guidance of a well-known NLS. Besides, no NLS sequence in human GSDMD was predicted by online NLS Prediction softwares, this negative result is in accordance with the cytoplasm localization of GSDMD in the cell culture conditions in vitro. These results also indirectly demonstrated that GSDMD itself does not have NLS. We also found that GSDMD can promote apoptosis in GSDMD-NLS overexpression group with the well-known NLS tag. These results support the clinical value of our research, GSDMD nuclear translocation promotes apoptosis of CRC cells and enhances chemotherapeutic sensitivity. Since the

NLS of GSDMD is still unknown. I hypothesized that there could be an accessory protein with the NLS sequence that can guide GSDMD nuclear translocation. For the next steps, we will perform Co-IP and MS to identify the potential accessory protein of GSDMD. Previous studies have found that targeted nuclear localized protein and its specific NLS sequence interaction can be affected directly by phosphorylation increasing the affinity of the interaction with importins, or by targeting sequence masking through phosphorylation or specific protein binding [96]. These studies support my finding, translocation of GSDMD into nucleus is ATM activation dependent. In conclusion, all these above revealed our model of "Chemotherapy—DNA damage—ATM phosphorylation—GSDMD nuclear translocation—PARP-1 blockage—DNA damage & Apoptosis" axis.

CHAPTER 5: CONCLUSIONS, PERSPECTIVES, AND FUTURE DIRECTIONS

Our study characterized a new mechanism of GSDMD in colorectal cancer. Our data indicated that instead of its reported role in pyroptosis, GSDMD is a key molecule mediating hypoxia and chemotherapy drugs-induced apoptosis via translocation from cytoplasm to nucleus. In cell nucleus, GSDMD directly interacts with PARP-1 to block the DNA repair function of the latter. Our finding is important for understanding cancer biology and the mechanisms underlying inter-patient variability in cancer patient prognosis as well as response to anti-cancer treatments.

Currently, it is controversial in terms of the role of GSDMD in CRC. Previously, one study has shown that downregulated GSDMD promotes proliferation of gastric cancer[64], while another study has shown that downregulation of GSDMD suppresses proliferation of non-small cell lung cancer [97]. The contrary functions of GSDMD above were both associated with pyroptosis. Another study in gastric cancer revealed that GSDMD cannot be cleaved under the treatment with chemotherapeutic drug 5-Fu [98]. Therefore, it was poorly explored whether GSDMD plays a role in tumorigenesis and progression. As is demonstrated by our working model in Figure 1.2, our data clearly revealed that GSDMD, beyond its role in pyroptosis, functions as a tumor suppressor depending on its subcellular localization. This positive nuclear staining of GSDMD shows a correlation with increased overall survival in colorectal cancer patients, suggesting the importance of GSDMD

nuclear translocation in the prognosis of cancer patients.

Mechanistically, we demonstrated that while GSDMD is predominately localized in cytoplasm under regular cell culture conditions in vitro, under certain stresses, such as hypoxia or chemotherapy drugs, GSDMD is translocated into nucleus where it directly interacts with nuclear PARP-1. More specifically, GSDMD interacts with the CA Domain of PARP-1 to inhibit its DNA Damage Repair-related function, enhancing apoptosis induced by chemotherapy drugs. This effect may be via interfering the single strand break (SSB)-related nucleotide excision repair, which further exacerbates the formation of DSBs, as characterized by the increased formation of γ-H2AX foci [120].

Our study also demonstrated that GSDMD is a direct biological inhibitor for PARP-1, via binding to the same domain where the PARP inhibitor Olaparib binds. As a potent PARP inhibitor, Olaparib induces synthetic lethality in BRCA1/2 deficient tumor cells by the formation of DSBs of DNA that leads to cell death [35]. Olaparib monotherapy represents a new therapeutic measure for patients with advanced, heavily pretreated germline BRCA mutation-associated ovarian cancer [105-107]. The combination of Platinum-based chemotherapeutic drugs and PARP inhibitor Olaparib has been approved by FDA for treating breast and ovarian cancers, especially the triple negative metastatic breast cancer and advanced ovarian cancer with “BRCAness”, i.e., cancer cells with BRCA1 or BRCA2 mutations that result in Homologous Recombination Repair defects [80, 81]. Nevertheless, combined application of Oxaliplatin and Olaparib has been reported to

increase the sensitivity of colorectal cancer to Oxaliplatin to induce more G2/M arrest and γ-H2AX foci formation and hence more cell death [109]. In our study, down-regulation of GSDMD contributes to Oxaliplatin chemotherapy resistance, while the resistance can be eliminated by the combined application with PARP inhibitor Olaparib, suggesting that GSDMD exerts at least in part, a function of PARP inhibitor Olaparib.

In conclusion, our data identified a novel, pyroptosis-independent function of GSDMD in colorectal cancer and demonstrated its role as a tumor suppressor by interacting with PARP-1 in a nuclear localization-dependent manner. These data indicated that nucleus localized GSDMD may be a useful marker for clinical outcomes of colorectal cancer and its subcellular localization pattern may be able to guide combinational therapy with PARP inhibitors and chemotherapy drugs in colorectal cancer.

On the one hand, GSDMD is widely expressed in the innate immune cells and different types of cancer cells [64, 97]. On the other hand, PARP-1 is highly and stably expressed in the cell nucleus of all types of eukaryotic cells, PARP-1 can even work as the Western blot loading control, like the other loading controls such as β-actin, β-tubulin or GAPDH (Housekeeping genes) [67-69]. Thus, due to the widely and stably expression pattern of GSDMD and PARP-1 in various types of cancer cells, I hypothesize that GSDMD could also inhibit PARP-1's DNA damage repair function in many other types of cancer cells, such as lung cancer, gastric cancer, ovarian cancer, breast cancer, etc. GSDMD could have the non-pyroptotic function in many other types of cancer cells. Full-length

GSDMD nuclear translocation could facilitate chemotherapy-induced apoptosis through inhibiting PARP-1-mediated DNA Repair and enhance chemotherapeutic sensitivity in various types of cancer cells. All these hypotheses extend the potential clinical application values of our findings in colorectal cancer to many other types of cancer.

For the future directions, our data warranted continued study to examine whether platinum-based chemotherapeutic drugs combined with Olaparib can achieve a better response, especially in colorectal cancer with negative nuclear localization of GSDMD. Besides, we demonstrated that nuclear localization of GSDMD showed inter-patient difference, which was significantly associated with cancer patient survival. The underlying mechanisms for this inter-individual variability and difference remain unknown. It should be noted that protein nucleocytoplasmic transporting is regulated by a wide range of stimuli in the short-term and can be also affected by many cellular and pathological conditions such as cell differentiation, development, transformation, infection, and certain diseases [121, 122]. It is possible that unknown factors among about 25% cases of CRC patients prevent the induction of GSDMD translocation, which further leads to unfavorable clinical outcomes. Future studies are needed to identify these factors, so that better therapeutic outcomes can be achieved. In our working plan, we would like to elucidate the underlying mechanism of GSDMD nuclear translocation, to account for the forming reason of 20-25% nuclear GSDMD negative cases in clinics. I hypothesize that this group of CRC patients has GSDMD or GSDMD accessory protein mutation. It is also important to explore the

potential value of using nuclear staining of GSDMD as a prognostic biomarker for colorectal cancers.

In addition, no NLS (Nuclear Localization Signal) sequence in human GSDMD was predicted by online Nuclear Localization Signal Prediction softwares. The NLS of GSDMD remains unknown. This is a deficiency in my research. Previously, one study has shown that Human immunodeficiency virus type 1 (HIV-1) virus fuses with the cell surface membrane introducing genetic material and virion proteins, which include gag proteins that comprise the matrix and nucleocapsid, the latter containing significant quantities of Viral protein R (Vpr). Vpr is a 96 amino acid, 14 kDa protein that contains NLS sequence. Vpr's structure allows for direct binding to many cellular proteins, which likely enables Vpr to mediate functions such as nuclear import. Vpr mediates nuclear localization by binding to Importin-α. Vpr containing NLS works as the nuclear translocation accessory protein of HIV-1 viral pre-integration protein complex (PIC). Vpr promotes the binding of the PIC, including viral proteins matrix antigen, integrase and proviral DNA to importins and nucleoporins, thereby facilitating nuclear entry of HIV-1 provirus into the nucleus of non-dividing cells [123]. Moreover, another study by Dr. Weidong Yang's team used super-resolution microscopy to show that β-catenin import requires Custos as a docking protein. Custos containing NLS sequence and β-catenin form a complex in the cytoplasm and move together through the nuclear pore complex into the nucleus, where they dissociate in the nucleus.

If GSDMD functions as a cargo during nuclear translocation process, I hypothesize that there are three possibilities that could cause GSDMD nuclear translocation. For the first possibility, GSDMD may have hydrophobic domains on its surface that could help GSDMD interacts with nuclear pore complex and guide nuclear translocation. I will further check the hydrophobic amino acids sequence of GSDMD and perform confocal microscopy and SPEED microscopy to verify the potential hydrophobic protein interactions. For the second possibility, there may be a current unknown NLS sequence or Non-classical NLS sequence in GSDMD that could guide GSDMD nuclear translocation. For the third possibility, there may be an accessory protein containing NLS sequence that could guide GSDMD nuclear translocation. For the next step, we will perform Co-immunoprecipitation and Mass spectrometry of GSDMD once again to identify the potential accessory protein of GSDMD that can guide GSDMD nuclear translocation. Moreover, we found that GSDMD nuclear translocation is guided by Importin β. Next, we will perform GST (Glutathione S-transferase) pull down assay to verify the interaction among GSDMD, accessory protein with the NLS and Importin β family members, including Importin β1 and Importin β2. Knockdown of Importin β1 or Importin β2, or Import factor, RanGTP, inhibitor, induced blockage of protein nuclear translocation could be used to further verify the interactions among GSDMD, nuclear translocation accessory protein and Importin β family members. After that, GSDMD's accessory protein truncate assay allows us to detect the approximate location of NLS. We will build GSDMD's

accessory protein's truncate plasmids. We will generate GSDMD's accessory protein truncates fused with GFP. After cell transfection, the localization of the GSDMD's accessory protein truncate in CRC cells could then be visualized by fluorescence microscopy. Finally, NLS mutation assay allows us to detect the exact location of NLS. We could perform an alanine substitution mutation of NLS, we will add one or a few amino acids into NLS sequence to make NLS loss of function. Cells will be transfected with wild type or mutants of NLS with GFP tag. The localization of the GSDMD in CRC cells could then be visualized by fluorescence microscopy.

Due to the molecular weight of GSDMD (53 kDa), it is hard to completely exclude the possibility of passive diffusion induced GSDMD nuclear accumulation. If GSDMD accumulates in the nucleus by passive diffusion, I hypothesize that some nuclear tension events could guides this GSDMD nuclear accumulation process. Some co-factors in the nucleus could enhance GSDMD nuclear accumulation. We found that GSDMD interacts with PARP-1 in the nucleus, so PARP-1 is one potential candidate co-factor. Besides, we found that GSDMD nuclear accumulation is ATM kinase activation dependent. Thus, some kinases could also be the candidate co-factors in the nucleus that guide GSDMD nuclear accumulation. These possibilities and hypotheses are involved in our further research.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA: a cancer journal for clinicians. 2020;70(1):7-30.

2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018;68(6):394-424.

3. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. CA: a cancer journal for clinicians. 2016;66(2):115-32.

4. Falcone A, Ricci S, Brunetti I, Pfanner E, Allegrini G, Barbara C, et al. Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007;25(13):1670-6.

5. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, et al. Colorectal cancer statistics, 2020. CA: a cancer journal for clinicians. 2020;70(3):145-64.

6. Brenner H, Kloor M, Pox CP. Colorectal cancer. Lancet (London, England). 2014;383(9927):1490-502.

7. Weitz J, Koch M, Debus J, Höhler T, Galle PR, Büchler MW. Colorectal cancer. Lancet

(London, England). 2005;365(9454):153-65.

8. Dariya B, Aliya S, Merchant N, Alam A, Nagaraju GP. Colorectal Cancer Biology, Diagnosis, and Therapeutic Approaches. Critical reviews in oncogenesis. 2020;25(2):71-94.

9. Mármol I, Sánchez-de-Diego C, Pradilla Dieste A, Cerrada E, Rodriguez Yoldi MJ. Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. International journal of molecular sciences. 2017;18(1).

10. Mäkinen MJ. Colorectal serrated adenocarcinoma. Histopathology. 2007;50(1):131-50.

11. Carethers JM, Jung BH. Genetics and Genetic Biomarkers in Sporadic Colorectal Cancer. Gastroenterology. 2015;149(5):1177-90.e3.

12. O'Brien MJ, Yang S, Mack C, Xu H, Huang CS, Mulcahy E, et al. Comparison of microsatellite instability, CpG island methylation phenotype, BRAF and KRAS status in serrated polyps and traditional adenomas indicates separate pathways to distinct colorectal carcinoma end points. The American journal of surgical pathology. 2006;30(12):1491-501.

13. Wang X, Amitay E, Harrison TA, Banbury BL, Berndt SI, Brenner H, et al. Association Between Smoking and Molecular Subtypes of Colorectal Cancer. JNCI cancer spectrum. 2021;5(4).

14. Velho S, Moutinho C, Cirnes L, Albuquerque C, Hamelin R, Schmitt F, et al. BRAF, KRAS and PIK3CA mutations in colorectal serrated polyps and cancer: primary or

secondary genetic events in colorectal carcinogenesis? BMC cancer. 2008;8:255.

15. Kambara T, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut. 2004;53(8):1137-44.

16. Goel A, Nagasaka T, Arnold CN, Inoue T, Hamilton C, Niedzwiecki D, et al. The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. Gastroenterology. 2007;132(1):127-38.

17. Dahlin AM, Palmqvist R, Henriksson ML, Jacobsson M, Eklöf V, Rutegård J, et al. The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16(6):1845-55.

18. Kim JH, Kang GH. Molecular and prognostic heterogeneity of microsatellite-unstable colorectal cancer. World journal of gastroenterology. 2014;20(15):4230-43.

19. Bae JM, Kim JH, Kang GH. Epigenetic alterations in colorectal cancer: the CpG island methylator phenotype. Histology and histopathology. 2013;28(5):585-95.

20. Lee S, Cho NY, Yoo EJ, Kim JH, Kang GH. CpG island methylator phenotype in colorectal cancers: comparison of the new and classic CpG island methylator phenotype marker panels. Archives of pathology & laboratory medicine. 2008;132(10):1657-65.

21. Migliore L, Migheli F, Spisni R, Coppedè F. Genetics, cytogenetics, and epigenetics of colorectal cancer. Journal of biomedicine & biotechnology. 2011;2011:792362.

22. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487(7407):330-7.

23. Fakih MG, Padmanabhan A. CEA monitoring in colorectal cancer. What you should know. Oncology (Williston Park, NY). 2006;20(6):579-87; discussion 88, 94, 96 passim.

24. Campos-da-Paz M, Dórea JG, Galdino AS, Lacava ZGM, de Fatima Menezes Almeida Santos M. Carcinoembryonic Antigen (CEA) and Hepatic Metastasis in Colorectal Cancer: Update on Biomarker for Clinical and Biotechnological Approaches. Recent patents on biotechnology. 2018;12(4):269-79.

25. Ladabaum U, Dominitz JA, Kahi C, Schoen RE. Strategies for Colorectal Cancer Screening. Gastroenterology. 2020;158(2):418-32.

26. Shaukat A, Levin TR. Current and future colorectal cancer screening strategies. Nature reviews Gastroenterology & hepatology. 2022:1-11.

27. Lech G, Slotwinski R, Krasnodebski IW. The role of tumor markers and biomarkers in colorectal cancer. Neoplasma. 2014;61(1):1-8.

28. Lech G, Słotwiński R, Słodkowski M, Krasnodębski IW. Colorectal cancer tumour markers and biomarkers: Recent therapeutic advances. World journal of gastroenterology. 2016;22(5):1745-55.

29. Yamashita K, Watanabe M. Clinical significance of tumor markers and an emerging perspective on colorectal cancer. Cancer science. 2009;100(2):195-9.

30. Biller LH, Schrag D. Diagnosis and Treatment of Metastatic Colorectal Cancer: A

Review. Jama. 2021;325(7):669-85.

31. Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, et al. Colorectal cancer. Nature reviews Disease primers. 2015;1:15065.

32. Johdi NA, Sukor NF. Colorectal Cancer Immunotherapy: Options and Strategies. Frontiers in immunology. 2020;11:1624.

33. Piawah S, Venook AP. Targeted therapy for colorectal cancer metastases: A review of current methods of molecularly targeted therapy and the use of tumor biomarkers in the treatment of metastatic colorectal cancer. Cancer. 2019;125(23):4139-47.

34. Linnekamp JF, Wang X, Medema JP, Vermeulen L. Colorectal cancer heterogeneity and targeted therapy: a case for molecular disease subtypes. Cancer research. 2015;75(2):245-9.

35. Zhai Z, Yu X, Yang B, Zhang Y, Zhang L, Li X, et al. Colorectal cancer heterogeneity and targeted therapy: Clinical implications, challenges and solutions for treatment resistance. Seminars in cell & developmental biology. 2017;64:107-15.

36. Gutting T, Burgermeister E, Härtel N, Ebert MP. Checkpoints and beyond - Immunotherapy in colorectal cancer. Seminars in cancer biology. 2019;55:78-89.

37. Kather JN, Halama N, Jaeger D. Genomics and emerging biomarkers for immunotherapy of colorectal cancer. Seminars in cancer biology. 2018;52(Pt 2):189-97.

38. Wolpin BM, Mayer RJ. Systemic treatment of colorectal cancer. Gastroenterology. 2008;134(5):1296-310.

39. Cheung-Ong K, Giaever G, Nislow C. DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology. Chemistry & biology. 2013;20(5):648-59.

40. Hammond WA, Swaika A, Mody K. Pharmacologic resistance in colorectal cancer: a review. Therapeutic advances in medical oncology. 2016;8(1):57-84.

41. Nagata S, Tanaka M. Programmed cell death and the immune system. Nature reviews Immunology. 2017;17(5):333-40.

42. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology. 2007;35(4):495-516.

43. Bedoui S, Herold MJ, Strasser A. Emerging connectivity of programmed cell death pathways and its physiological implications. Nature reviews Molecular cell biology. 2020;21(11):678-95.

44. Yang F, He Y, Zhai Z, Sun E. Programmed Cell Death Pathways in the Pathogenesis of Systemic Lupus Erythematosus. Journal of immunology research. 2019;2019:3638562.

45. Fang Y, Tian S, Pan Y, Li W, Wang Q, Tang Y, et al. Pyroptosis: A new frontier in cancer. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2020;121:109595.

46. Yu P, Zhang X, Liu N, Tang L, Peng C, Chen X. Pyroptosis: mechanisms and diseases. Signal transduction and targeted therapy. 2021;6(1):128.

47. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature. 2015;526(7575):660-5.

48. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, et al. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature. 2015;526(7575):666-71.

49. He WT, Wan H, Hu L, Chen P, Wang X, Huang Z, et al. Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. Cell research. 2015;25(12):1285-98.

50. Tamura M, Tanaka S, Fujii T, Aoki A, Komiyama H, Ezawa K, et al. Members of a novel gene family, Gsdm, are expressed exclusively in the epithelium of the skin and gastrointestinal tract in a highly tissue-specific manner. Genomics. 2007;89(5):618-29.

51. Xu B, Jiang M, Chu Y, Wang W, Chen D, Li X, et al. Gasdermin D plays a key role as a pyroptosis executor of non-alcoholic steatohepatitis in humans and mice. Journal of hepatology. 2018;68(4):773-82.

52. Khanova E, Wu R, Wang W, Yan R, Chen Y, French SW, et al. Pyroptosis by caspase11/4-gasdermin-D pathway in alcoholic hepatitis in mice and patients. Hepatology (Baltimore, Md). 2018;67(5):1737-53.

53. Kovacs SB, Miao EA. Gasdermins: Effectors of Pyroptosis. Trends in cell biology. 2017;27(9):673-84.

54. Ramos-Junior ES, Morandini AC. Gasdermin: A new player to the inflammasome game. Biomedical journal. 2017;40(6):313-6.

55. Ruan J. Structural Insight of Gasdermin Family Driving Pyroptotic Cell Death.

Advances in experimental medicine and biology. 2019;1172:189-205.

56. Feng S, Fox D, Man SM. Mechanisms of Gasdermin Family Members in Inflammasome Signaling and Cell Death. Journal of molecular biology. 2018;430(18 Pt B):3068-80.

57. Liao XX, Dai YZ, Zhao YZ, Nie K. Gasdermin E: A Prospective Target for Therapy of Diseases. Frontiers in pharmacology. 2022;13:855828.

58. Zuo Y, Chen L, Gu H, He X, Ye Z, Wang Z, et al. GSDMD-mediated pyroptosis: a critical mechanism of diabetic nephropathy. Expert reviews in molecular medicine. 2021;23:e23.

59. Zhao L, Li L, Xue M, Liu X, Jiang C, Wang W, et al. Gasdermin D Inhibits Coronavirus Infection by Promoting the Noncanonical Secretion of Beta Interferon. mBio. 2022;13(1):e0360021.

60. Liu X, Ding S, Liu P. The Roles of Gasdermin D in Coronavirus Infection and Evasion. Frontiers in microbiology. 2021;12:784009.

61. Komiyama H, Aoki A, Tanaka S, Maekawa H, Kato Y, Wada R, et al. Alu-derived cis-element regulates tumorigenesis-dependent gastric expression of GASDERMIN B (GSDMB). Genes & genetic systems. 2010;85(1):75-83.

62. Saeki N, Usui T, Aoyagi K, Kim DH, Sato M, Mabuchi T, et al. Distinctive expression and function of four GSDM family genes (GSDMA-D) in normal and malignant upper gastrointestinal epithelium. Genes, chromosomes & cancer. 2009;48(3):261-71.

63. Saeki N, Kim DH, Usui T, Aoyagi K, Tatsuta T, Aoki K, et al. GASDERMIN, suppressed frequently in gastric cancer, is a target of LMO1 in TGF-beta-dependent apoptotic signalling. Oncogene. 2007;26(45):6488-98.

64. Wang WJ, Chen D, Jiang MZ, Xu B, Li XW, Chu Y, et al. Downregulation of gasdermin D promotes gastric cancer proliferation by regulating cell cycle-related proteins. Journal of digestive diseases. 2018;19(2):74-83.

65. Sborgi L, Rühl S, Mulvihill E, Pipercevic J, Heilig R, Stahlberg H, et al. GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death. The EMBO journal. 2016;35(16):1766-78.

66. Ding J, Wang K, Liu W, She Y, Sun Q, Shi J, et al. Pore-forming activity and structural autoinhibition of the gasdermin family. Nature. 2016;535(7610):111-6.

67. Heitz F, Harter P, Ewald-Riegler N, Papsdorf M, Kommoss S, du Bois A. Poly(ADP-ribosyl)ation polymerases: mechanism and new target of anticancer therapy. Expert review of anticancer therapy. 2010;10(7):1125-36.

68. Wang Y, Luo W, Wang Y. PARP-1 and its associated nucleases in DNA damage response. DNA repair. 2019;81:102651.

69. Hanzlikova H, Kalasova I, Demin AA, Pennicott LE, Cihlarova Z, Caldecott KW. The Importance of Poly(ADP-Ribose) Polymerase as a Sensor of Unligated Okazaki Fragments during DNA Replication. Molecular cell. 2018;71(2):319-31.e3.

70. Henning RJ, Bourgeois M, Harbison RD. Poly(ADP-ribose) Polymerase (PARP) and

PARP Inhibitors: Mechanisms of Action and Role in Cardiovascular Disorders. Cardiovascular toxicology. 2018;18(6):493-506.

71. Bartha E, Solti I, Kereskai L, Lantos J, Plozer E, Magyar K, et al. PARP inhibition delays transition of hypertensive cardiopathy to heart failure in spontaneously hypertensive rats. Cardiovascular research. 2009;83(3):501-10.

72. Pandey N, Black BE. Rapid Detection and Signaling of DNA Damage by PARP-1. Trends in biochemical sciences. 2021;46(9):744-57.

73. Maluchenko NV, Feofanov AV, Studitsky VM. PARP-1-Associated Pathological Processes: Inhibition by Natural Polyphenols. International journal of molecular sciences. 2021;22(21).

74. Dantzer F, Amé JC, Schreiber V, Nakamura J, Ménissier-de Murcia J, de Murcia G. Poly(ADP-ribose) polymerase-1 activation during DNA damage and repair. Methods in enzymology. 2006;409:493-510.

75. Pacher P, Szabó C. Role of poly(ADP-ribose) polymerase 1 (PARP-1) in cardiovascular diseases: the therapeutic potential of PARP inhibitors. Cardiovascular drug reviews. 2007;25(3):235-60.

76. Virág L. Structure and function of poly(ADP-ribose) polymerase-1: role in oxidative stress-related pathologies. Current vascular pharmacology. 2005;3(3):209-14.

77. Coleman RL, Sill MW, Bell-McGuinn K, Aghajanian C, Gray HJ, Tewari KS, et al. A phase II evaluation of the potent, highly selective PARP inhibitor veliparib in the treatment

of persistent or recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer in patients who carry a germline BRCA1 or BRCA2 mutation - An NRG Oncology/Gynecologic Oncology Group study. Gynecologic oncology. 2015;137(3):386-91.

78. Oza AM, Tinker AV, Oaknin A, Shapira-Frommer R, McNeish IA, Swisher EM, et al. Antitumor activity and safety of the PARP inhibitor rucaparib in patients with high-grade ovarian carcinoma and a germline or somatic BRCA1 or BRCA2 mutation: Integrated analysis of data from Study 10 and ARIEL2. Gynecologic oncology. 2017;147(2):267-75.

79. Dörsam B, Seiwert N, Foersch S, Stroh S, Nagel G, Begaliew D, et al. PARP-1 protects against colorectal tumor induction, but promotes inflammation-driven colorectal tumor progression. Proceedings of the National Academy of Sciences of the United States of America. 2018;115(17):E4061-e70.

80. Rottenberg S, Jaspers JE, Kersbergen A, van der Burg E, Nygren AO, Zander SA, et al. High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(44):17079-84.

81. Basourakos SP, Li L, Aparicio AM, Corn PG, Kim J, Thompson TC. Combination Platinum-based and DNA Damage Response-targeting Cancer Therapy: Evolution and Future Directions. Current medicinal chemistry. 2017;24(15):1586-606.

82. Maréchal A, Zou L. DNA damage sensing by the ATM and ATR kinases. Cold Spring

Harbor perspectives in biology. 2013;5(9).

83. Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. Nature reviews Cancer. 2003;3(3):155-68.

84. Di Domenico EG, Romano E, Del Porto P, Ascenzioni F. Multifunctional role of ATM/Tel1 kinase in genome stability: from the DNA damage response to telomere maintenance. BioMed research international. 2014;2014:787404.

85. Zakian VA. ATM-related genes: what do they tell us about functions of the human gene? Cell. 1995;82(5):685-7.

86. Wenzel ES, Singh ATK. Cell-cycle Checkpoints and Aneuploidy on the Path to Cancer. In vivo (Athens, Greece). 2018;32(1):1-5.

87. Alao JP, Sunnerhagen P. The ATM and ATR inhibitors CGK733 and caffeine suppress cyclin D1 levels and inhibit cell proliferation. Radiation oncology (London, England). 2009;4:51.

88. Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, Orr AI, et al. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. Cancer research. 2004;64(24):9152-9.

89. Stankovic T, Stewart GS, Fegan C, Biggs P, Last J, Byrd PJ, et al. Ataxia telangiectasia mutated-deficient B-cell chronic lymphocytic leukemia occurs in pregerminal center cells and results in defective damage response and unrepaired chromosome damage. Blood. 2002;99(1):300-9.

90. Starostik P, Manshouri T, O'Brien S, Freireich E, Kantarjian H, Haidar M, et al. Deficiency of the ATM protein expression defines an aggressive subgroup of B-cell chronic lymphocytic leukemia. Cancer research. 1998;58(20):4552-7.

91. Byrski T, Huzarski T, Dent R, Marczyk E, Jasiowka M, Gronwald J, et al. Pathologic complete response to neoadjuvant cisplatin in BRCA1-positive breast cancer patients. Breast cancer research and treatment. 2014;147(2):401-5.

92. Weber AM, Ryan AJ. ATM and ATR as therapeutic targets in cancer. Pharmacology & therapeutics. 2015;149:124-38.

93. Wagstaff KM, Jans DA. Importins and beyond: non-conventional nuclear transport mechanisms. Traffic (Copenhagen, Denmark). 2009;10(9):1188-98.

94. Poon IK, Jans DA. Regulation of nuclear transport: central role in development and transformation? Traffic (Copenhagen, Denmark). 2005;6(3):173-86.

95. Pemberton LF, Paschal BM. Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic (Copenhagen, Denmark). 2005;6(3):187-98.

96. Jans DA, Xiao CY, Lam MH. Nuclear targeting signal recognition: a key control point in nuclear transport? BioEssays : news and reviews in molecular, cellular and developmental biology. 2000;22(6):532-44.

97. Gao J, Qiu X, Xi G, Liu H, Zhang F, Lv T, et al. Downregulation of GSDMD attenuates tumor proliferation via the intrinsic mitochondrial apoptotic pathway and inhibition of EGFR/Akt signaling and predicts a good prognosis in non‐small cell lung cancer.

Oncology reports. 2018;40(4):1971-84.

98. Wang Y, Yin B, Li D, Wang G, Han X, Sun X. GSDME mediates caspase-3-dependent pyroptosis in gastric cancer. Biochemical and biophysical research communications. 2018;495(1):1418-25.

99. Fukushima A. DiffCorr: an R package to analyze and visualize differential correlations in biological networks. Gene. 2013;518(1):209-14.

100. Alvarenga TC, Lima RR, Bueno Filho JSS, Simão SD, Mariano FCQ, Alvarenga RR, et al. Application of Bayesian networks to the prediction of the AMEn: a new methodology in broiler nutrition. Translational animal science. 2021;5(1):txaa215.

101. Xu Z, Zhou Y, Cao Y, Dinh TL, Wan J, Zhao M. Identification of candidate biomarkers and analysis of prognostic values in ovarian cancer by integrated bioinformatics analysis. Medical oncology (Northwood, London, England). 2016;33(11):130.

102. Bouchard VJ, Rouleau M, Poirier GG. PARP-1, a determinant of cell survival in response to DNA damage. Experimental hematology. 2003;31(6):446-54.

103. Langelier MF, Planck JL, Roy S, Pascal JM. Structural basis for DNA damage-dependent poly(ADP-ribosyl)ation by human PARP-1. Science (New York, NY). 2012;336(6082):728-32.

104. Helleday T. The underlying mechanism for the PARP and BRCA synthetic lethality: clearing up the misunderstandings. Molecular oncology. 2011;5(4):387-93.

105. Kim G, Ison G, McKee AE, Zhang H, Tang S, Gwise T, et al. FDA Approval Summary: Olaparib Monotherapy in Patients with Deleterious Germline BRCA-Mutated Advanced Ovarian Cancer Treated with Three or More Lines of Chemotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(19):4257-61.

106. Kaufman B, Shapira-Frommer R, Schmutzler RK, Audeh MW, Friedlander M, Balmaña J, et al. Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(3):244-50.

107. Matulonis UA, Penson RT, Domchek SM, Kaufman B, Shapira-Frommer R, Audeh MW, et al. Olaparib monotherapy in patients with advanced relapsed ovarian cancer and a germline BRCA1/2 mutation: a multistudy analysis of response rates and safety. Annals of oncology : official journal of the European Society for Medical Oncology. 2016;27(6):1013-9.

108. Leichman L, Groshen S, O'Neil BH, Messersmith W, Berlin J, Chan E, et al. Phase II Study of Olaparib (AZD-2281) After Standard Systemic Therapies for Disseminated Colorectal Cancer. The oncologist. 2016;21(2):172-7.

109. Xu K, Chen Z, Cui Y, Qin C, He Y, Song X. Combined olaparib and oxaliplatin inhibits tumor proliferation and induces G2/M arrest and γ-H2AX foci formation in colorectal cancer. OncoTargets and therapy. 2015;8:3047-54.

110. Wong RS. Apoptosis in cancer: from pathogenesis to treatment. Journal of experimental & clinical cancer research : CR. 2011;30(1):87.

111. Mohammad RM, Muqbil I, Lowe L, Yedjou C, Hsu HY, Lin LT, et al. Broad targeting of resistance to apoptosis in cancer. Seminars in cancer biology. 2015;35 Suppl(0):S78-s103.

112. Shi J, Gao W, Shao F. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. Trends in biochemical sciences. 2017;42(4):245-54.

113. Bochum S, Berger S, Martens UM. Olaparib. Recent results in cancer research Fortschritte der Krebsforschung Progres dans les recherches sur le cancer. 2018;211:217-33.

114. Min A, Im SA. PARP Inhibitors as Therapeutics: Beyond Modulation of PARylation. Cancers. 2020;12(2).

115. Lane RJ, Khin NY, Pavlakis N, Hugh TJ, Clarke SJ, Magnussen J, et al. Challenges in chemotherapy delivery: comparison of standard chemotherapy delivery to locoregional vascular mass fluid transfer. Future oncology (London, England). 2018;14(7):647-63.

116. Jans DA, Martin AJ, Wagstaff KM. Inhibitors of nuclear transport. Current opinion in cell biology. 2019;58:50-60.

117. Çağatay T, Chook YM. Karyopherins in cancer. Current opinion in cell biology. 2018;52:30-42.

118. Smith AE, Kalderon D, Roberts BL, Colledge WH, Edge M, Gillett P, et al. The nuclear

location signal. Proceedings of the Royal Society of London Series B, Biological sciences. 1985;226(1242):43-58.

119.Kalderon D, Roberts BL, Richardson WD, Smith AE. A short amino acid sequence able to specify nuclear location. Cell. 1984;39(3 Pt 2):499-509.

120. Rothkamm K, Barnard S, Moquet J, Ellender M, Rana Z, Burdak-Rothkamm S. DNA damage foci: Meaning and significance. Environmental and molecular mutagenesis. 2015;56(6):491-504.

121. Mathew C, Ghildyal R. CRM1 Inhibitors for Antiviral Therapy. Frontiers in microbiology. 2017;8:1171.

122. Kim HJ, Taylor JP. Lost in Transportation: Nucleocytoplasmic Transport Defects in ALS and Other Neurodegenerative Diseases. Neuron. 2017;96(2):285-97.

123. Kogan M, Rappaport J. HIV-1 accessory protein Vpr: relevance in the pathogenesis of HIV and potential for therapeutic intervention. Retrovirology. 2011;8:25.