

Gastrointestinal Cancers

Jose Andres Morgado-Diaz, PhD
Editor

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Edited by

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FOREWORD

Gastrointestinal cancers represent a heterogeneous group of diseases of the gastrointestinal tract. These include cancers of the colon, rectum, stomach, pancreas, esophagus, anus, gallbladder, liver, and bile duct. There is an interplay of various non-modifiable and modifiable risk factors that foster the conversion of normal cells to precursor cells, precursor cells to premalignant cells, and premalignant cells to malignant cells. The non-modifiable risk factors are mostly genetic and aging, whereas some of the key modifiable risk factors are smoking, excessive alcohol consumption, and obesity. The initial trigger by these risk factors may be specific for each cancer, but a shared feature among gastrointestinal cancers is increased mortality and morbidity due to late-stage detection, and poor survival following metastasis. Despite significant advances in our understanding of molecular pathogenesis and the development of targeted therapies, gastrointestinal cancers continue to be a leading cause of cancer-related deaths.

Gastrointestinal cancers are diverse in etiology and clinical management. The chapters of this book explore the clinically relevant aspects of this diversity under three broad categories: epidemiology and pathology, early diagnosis and prognosis, and surgical management. The etiological aspects focus on stomach cancer while the pathological aspects provide an overview of colorectal cancer, how primary colorectal cancer becomes metastatic through epithelial mesenchymal transition, and how macrophage-derived extracellular vesicles drive tumor development and enable the progression of most gastrointestinal cancers. Chapters on early detection and prognosis emphasize on biomarker discovery, both at genetic and proteomic level, and how these can be used to effectively predict the origin, progress, prognosis, and treatment response of gastrointestinal cancers in general and pancreatic cancer in particular. Given that the gastrointestinal tract is solely responsible for the processing of the diet we consume, the impact of diet that we consume cannot be ignored. There is a dedicated chapter that covers the role of diet and lifestyle on colorectal cancer incidence and survival. Despite various treatment modalities, for localized cancers, surgery is still the best form of curative treatment, and the role of surgical management of gastrointestinal stromal tumors and esophageal cancers are elegantly summarized in two chapters.

Such a broad spectrum of diseases like gastrointestinal cancers cannot be covered in a single book; however, the contents of this book provide the readers with an overview of several important aspects of gastrointestinal cancers and may be of interest to healthcare professionals interested in gastrointestinal cancers.

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PREFACE

Gastrointestinal cancers are a leading cause of death among patients worldwide. The polygenic and heterogeneous nature of gastrointestinal cancers are characterized by alterations in multiple molecular pathways throughout their development, posing a big challenge for patient risk stratification and treatment options. Gastrointestinal cancers are common, and treatments are more effective when the cancers are detected at an early stage, which, unfortunately, is a challenge. About 10% of the gastrointestinal cancers are attributed to various genetic risk factors whereas the remaining 90% are sporadic, which further complicates timely diagnosis and subsequent development of management strategies, necessitating the need for a better understanding of the origin, and the development of better treatment strategies for gastrointestinal cancer. This book, contributed by an international team of clinicians and basic scientists, provides select, clinically significant aspects of gastrointestinal cancer.

The first three chapters focus on colorectal cancer, the most predominant of gastrointestinal cancers. *Chapter 1* provides an overview of colorectal cancer with emphasis on epidemiology, etiology, pathogenesis, clinical manifestations, diagnosis, treatment, prognosis, and prevention strategies. Identifying modifiable risk factors to reduce the incidence and morbidity of colorectal cancer is beneficial on an individual and public health level. There is a great emphasis on the role of lifestyle and diet as risk factors for colorectal cancer. As the diet we consume is processed by the gastrointestinal system, the putative role of the diet in colorectal cancer cannot be underestimated. *Chapter 2* summarizes our understanding of the role of lifestyle and diet on colorectal cancer incidence and survival and argues that lifestyle modification is essential for prevention and treatment of colorectal cancer for improved patient outcomes. Metastasis is the major cause of cancer-related deaths. The process of metastasis is multifactorial, and one such mechanism is epithelial-mesenchymal transition. It is a morphogenetic event in which cancer cells lose their epithelial characteristics and gain mesenchymal features with an increased migratory and invasive potential. *Chapter 3* discusses the fundamentals of epithelial-mesenchymal transition, how it leads to cancer progression, metastasis, resistance to radiotherapy and chemotherapy, and how various components of the epithelial mesenchymal transition can be used as potential markers or therapeutic targets for metastasis inhibition, along with the obstacles in the development of drugs targeting epithelial mesenchymal transition.

Gastrointestinal stromal tumors are mesenchymal tumors, thought to arise from the interstitial cells of Cajal. Gastrointestinal stromal tumors are mostly formed in the stomach and the small intestine. These tumors do not have specific endoscopic or radiological features. The treatment for confirmed gastrointestinal stromal tumors is surgery if the lesion is resectable with no metastases, or therapy with tyrosine kinase inhibitors if the lesion is unresectable, metastatic, or recurrent. *Chapter 4* discusses the clinicopathological features of gastrointestinal stromal tumors and describes the standard minimally invasive management techniques. Macrophages have a differential role in tumor biology: the M1 macrophages are anti-tumoral, and the

M2 macrophages are pro-tumoral. A subpopulation of these macrophages is described as tumor-associated macrophages and several studies have described the importance of extracellular vesicles derived from tumor-associated macrophages in the advancement and progression of gastrointestinal cancers. *Chapter 5* highlights the role of macrophage-derived extracellular vesicles in gastric, hepatic, pancreatic, and colorectal tumors. It discusses the importance of molecules and cell signaling pathways involved in this context and emphasizes the relevant role of these extracellular vesicles in tumor development. Gastrointestinal tumors are highly heterogeneous, characterized by alterations in multiple molecular pathways. Harnessing these molecular pathways can help the development of innovative therapeutic strategies. To this end, *Chapter 6* describes the prognostic and predictive potential of a multigene signature in gastrointestinal cancer. A validated panel of prognostic gene signature and score system that robustly and reliably predicts overall survival in gastric cancer patients, and how this signature can identify gastric cancer patients who may benefit from adjuvant FOLFOX chemotherapy are explored.

Pancreatic ductal adenocarcinoma has one of the worst survival rates among adult cancers, with only 11% in the United States surviving five years after diagnosis. The majority of patients are diagnosed with late-stage disease, since early-stage pancreatic ductal adenocarcinoma is typically either asymptomatic or presents with non-specific symptoms. By providing physicians with actionable information early enough for the cancer to be removed surgically, the overall 5-year pancreatic ductal adenocarcinoma survival rate could increase from 11% to over 50%. In *Chapter 7*, the authors describe the development and clinical implementation of a proteomic, multi-biomarker blood test for the early detection of pancreatic ductal adenocarcinoma. *Chapter 8* examines the incidence and mortality rate of stomach cancer worldwide, with emphasis on figures in Brazil. Data on new cases and deaths due to stomach cancer for 2020 and projections for 2040 are evaluated. Although both incidence and mortality rates currently exhibit a downward trend in most countries, including Brazil, the number of new cases and deaths each year is not negligible, indicating the need for continued actions to reduce exposure to stomach cancer risk factors and the expansion of early diagnoses with timely treatment. Esophageal cancer is currently the eighth most common cancer, and the sixth leading cause of death from cancer in the world due to its highly aggressive nature. The advent of minimally invasive surgical techniques has reduced morbidity and mortality of esophagectomy without compromising the oncological outcomes. *Chapter 9* provides a comprehensive account of McKeown mini-invasive esophagectomy technique.

I thank the authors for their dedication and intellectual contribution in bringing this book to fruition. I am confident the book will be a valuable resource to clinicians and basic scientists alike.

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Colorectal Cancer: An Overview

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Abstract: Colorectal cancer is a multifactorial disease. It is the third most frequently diagnosed cancer, and the second most common cause of cancer-related deaths worldwide. The etiology of colorectal cancer remains unclear. Although early diagnosis can significantly improve the prognosis, colorectal cancer patients often have no typical clinical manifestations, or display only non-specific signs in the early stage, resulting in a low early diagnosis rate. Multiple treatment modalities, depending on the stage of the tumor and patient characteristics, are available. These include surgery, chemotherapy, radiotherapy, molecular targeted therapy, immunotherapy, and other programs. This chapter provides an overview of colorectal cancer. Epidemiology, etiology, pathogenesis, clinical manifestations, diagnosis, treatment, prognosis, and prevention strategies of colorectal cancer are discussed.

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Keywords: clinical manifestation of colorectal cancer; diagnosis of colorectal cancer; epidemiology of colorectal cancer; etiology of colorectal cancer; treatment of colorectal cancer

INTRODUCTION

The adult large intestine includes the colon, rectum, and anal canal. The colon can be divided into the right colon (cecum, ascending colon, and right 2/3 transverse colon) and left colon (left 1/3 transverse colon, descending colon, and sigmoid colon). The blood supply of the colon is mainly from the mesenteric artery; the veins are accompanied by the arteries of the same name, and the lymphatic network drains through the regional lymph nodes (Figure 1) (1, 2). The colon is innervated by the vagus and pelvic nerves. The function of the right

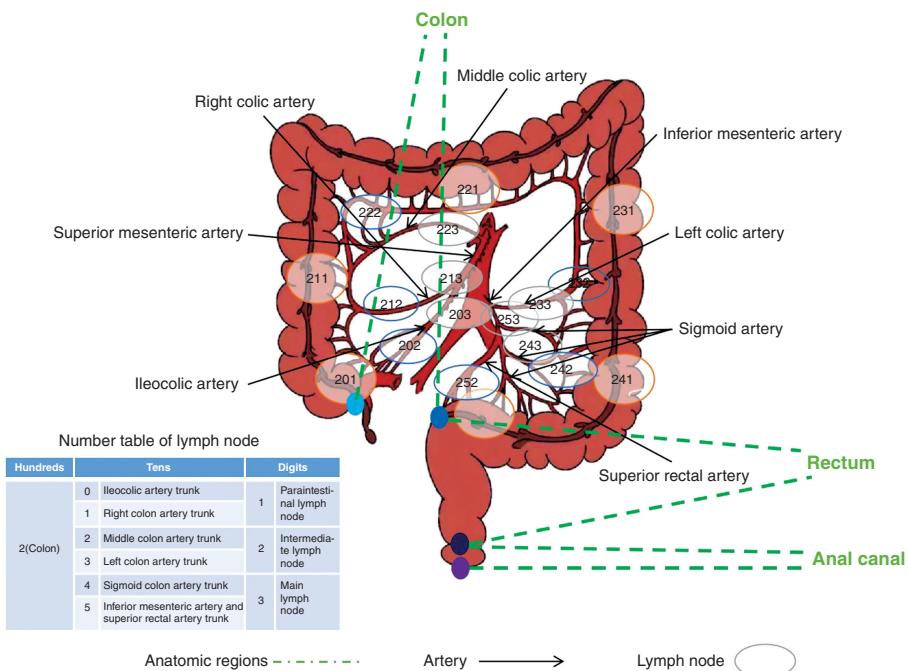


Figure 1. Anatomy, blood supply, and lymph node drainage of the large intestine. For anatomic regions, the three major parts (colon, rectum, and anal canal) relevant to colorectal cancer are labelled. For blood supply, only arterial supply is highlighted. The oval shape represents region(s) of lymph node groups or nodal stations. Usually, the lymph node stations of the large intestine are indicated with a three-digit number in the 200s. The simplest explanation for understanding the numbers, from left to right, is as follows: the first digit, 2, represents the anatomical group (in this case, the large intestine – hundreds in the table) the second middle digit represents the blood supply (in this case, the arteries—tens in the table) and the third digit represents lymph node group (oval shape—digits in the table). Concept for the figure is based on the Japanese Classification of Colorectal, Appendiceal, and Anal Carcinoma: the 3rd English Edition.

colon is mainly to absorb water and some nutrients, while the main function of the left colon is to store and excrete feces. Notably, the colon secretes gastrointestinal hormones and alkaline mucus substances.

The rectum joins the sigmoid colon, and the lower end joins the anal canal at the dentate line. The rectal blood supply mainly comes from the superior and inferior rectal arteries. Venous reflux mainly flows from the superior rectal vein to the liver. The regional lymph nodes of the rectum include pararectal lymph nodes, superior and inferior rectal artery lymph nodes, etc. The rectum is innervated by the autonomic nerves, its main physiological function is defecation. In addition, it also absorbs a small amount of water, salt, glucose, and some drugs (3).

Based on sites of onset, rectal cancer accounts for 49.66%, colon cancer accounts for 49.09%, and both sites combined account for 1.25% (4). Among colon cancers, the most common sites are the sigmoid colon (55%), followed by the ascending colon (23.3%), transverse colon (8.5%), descending colon (8.1%), cecum (8.0%), and crossing site (2.1%) (4). This chapter provides an overview of various aspects of colorectal cancer (CRC).

EPIDEMIOLOGY

CRC is the third most diagnosed cancers worldwide, of which males rank third and females rank second (5). CRC is the second most common cause of cancer-related deaths worldwide (5). In China, the incidence of CRC in the whole population ranks second among all tumors, and the age-standardized incidence is 23.7 per 100,000, among which male and female patients rank third, and the age-standardized incidence is 28.1 per 100,000 and 19.4 per 100,000 persons, respectively (6). Correlation between migration, religious factors, and the CRC suggests that the incidence of CRC is strongly related to environmental factors, lifestyle, and diet, but has no obvious relationship with ethnicity. The incidence of CRC differs by regions, with the highest incidence reported in North America, Western Europe, and Oceania, and the lowest incidence in Africa, Asia, and South America. In the mainland of China, the CRC incidence also follows an east-to-west gradient (6). The incidence of CRC is higher in the economically developed eastern coastal areas, while the incidence is relatively lower in the economically backward western areas (6). The incidence and mortality of second-generation immigrants from low-incidence areas of CRC to high-incidence areas are similar to those of residents (7).

The risk of CRC increases with age. The incidence and mortality of CRC are low until the age of 45 and increase significantly after that, peaking in the age group over 80, but a significant number of cases still occur in adolescents. CRC patients under the age of 30 account for 10–20% in China and the age of onset is 12–18 years earlier than in western countries (4, 8).

Notably, the morbidity and mortality of CRC have shifted over time. The change is different by regions. The rising rate of the original high-incidence regions slows down or decreases, while the low-incidence regions show an increasing trend, such as China. In the past 30 years, the incidence and mortality of CRC have been increasing year by year in China, especially in cities. This may be related to an aging population, changing lifestyles, and changing environments (3, 6).

In general, the epidemiological characteristics of CRC in China can be summarized as follows (3, 4): (i) higher in males than in females, and the male to female ratio is about 1.3:1; (ii) the median age of onset is 58 years; (iii) rectal cancer is more common than colon cancer; (iv) in economically developed regions, the most common site of colon cancer has changed from rectum to colon, and the proportion of right colon cancer has increased significantly.

ETIOLOGY

The etiology of CRC remains unclear, but it may be related to the following factors:

Genetic factors: About 20% of CRC cases are related to genetic factors, and investigations have shown a three-fold increased risk of cancer in the first-generation relatives of CRC patients. Familial Adenomatous Polyposis (FAP) has been identified as a genetic syndrome that predisposes to CRC, and the Mismatch Repair Gene (MMR) has also been linked to inherited CRC (9).

Dietary factors: It is currently believed that high fat, high animal protein, and low cellulose diet are related to the incidence of CRC. Excessive fat intake will promote bile secretion, bile acid decomposition, increased intestinal carcinogens, and the activity of intestinal anaerobic bacteria (10).

Non-cancerous diseases: Non-cancerous diseases such as colorectal polyps, colorectal adenomas, ulcerative colitis and Crohn's disease, etc. can contribute to CRC. Research shows that about 3–5% of ulcerative colitis patients will develop CRC, and the incidence of malignant transformation is greater than 10% in patients with ulcerative colitis lasting more than 20 years. About 15–40% of colon cancers originate from colonic polyps, with a precancerous course of 2–5 years. Adenomas less than 1 cm in diameter have a less than 2% chance of becoming cancerous, while those larger than 3 cm have a more than 40% chance of becoming cancerous (3, 11).

Other factors: Carcinogenic exposure and lifestyle, such as sedentary and overweight, are risk factors for CRC, and the incidence of sigmoid and rectal cancer is higher in patients undergoing pelvic radiation therapy (3).

PATHOGENESIS

CRC is a multi-factorial disease. The epithelial cells of colorectal mucosa can undergo hyperplasia, atypical hyperplasia (mild, moderate, severe) and adenomas, that can eventually develop into carcinoma (12). This process is usually initiated by carcinogenic factors, causing structural changes in DNA, leading to the malignant transformation of cells into cancer. Morphology includes epithelial hyperplasia, atypical hyperplasia, adenoma formation, carcinoma in situ, and invasive carcinoma (12). In 1990, Fearon and Vogelstein proposed a molecular event model for the occurrence and development of CRC (13). With the development of research, three molecular mechanisms related to the occurrence and

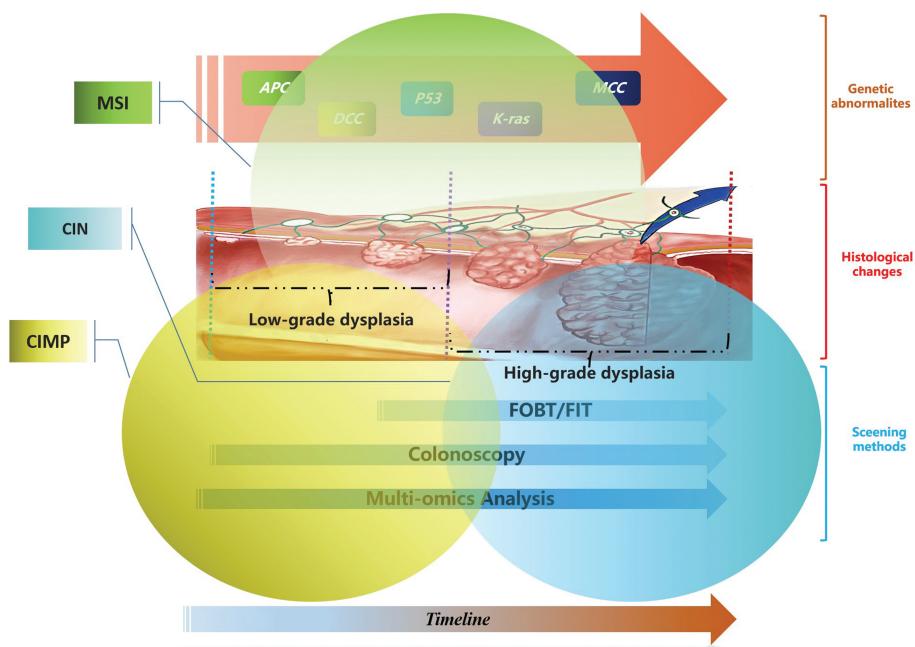


Figure 2. Genetic abnormalities, histological changes, and screening methods in the occurrence and development process of CRC. CIMP, CpG island methylator phenotype; CIN, chromosomal instability; FIT, fecal immunochemical test; FOBT, fecal occult blood test; MSI, microsatellite instability.

development of CRC have been confirmed: (i) chromosomal instability that mainly occurs in FAP (14); (ii) genetic mutations such as in Lynch syndrome and other sporadic MMR mutations (15); and (iii) hypermethylation of CPG islands in specific gene promoter regions (16). These processes are often associated with abnormal changes in multiple genes such as *APC*, *DCC*, *P53*, *K-ras*, *c-MYC*, *MCC*, and MMR-related genes (*hMLH1*, *hMLH3*, *hMSH2*, *hMSH3*, *hMSH6*, *hPMS1*, and *hPMS2*), Figure 2. It is worth noting that these abnormal molecular signaling pathways are not mutually exclusive, and multiple abnormal molecular pathways are co-existing in some CRC patients (17–19).

PATHOLOGICAL TYPE AND METASTASIS

In early stages, CRC is confined to the intestinal mucosa and submucosa (12). Lymphatic metastasis usually does not occur in early CRC (12). When the tumor breaks through the submucosa, lymphatic metastasis occurs in about 10% of patients. The gross types of CRC mainly include uplift, ulceration, and infiltration (12). The pathological histological types mainly include papillary adenocarcinoma, tubular adenocarcinoma, mucinous adenocarcinoma, signet-ring cell carcinoma, undifferentiated carcinoma, adenosquamous carcinoma, squamous cell carcinoma, and carcinoid carcinoma (12). Adenocarcinoma has the highest

incidence, accounting for more than 90% (colon cancer). Ji et al. analyzed the clinical data of 1092 primary CRC patients and found that adenocarcinoma accounted for 93.4%, mucinous adenocarcinoma and signet ring cell carcinoma accounted for 3.9% and 0.6% respectively, and carcinoid accounted for 0.2% (20). CRC spreads and metastasizes mainly through the following four pathways:

Local invasion: The tumor infiltrates into the local area of the primary lesion and adjacent structures.

Lymphatic metastasis: The neoplastic cells use the intramucosal lymphatic system to reach regional lymph nodes, eventually causing distant lymph node metastasis; about 60% of CRC metastasis occurs through this route (3).

Hematogenous metastasis: The cancer cells spread via the blood vessels. The most common target organs of CRC through hematogenous metastasis are liver and lung; about 30% of CRC are transferred through this route (3).

Implantation and metastasis: After the cancer cells fell off, they are implanted in the abdomen and pelvic peritoneum to form metastatic foci (3).

CLINICAL MANIFESTATIONS

Early CRC is often asymptomatic (19). With the progress of the disease, the following symptoms will generally occur.

Hematochezia: In small amount of hematochezia, general stool has no visible changes, but fecal occultation test can be positive; blood stool, mucus blood stool, or jam-like stool may appear when there is a lot of blood in the stool (3).

Intestinal obstruction: It is often a characteristic of advanced CRC; abdominal pain, abdominal distention, nausea, vomiting, exhaustion, and defecation will occur when intestinal obstruction caused by the enlargement of the mass (3).

Abdominal mass: It usually occurs in the right colon cancer; this symptom is a mass enlargement to a certain extent, palpable abdominal mass.

Systemic symptoms: CRC generally has no obvious symptoms at the early stage, so the course of the disease is relatively long, leading to tumor proliferation, cachexia, anemia, emaciation, and other symptoms.

Due to the different anatomical and physiological functions of colon and rectum, the clinical manifestations of tumors in different anatomical sites are also different. Generally, abdominal mass and systemic symptoms are more common in right colon cancer, blood stool and obstruction are more common in left colon cancer, and changes in defecation habits are more common in rectal cancer (3).

DIAGNOSIS

Most CRC patients have no obvious clinical symptoms at the early stage, resulting in a low early diagnosis rate. Many patients are diagnosed at an advanced stage,

losing the opportunity for radical treatment. In general, it is clinically recommended that those over 20 years of age with the following symptoms require further investigation: (i) recent persistent abdominal discomfort, such as abdominal pain, gas, etc.; (ii) changes in defecation habits, such as diarrhea, constipation, or both, and changes in stool shape; (iii) blood in stool or mucous blood stool; (v) unexplained anemia or progressive weight loss; and (vi) abdominal mass (21).

Further examination includes routine physical and other examinations. Digital rectal examination is recommended for patients suspected of rectal cancer. Other examinations include: (i) fecal occult blood test (FOBT)—positive results can be obtained with about 5 ml of bleeding in the digestive tract, so it has a certain clinical value for the screening and diagnosis of CRC (3); (ii) tumor markers—there are no specific tumor markers for CRC but currently, CEA (carcinoembryonic antigen) and CA19–9 (carbohydrate Antigen 19–9) are commonly used, and the sensitivity of combined detection of CEA and CA19–9 is higher than that of each separately, which has important clinical significance for the evaluation of therapeutic effect and monitoring of disease recurrence (3); (iii) endoscopy—colorectal endoscopy directly observes the position, size, and shape of the lesion, and the most important is feasible pathological tissue biopsy; (iv) X-ray—X-ray examination after barium enema can find signs of filling defect and mucosal destruction at the tumor site. Gas-barium double-contrast acts on the detection of colon cancer with small lesion, but it is not suitable for patients with intestinal obstruction; (v) ultrasound—ultrasonography has a certain effect on the detection of intestinal masses and abdominal lymph nodes; (vi) computed tomography (CT)—CT is of great diagnostic value for displaying the size of lesions, the relationship with adjacent tissues and organs, abdominal lymph nodes, and other conditions, which can assist in clinical staging; (vii) nuclear magnetic resonance (NMR)—similar to CT, but higher tissue resolution than CT examination, especially for pelvic lesions, such as rectal cancer, preoperative evaluation of great clinical value; and (viii) positron emission computed tomography (PET/CT)—it provides information on the anatomical site and metabolic characteristics of the tumor, and has great guidance for the diagnosis, preoperative staging, and recurrence assessment of CRC (3, 21, 22).

TREATMENT

The treatment principle of CRC is an individualized comprehensive treatment based on surgery, supplemented by chemotherapy, radiotherapy, molecular targeted therapy, immunotherapy, and other programs (21, 23). Studies have shown that the 5-year survival rate of patients with early CRC, who receive surgery-based treatment, is more than 90% (24). Therefore, surgery remains the cornerstone of CRC treatment. For initially inoperable CRC patients, and some CRC patients with metastasis, the feasibility of surgery should be evaluated after neoadjuvant therapy to strive for the opportunity of surgical treatment. The surgical methods mainly include radical surgery and palliative surgery (21).

CRC chemotherapy mainly includes neoadjuvant chemotherapy, adjuvant chemotherapy after radical surgery, and palliative chemotherapy (25). Neoadjuvant chemotherapy is often used in combination with radiotherapy, which can reduce the clinical stage of the tumor, strive for the opportunity of surgery, improve the

quality of life of patients, and reduce postoperative recurrence. Adjuvant chemotherapy can eliminate the remaining tumor cells after radical surgery and further consolidate the effect of radical surgery. The purpose of palliative chemotherapy is to improve the quality of life and prolong the survival time of patients with advanced CRC. Commonly used chemotherapy drugs include fluorouracil, irinotecan, oxaliplatin, and raltitrexed. The above chemotherapy drugs are often used in combination, and commonly used combination chemotherapy regimens include FOLFOX, FOLFIRI, CAPEOX, FOLFOXIRI, etc. (26).

The application of molecularly targeted drugs has brought significant benefits to CRC patients. Currently, molecularly targeted drugs used in the clinical treatment of CRC mainly include two types, one is anti-angiogenesis drugs represented by bevacizumab and the other is anti-epidermal growth factor drugs represented by cetuximab. Clinically, molecular-targeted drugs are recommended to be used in combination with chemotherapeutic drugs, because they are non-cytotoxic drugs and have relatively mild adverse reactions, which generally do not significantly increase the adverse reactions of chemotherapy (23).

Radiotherapy is mainly used for rectal cancer patients, which can improve local control rate, improve quality of life, and prolong survival. The radiotherapy schemes for rectal cancer mainly include preoperative radiotherapy, preoperative concurrent chemoradiotherapy, and postoperative concurrent chemoradiotherapy combined with adjuvant chemotherapy (27).

Recent studies have shown that immunotherapy can prolong survival in CRC patients (28). Other treatments include hyperthermia combined with chemotherapy or radiotherapy, biotherapy, traditional Chinese medicine, local cryotherapy, radiofrequency therapy, and palliative care (21).

PREVENTION AND PROGNOSIS

According to the pathogenic factors of CRC, the prevention methods of CRC include: (i) diet—intake of fresh vegetables, fruits, crude fiber food, appropriate minerals and trace elements such as calcium, magnesium, and vitamin D (25); (ii) lifestyle management—quitting smoking, limiting alcohol consumption, proper exercise, and weight control; (iii) active treatment of benign colorectal diseases, such as polyps, adenomas, ulcerative colitis, and Crohn's disease; (iv) regular screening—early screening to prevent CRC is very important (29).

In the literature, primary prevention has been reported to play a 35% role in reducing CRC mortality, secondary prevention through early screening for CRC has been reported to play a 53% role, and prescriptive treatment of patients diagnosed with CRC has been reported to play a 12% role (29–31).

The disease stage at initial diagnosis is the most important prognostic indicator of CRC. It has been reported that the 5-year survival rate of patients with localized CRC, who can be surgically resected, is about 90%, while the 5-year survival rate of advanced CRC patients who lose the opportunity of surgical treatment is reduced to about 10% (32). The natural course of progression from normal colorectal epithelial cells to benign lesions such as adenomas and eventually to cancer is generally 5–10 years (24). This process often involves abnormal changes of multiple genes, such as APC, DCC, P53, K-RAS, C-MYC, BRAF, MCC, and MMR-related genes. Early screening to detect limited-stage lesions in this time

window and clinical intervention is crucial to improve the prognosis of CRC patients (24, 33).

Currently, common screening and diagnosis methods for CRC include FOBT, fecal immunohistochemistry (FIT), tumor markers, and colonoscopy. FOBT is an economical and non-invasive screening method, but its sensitivity to the diagnosis of CRC is less than 50%. Although the sensitivity and specificity of FIT are higher than FOBT (78% and 96%, respectively) (34), and diet restriction is not required, the detection results of the FIT are susceptible to non-hemorrhagic tumors and hemorrhagic non-tumor diseases (35, 36). On the other hand, patients, and their family members' aversion to handling stool specimens restrict the development of this examination. Data from the UK study showed that only 50% of patients undergoing FOBT had a specimen sent in (37). Although more user-friendly DNA-based stool tests have been introduced in the UK, the delivery of fecal samples remains a major challenge. Blood samples can dynamically reflect the physiological and pathological state of the body in real-time, and it is easy to obtain. CRC markers in blood samples mainly include CEA and CA19–9, which are currently clinically used to evaluate the efficacy of anti-tumor therapy and monitor the recurrence of the disease with low sensitivity and specificity (40–70% and 73–90%, respectively) (37, 38). It has been reported that CEA fluctuates greatly in healthy individuals, and its variation in the same individual can reach 30%, leading to controversy over its value in screening asymptomatic people and diagnosing CRC (39). Therefore, CEA and CA19–9 are not suitable as screening and diagnostic markers for CRC. Colonoscopy can visually observe the shape, size, and location of colorectal lesions. Most importantly, it can obtain pathological biopsy specimens, which is an important means for CRC screening and diagnosis (32, 40). In the light of large proportion of CRC cases and that deaths could be prevented by screening and early detection and removal of colorectal adenomas or early stage CRC, colonoscopy screening could reduce mortality from colon cancer (41). However, colonoscopy is an invasive examination method that requires intestinal preparation, leading to poor patient compliance and potential examination-related risks, such as intestinal perforation (32). The accuracy of colonoscopy varies greatly due to technology-related factors (such as operator experience, bowel preparation, and examination duration) and disease-related factors (such as lesion size, number, and anatomical site) (19). Histology, genetic abnormalities, and screening methods in the occurrence and development of CRC are shown in Figure 2.

CONCLUSION

In conclusion, the incidence and mortality of CRC is high and increasing year by year. Although early diagnosis can significantly improve the prognosis, CRC patients often have no typical clinical manifestations or exhibit only non-specific signs in the early stage, and there are shortcomings in the currently used clinical screening and diagnosis methods, resulting in a low rate of early diagnosis of CRC. Therefore, it is of great value for the diagnosis and treatment of CRC to find CRC screening and diagnosis methods with safety, compliance, high sensitivity and specificity, and a good economic benefit ratio (22, 38).

Conflict of Interest: The authors declare no potential conflict of interest with respect to research, authorship and/or publication of this chapter.

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The Role of Diet and Lifestyle in Colorectal Cancer Incidence and Survival

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Abstract: Colorectal cancer is one of the most prevalent malignancies in the population, resulting in significant morbidity and mortality world-wide. Identifying modifiable risk factors to reduce the incidence and morbidity of colorectal cancer is beneficial on an individual and public health level. Protective lifestyle factors against colorectal cancer incidence includes high levels of physical activity, healthy diets rich in fruits and vegetables, fish, dietary fiber, dairy, and all essential vitamins and minerals. Risk factors for increased colorectal cancer incidence includes a diet high in red and processed meat, alcohol, and tobacco. The evidence regarding the influence of specific vitamins and minerals is still evolving, as well as the etiology behind their mechanism of action in colorectal pathogenesis. Ongoing epidemiological studies are underway to determine the effects of

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various lifestyle factors on colorectal cancer survival. Overall, lifestyle modification is essential for prevention and treatment of colorectal cancer for improved patient outcomes.

Keywords: colorectal cancer incidence; diet and colorectal cancer; lifestyle and colorectal cancer; red meat and colorectal cancer; survival in colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer world-wide and the second-leading cause of overall cancer deaths (1). These cancers develop most commonly from polyps within the colon or rectum that undergo a dysplastic process to progress to adenocarcinomas through the adenoma-carcinoma sequence (2). Although there are many genetic syndromes associated with familial CRC, the vast majority of CRC, approximately 70%, occur sporadically. The development of these non-hereditary tumors is influenced by a variety of environmental factors. As CRC is a common and morbid disease, there has been extensive epidemiological investigation to identify the modifiable risk factors associated with both the incidence of CRC and factors influencing cancer survival. Through a comprehensive review of current literature, this chapter outlines how physical activity, diet, vitamin and minerals, and other lifestyle factors influence the incidence and survival of CRC.

PHYSICAL ACTIVITY

The benefit of daily physical activity of moderate intensity for 30 minutes or more is well established in improvement of overall health. There have been many observational studies that have demonstrated the protective effects of high levels of daily physical activity in the prevention of CRC. There is strong evidence to support an approximate 20% relative risk reduction of developing CRC with high levels of recreational physical activity, in a dose-response relationship (3, 4). A 42% improvement in both all-cause and colon-cancer specific mortality in CRC patients with high levels of physical activity after cancer diagnosis has also been demonstrated (3).

On the other side of the same coin, there has been increasing evidence to suggest that individuals who live a sedentary lifestyle, meaning spending most of the day sitting without whole body movement, have an increased risk of CRC (5). With the turn of the Computer Age, an increasing proportion of Western populations have shifted into occupations requiring less physical labor, which has caused a societal shift to a more sedentary lifestyle, like sitting at an office computer. This is a concern as approximately 80% of a person's day may be spent stationary even with fulfilling the recommended amount of daily physical activity. Pooled epidemiological data has shown a 44% increased risk of colon cancer with high rates of occupational sitting time (4). Similar trends are seen in increased hours of recreational sitting time, like watching television. Overall, higher energy in both work

and leisure time is associated with decreased risk of CRC, in a dose-response relationship.

The etiology behind the inverse relationship of physical activity and CRC is not yet clearly understood. Currently, there is a paucity of clinical trials examining this relationship. However, it is likely that the pathophysiology behind the association with increased physical activity and decreased CRC risk and mortality is multifactorial. It is well established that the increased body composition of adipose tissue found in obesity increases overall cancer risk and mortality through increased levels of sex hormones, chronic inflammation, and insulin that promote carcinogenesis (5). Thus, as increased levels of physical activity decrease the rates of obesity, it may also decrease the risk of CRC. Additionally, sedentary lifestyles even in the absence of obesity have been correlated with increased glucose levels and insulin resistance, ultimately leading to high blood circulating levels of insulin (4). Hyperinsulinemia is an independent risk factor for CRC as insulin behaves as a growth factor for cells within the colonic mucosa and has been demonstrated to stimulate colon cancer cells, including stimulating proliferation of metastatic disease (6, 7). Finally, physical activity increases colonic transit time by stimulating the vagal innervation that promotes peristalsis of the intestinal system, which reduces the contact time between food and potential carcinogens with the colonic mucosa, potentially providing an additional protective effect against carcinogenesis (8).

DIET

Healthy dietary habits are essential for maintenance of overall health. Increasing epidemiological research has been focused on how diet affects a multitude of diseases, especially in malignancy. The colon and rectum are particularly susceptible to influence from the diet as it acts as one of the body's first barriers for dietary consumption. Red and processed meat has been faulted as the highest risk foods for increased CRC risk (9). Conversely, there are many proposed protective dietary factors against CRC including fruits and vegetables, fiber, and dairy products, as well as some specific diets and various vitamin supplements. We explore these positive and negative associations in the following section.

Red and processed meat

The typical Western diet consists substantially of processed foods and red meat. Red meat, including beef, lamb, pork, mutton, horse, or goat, is classified as meat from mammalian muscle tissue containing a high concentration of myoglobin (10). When this type of meat undergoes oxidization, it produces oxymyoglobin which appears red, from which the name is derived. When meat products are prepared through salting, curing, fermenting, or smoking before being consumed is it defined as a processed meat (11). Although red meat has significant nutritional benefits, especially regarding its high vitamin and iron content, red and processed meats have been deemed carcinogenic to the human body by the International Agency for Research on Cancer (12). As the gastrointestinal tract mucosa interacts first and foremost with these meats, there has been an abundance of observational data

linking red and processed meats to CRC. The daily meat intake of the average consumer of a Westernized diet is estimated to be around 220g/day (13). It has been demonstrated in systematic reviews of primarily observational studies (14, 15) that diets high in red and processed meats are associated with an increased risk of developing CRC. Increased CRC risk as high as 10–16% has been proposed for each 100g/day increase of dietary red meat and 16–22% for each 50g/day increase in processed meats (14, 15). These trends are supported by experimental studies in animal models (16). However, it is worth noting there is a paucity of experimental studies in clinical research to date and the evidence currently from observational data is highly subjected to other confounding dietary factors. Similarly, in the current data, some results lose significance when evaluating colon and rectal cancers individually (14). Thus, more experimental research is encouraged in this area to truly determine the absolute CRC risk with high red and processed meat intake, but the overall increased cancer risk is undeniable. There has not been any evidence indicating improved mortality with decreased servings of red or processed meat after CRC diagnosis.

White meat, like lean pork and poultry, are not associated with increased CRC risk (17). Therefore, it has been proposed that the heme-containing proteins in the myoglobin rich red meats may promote malignant transformation of epithelial cells in the colonic mucosa through lipid peroxidation, free radical formation, cytotoxicity, and cell hyperplasia (15). Similarly, the process of curing and smoking meat results in the production of carcinogenic compounds like N-nitroso-compounds and polycyclic aromatic hydrocarbons, and cooking meat at high temperatures produce heterocyclic aromatic amines and polycyclic aromatic hydrocarbons (11). These compounds have been linked with increased risk of developing precursor adenomatous colonic polyps that may undergo malignant transformation to develop CRC (18).

Dietary fiber

Diets high in dietary fiber, including whole grains, have been shown to have many health benefits. Whole grains are associated with protective effects against CRC, with 90g/day correlating to a 12–17% decreased risk (9, 19, 20). With each 10g/day of total dietary fiber, a 10–17% CRC risk reduction is observed. This relationship is likely due to dietary fiber decreasing colonic transit time, resulting in decreased exposure to dietary carcinogens, such as processed meats as discussed above. However, it is prudent to note that the evidence for this relationship between dietary fiber and CRC risk is weak.

Pescatarian and other specific diets

Dietary choices are highly influenced by social factors including culture, geography, ethics, and socioeconomic status. A classic comparison in nutrition literature is the Western vs Mediterranean Diets. The Westernized diet refers to a shift towards processed and high fat- and sugar-containing foods, lacking in fresh fruit and vegetables (21). As discussed previously, this type of diet is prone to increased adipose tissue leading to obesity and insulin resistance, which ultimately has been associated with increased CRC risk and worsened CRC survival (5, 6). Conversely, the Mediterranean diet, based on the traditional diet in European countries off the

Mediterranean Sea, consists of whole grains, a variety of fruits and vegetables, low-fat or fermented dairy products, olive oil, and protein sources from fish, white meat, and eggs (21). Other cultural-based diets, including the Healthy Nordic and Traditional Asian diets, or diets designed to improve medical comorbidities like the Dietary Approaches to Stop Hypertension or “DASH” diet, reflect similar composition to the Mediterranean diet. Recent evidence suggests that adherence to the Mediterranean diet is associated with a reduced risk of CRC by 8–17% (22, 23), corresponding to a linear dose-response association of a 4% CRC risk reduction per increasing adherence to the Mediterranean diet (23). No significant associations can be made on CRC-mortality based on current evidence.

Pescatarian diets refer to dietary choices including fish, dairy, grains, fruits, and vegetables, which are similar in composition to the aforementioned diets. Pescatarian diets have been associated with a CRC risk reduction as high as 33% (9). This may be due to the decreased risk in CRC associated with high intake of vegetables and fish. For each increase of 100g/day of vegetable consumption, a CRC risk reduction of 3–10% has been observed (19, 24). Similar results have been seen in the high intake of legumes of 50g/day (9, 24), but the data is conflicted (19, 20). Additionally, high soy intake, which is often a common protein source in vegetarian diets, has some limited data to support a 8–15% reduced risk of CRC incidence (24). On the hand, there is some evidence that high dietary intake of fish of 100g/day has been linked to a 11% decreased risk of CRC development (20), which is supported by some other meta-analysis, but the evidence remains split (24) so only weak recommendations can be made at this time.

Dairy products

Dairy products, including milk, yogurt, butter, and cheese, are a key component for a well-balanced diet as they provide essential nutrients including protein, calcium, and phosphorus. Higher rates of total dairy intake have been linked to decreased incidence of CRC (9). People with high daily intakes of 400g of dairy products per day had an 8–13% reduced risk of CRC, and daily milk intake of 200g/day had a 6–10% reduced risk (20, 25). These inverse relationships have been demonstrated in a dose-response manner and are also demonstrated for cheese (50g/day increments) and fermented milk (200g/day increments) each with a 7% reduced risk of CRC (25, 26). Similar trends have been seen for yogurt consumption (9, 25). This reduced incidence is likely related further data demonstrating a 12% reduced risk of precursor adenomatous and serrated colonic polyps associated with total dairy intake in a dose-response association (27, 28).

There is less overall evidence evaluating dietary dairy intake and CRC-related mortality, but a recent meta-analysis found a 29% reduced CRC-mortality with high total dairy consumption, particularly in the Western population (26). As dairy products act as the primary dietary intake of calcium in most populations, it is purposed that the improved survival is related to calcium intake. This relationship will be further explored in the next section.

Calcium and Vitamin D

Dietary calcium, as described above, is primarily attained through dairy products or alternatively plant-based dairy alternatives. Vitamin D intake is unique as the

metabolism is dependent on both dietary intake and synthesis from skin sun exposure. Intestinal uptake of dietary calcium is aided by vitamin D through the vitamin D receptor in the intestinal cell membranes in the distal small bowel and colon (29). Thus, discussion of the influence of dietary calcium on CRC cannot be completed without the inclusion of vitamin D.

There is strong evidence to support that high dietary calcium intake, deemed at 300–400mg/day, has a significant protective effect in CRC by decreasing risk by 5–6% in a linear dose-response relationship (30, 31). Similarly, vitamin D demonstrates a linear dose-response decrease in CRC risk with each dietary supplementation of 100 units/day by 4%. High levels of the circulating form of dietary or supplemental vitamin D, 25(OH)D, is shown to reduce the risk of colorectal adenoma formation by 20%, with each increase of 200 units/day corresponding to a 10% reduced risk of adenomas (30). This protective effect is amplified when combined with high calcium intake. When both are combined there is evidence of decreasing the risk of adenomatous polyps by as much as 37% and 32% for CRC (30). There has been some additional data showing elemental calcium supplementation of 1200–2000mg/day may decrease the risk of recurrent adenomatous polyps by 23%, with a treatment benefit for each 1 in 20 patients supplemented (32). Furthermore, there is some evidence suggesting that in CRC patients, high levels of circulating 25(OH)D to be associated with improved clinical outcomes and survival, including an improved CRC-specific survival of 35% (30, 33). However, data in this area is still limited.

It has been proposed that calcium and vitamin D work synergistically to protect the colonic mucosa by sequestering fatty acids and bile salts from colonic epithelial cells, compounds that have been indicated to promote carcinogenesis in CRC (34). This in turn may decrease proliferation of the epithelium, reducing colonic toxicity, which may reduce the formation of adenomatous polyps. Additionally, there is evidence to support that calcium may act on mediators along the adenoma-carcinoma sequence during the transformation of pre-cancerous polyps to CRC. Approximately 70% of non-hereditary CRC development is secondary to chromosomal instability that takes place in precursor polyps through mutations in tumor suppressor genes, including the adenomatous polyposis coli gene and KRAS (2). Both calcium and vitamin D have been shown to decrease mutations in the genes mediating this pathway, decreasing the risk of malignant transformation (26, 30). Independently, vitamin D deficiency has been indicated to promote cell proliferation, invasion, metastasis, and angiogenesis, which may reflect the decreased rates of CRC mortality during vitamin D supplementation described above (33). Further mechanisms underlying this relationship is an area of active experimental and epidemiological research.

Folate

Folate, or the supplemental form folic acid, is a B vitamin found in leafy green vegetables and is essential for the prevention of birth defects and can cause significant neurological symptoms in deficient states, more commonly in patients with heavy alcohol use. The current evidence linking folate and CRC risk is conflicting. There is some new data showing a 12% decreased risk of CRC with folic acid supplementation in moderate-heavy alcohol drinkers, but not in the general population (35).

Although there has been some evidence linking a decreased risk of CRC and folate in the general population (9, 24), there remains significant conflicting results (36) so that no association can be made with this current level of evidence.

Vitamin B6

Vitamin B6, or pyridoxine, is a water-soluble vitamin that, when in its active form of pyridoxal 5'-phosphate, acts as a co-enzyme in innumerable metabolic enzymatic reactions (37). There are a few studies to support an inverse relationship between vitamin B6 and CRC risk (9, 24, 37). High intake of vitamin B6 was associated with a 12% decreased risk of CRC (9, 24). There was a dose-response relationship with CRC and the active form of vitamin B6, where CRC risk decreased by 49% for every 100-pmol/mL increase in blood pyridoxal 5'-phosphate level (37). The pathogenesis behind this association may be related to the role of vitamin B6 in the 1-carbon metabolic pathway essential for DNA synthesis and methylation, which in CRC may inhibit carcinogenesis by reducing epithelial proliferation through this pathway (37).

Magnesium

Magnesium, an abundant mineral that is important for many bodily functions, has been implicated to have a protective effect against CRC risk, with a 7% decreased risk for each 50mg supplemented per day (24). Similar results were seen elsewhere (9), with the greatest benefits being observed with supplementation of 200–270mg/day (24, 38). The purposed etiology of this benefit is that magnesium has been shown to be active within cells of the colonic mucosa and may decrease epithelial proliferation through decreased oxidative stress, apoptosis, and inhibition of angiogenesis (38).

Garlic

Garlic is a plant a part of the onion family and typically used to flavor food. Incidentally it also has numerous purposed health benefits, including reduced risk of CRC. The World Cancer Research Fund/American Institute for Cancer Research has stated that garlic may be associated with reduced incidence of CRC with a demonstrated reduced risk of CRC as high as 34–44% for diets high in garlic intake (20). Similar results have been seen elsewhere (39), but conflicting results (24) prevent any strong recommendations. The underlying etiology remains unclear but is purposed to be related to its anti-inflammatory effects on the colonic mucosa which may inhibit proliferation and angiogenesis (39).

OTHER LIFESTYLE FACTORS

Many other lifestyle factors can influence the development and course of CRC. These other lifestyle factors are modifiable and should be identified early in patients who are otherwise high-risk for CRC, to potentially decrease the overall

risk of CRC incidence. Upon CRC diagnosis, these professionals within the health-care team should reassess for the presence of these factors to aid in cancer survival.

Coffee and other caffeine sources

Dietary coffee, tea, and other caffeinated beverages are highly prevalent in most diets. The evidence is unclear whether regular intake of these beverages affects CRC risk. Recent meta-analyses were unable to show an association between coffee consumption and CRC risk (20, 40), although there is some conflicting evidence in this area an older meta-analysis reported a dose-response relationships of 3–6% reduced CRC risk per 1 cup of coffee per day (41). There is similarly conflicting evidence regarding tea consumption and CRC risk (24). Overall, associations can be made definitively at this time due to the weak evidence.

Tobacco

The overall poor health effects from tobacco and cigarette smoking are well established and undebatable. Tobacco use is an established risk factor for CRC incidence and prognosis. Both previous and current cigarette smoking is associated with an increased risk of CRC at 17–25% (42, 43). This increased incidence is likely secondary to an increased risk of both adenomatous and serrated polyps with cigarette smoking (43). The carcinogens contained in cigarette smoke, including polycyclic aromatic hydrocarbons and heterocyclic amines among others, cause DNA mutations of mediators in the adeno-carcinoma sequence through carcinogen-metabolizing enzymes (44). Compared to never smokers, there was also an increased risk of mortality with CRC in former and current smokers at 15% and 40%, respectively. An increase in cigarette consumption of 1 pack (or 20 cigarettes) per day increased the risk and mortality of CRC to 17% and 40%, respectively, in a dose-response relationship (42). Therefore, smoking cessation should be heavily encouraged for all patients, but particularly high-risk for CRC and current CRC patients.

Alcohol

Increased alcohol consumption has been indicated in the increased risk of several different malignancies, including CRC. For each increase in total alcoholic drinks per day, defined as 10g/day, CRC risk increases by 6% (20). The risk with moderate (2–3 alcoholic drinks/day) and heavy (more than 4 alcoholic drinks/day) was associated with an increased risk of 21–52% (24, 45). There is sufficient evidence to say that heavy alcohol intake is a strong modifiable risk factor for CRC development and worsens both CRC and overall health outcomes (9). The current evidence demonstrating the effects of alcohol use on CRC mortality is controversial, but the consensus is to recommend the avoidance of moderate-heavy alcohol consumption in the context of cancer diagnosis as it may impair the efficacy of cancer treatment (46).

A summary of the current evidence linking these lifestyle modifications and CRC risk and morality is provided below in Table 1.

TABLE 1**Summary of associations between lifestyle exposures on CRC incidence and mortality**

| Exposure | Overall Effect | CRC Incidence | CRC Mortality | Level of Evidence |
|-------------------------|----------------|---|--|-------------------|
| Daily physical activity | Protective | 20% relative risk reduction (3, 4) | 42% improved all-cause and cancer-specific mortality (3) | Moderate |
| Sedentary Lifestyle | Harmful | 44% increased risk of CRC (4) | NED | Moderate |
| Red and processed meat | Harmful | 16% increased risk per 100g red meat/day (12) 22% increased risk per 50g processed meat/day (12) | NED | Strong |
| Dietary fiber | Protective | 10–17% decreased risk per 10g/day (9, 16, 17) | NED | Weak |
| Mediterranean Diet | Protective | 8–17% decreased risk (19, 20) | NED | Weak |
| Pescatarian Diet | Protective | 33% decreased risk (9) | NED | Weak |
| Dairy | Protective | 8–13% decreased risk per 400g/day | 29% reduced CRC-mortality (23) | Moderate |
| Calcium | Protective | 6% decreased risk per 300–400mg/day (27, 28) | NED | Moderate/Strong |
| Vitamin D | Protective | 4% decreased risk per 100units/day | 35% improved cancer-specific survival (27, 30) | Moderate |
| Folate | Protective* | 12% decreased risk in heavy alcohol users (32) | NED | Weak |
| Vitamin B6 | Protective | 12% decreased risk (9, 21) | NED | Weak |
| Magnesium | Protective | 7% decreased risk per 50mg/day | NED | Weak |
| Garlic | Protective | 34–44% decreased risk (17, 36) | NED | Weak |
| Coffee | U | NED | NED | Weak |
| Tobacco | Harmful | 17–25% increased risk (39, 40) | 40% increased mortality per pack of cigarettes/day (39) | Strong |
| Alcohol | Harmful | 6% increased risk with each 10g/day (or one alcoholic drink) (17, 21, 42) | Unclear association. Current literature is mixed. | Strong |

NED, Not Enough Data

*Controversy of overall benefit unclear in general population based on current evidence. Harmful exposures, including sedentary lifestyles, red/processed meats, tobacco, and alcohol, are well established in the literature, and accepted into clinical practice. On the other hand, the protective factors listed above still require further evaluation to produce stronger evidence to influence current medical recommendations.

CONCLUSION

Physical activity, diet, smoking, and alcohol intake are all modifiable lifestyle factors that can influence both CRC incidence and survival. The prudent healthcare professional should evaluate patients at high-risk of CRC from genetics or medical comorbidities and provide counselling to patients on these lifestyle behaviors. Ongoing epidemiological studies are required to further elucidate the relationship of some dietary and supplement factors on the protective benefit on CRC risk and mortality. Many of these supplements do not currently have strong enough evidence to be implemented into standard medical practice. Overall, the cornerstone of lifestyle modifications for CRC involves promoting an active lifestyle with a well-balanced diet and moderating or eliminating the intake of processed meats and alcohol.

Conflict of Interest: The authors declare no potential conflict of interest with respect to research, authorship and/or publication of this chapter.

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Epithelial-Mesenchymal Transition in Metastatic Colorectal Cancer

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Abstract: Epithelial-mesenchymal transition (EMT) is a morphogenetic event during which cells lose their epithelial characteristics, such as apicobasal polarity, and gain mesenchymal features with an increased migratory and invasive potential. A wide range of studies have shown that this event plays a crucial role in tumor progression and metastasis. The results of the studies also demonstrate participation of EMT in therapy resistance and in the development and maintenance of stemness potential in colorectal cancer. In addition, evidence from preclinical and early clinical studies have shown that EMT markers might serve as outcome predictors and potential therapeutic targets in colorectal cancer. In this chapter, we discuss the fundamentals of EMT, including cell-cell adhesion disruption and cell polarity loss, actin cytoskeleton reorganization, transcription factors, and post-translational modifications associated with EMT. We also discuss EMT-mediated mechanisms of resistance to radiotherapy and chemotherapy. Finally, we provide a summary of EMT components and their use

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as potential markers or therapeutic targets for metastasis inhibition, along with the obstacles in the development of drugs targeting EMT.

Keywords: actin cytoskeleton reorganization; cell polarity loss; cell-cell adhesion disruption; epithelial-mesenchymal transition; metastatic colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer (10%) and second most common cause of cancer-related deaths (9.4%) worldwide (1). Despite various advances in the screening, early detection, and management of established diseases, there is still a lack of innovative therapeutic treatments. Therefore, a herculean effort must be made to improve our understanding of this heterogeneous disease and develop an effective treatment to overcome this cancer by preventing metastasis to distant organs. Epithelial-mesenchymal transition (EMT), first described in embryogenesis, is a complex cellular process, in which cells transit between epithelial and mesenchymal phenotypes. Cancer is mainly characterized by the loss of apicobasal polarity and actin cytoskeleton disorganization. Consequently, epithelial features are lost in favor of mesenchymal features, increasing motility and invasiveness (2). Cells in this transition may also undergo the reverse process, Mesenchymal-Epithelial Transition (MET), which allows them to seed and form a secondary tumor (3).

Multiple signaling pathways, including TGF- β , Wnt, Notch, and FGF, can induce changes in epithelial cells by activating EMT-promoting transcription factors (EMT-TFs). EMT-TFs from the Snail, Twist, and ZEB families are responsible for repressing epithelial genes and activating mesenchymal genes (4). The role of EMT in the metastatic cascade has been controversial. Although studies indicate that it is involved in basal membrane rupture, intravasation, resistance to shear stress in blood vessels, and extravasation, some studies have shown that EMT is not essential for metastatic colonization (5). A probable explanation is the multiplicity of possible outcomes for cells undergoing EMT. There are several intermediate stages in this process that contribute to the formation of subpopulations that differ in proliferation, invasion, plasticity, and metastatic capabilities. The plasticity allows cells to undergo reversible changes between epithelial and mesenchymal features, adapting to diverse hostile conditions (6). In this context, studies have attributed that cells in EMT display an increased resistance to different therapeutic treatments, with consequent disease relapse leading to patient death (7). The properties, mechanisms and proteins that regulate cellular events involved in the EMT program could become promising markers and/or therapeutic targets for cancer therapy to prevent metastasis. The implications of use as markers will be addressed in this chapter.

EMT-ASSOCIATED CELLULAR ALTERATIONS

EMT involves a range of alterations in cell morphology, gene expression, and physiology. However, evidence has shown the existence of cell programming stages

between the two poles of EMT (complete epithelial or complete mesenchymal), in which there is incomplete suppression of pre-existing epithelial characteristics and incomplete acquisition of mesenchymal features. This gave rise to the concept of a hybrid intermediate stage known as partial EMT, which has been observed within tumors in a broad range of cancers, including CRC (6). Upon activation of EMT, tumor cells undergo a series of physical changes, including tight junction dissolution, disruption of apicobasal polarity, and reorganization of the cytoskeletal architecture. These events facilitate the dissemination of cells from their primary site, invasion of surrounding tissues, survival in the general circulation, and ultimately lead to the formation of metastases. Morphologically, this switch leads to a striking loss of the typical polygonal, cobblestone appearance of epithelial cells and the emergence of spindle-shaped fibrous cells that express mesenchymal cell markers (8). Here, we present evidence related to two of these cellular EMT-related events: cell-cell adhesion disruption and cell polarity loss, as well as actin cytoskeleton reorganization (Figure 1).

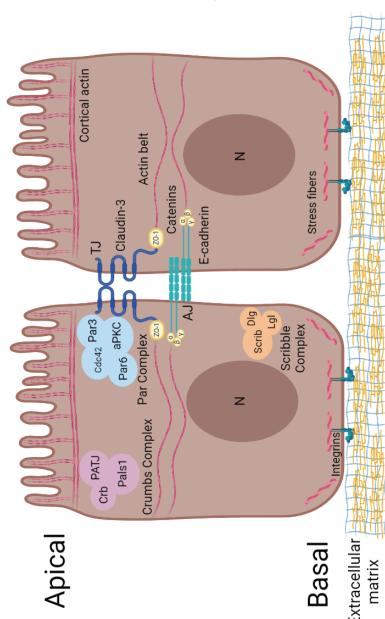
Cell-cell adhesion disruption and cell polarity loss

Colonic epithelial cells are cohesive sheets of polarized cells maintained by specialized intercellular junctions constituted by the apical junctional complex (AJC). AJC is connected to the actin cytoskeleton and maintains the dynamic properties of this complex, tissue architecture, and cell homeostasis (9).

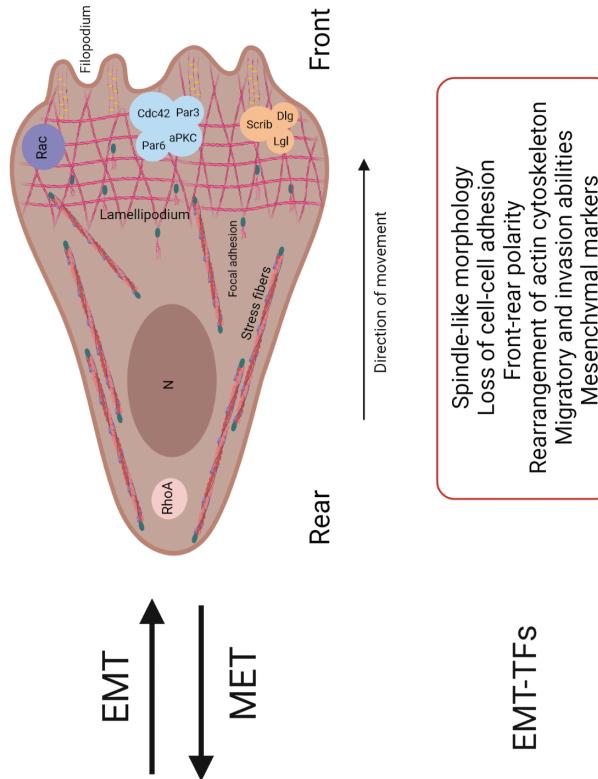
The establishment of apical-basal polarization is intimately linked to AJC, which is formed by adherens junctions (AJs) and tight junctions (TJs). TJs are composed of transmembrane proteins such as claudins, occludins, and junctional adhesion molecules (JAMs), whereas their cytoplasmic components are zonula occludens (ZOs) proteins. Basal to TJs, AJs bind cytoskeletal actin to the plasma membrane, creating an adhesive attachment between epithelial cells or the extracellular matrix and these cells. AJs consist of two basic adhesive units: the transmembrane component E-cadherins/nectins and the cytoplasmic component β -catenin/afadin complexes. Epithelial cells display an asymmetrical distribution of molecules, organelles, and structures, which is defined as cell polarity. Cell polarity is a fundamental process involved in many biological processes that contribute to normal tissue integrity and development. To allow correct sheet alignment, epithelial tissues are organized according to two polarity axes: apicobasal cell polarity (ABP) and planar cell polarity (PCP). The ABP orients cells from the free surface or the lumen to the basal lamina contributing to the acquisition of a cell shape that characterizes epithelial functions. The epithelial cell polarity is established and maintained through the concerted actions of three conserved polarity complexes: Par (Par3, Par6, aPKC and Cdc42), Crumbs (Crb, PATJ and Pals1), and Scribble (Scrib, Lgl and Dlg). ABP polarity is involved in diverse cellular pathways that control cell proliferation, apoptosis, and invasion (10, 11). Most proteins involved in the core ABP machinery exhibit alterations during epithelial transformation and are therefore implicated in human cancers (12).

Loss of cell polarity is one of the hallmarks of epithelial human cancers, and leads to tissue disorganization, increased proliferation, and metastasis (13). Indeed, loss of the polarity protein Pals1 in CRC cells enhances cell migration and invasion *in vitro* and increases the metastasis of transplanted tumor cells in mice (14). Additionally, loss of epithelial markers, including E-cadherin and

Epithelial



Mesenchymal



Polygonal morphology
Stable cell junctions
Cell-cell adhesion
Apico-basal polarity
Epithelial markers

EMT-TFs

Spindle-like morphology
Loss of cell-cell adhesion
Front-rear polarity
Rearrangement of actin cytoskeleton
Migratory and invasion abilities
Mesenchymal markers

Figure 1. Schematic of overall EMT process. During EMT, epithelial cells change morphology to spindle-like shape accompanied by loss of cell polarity and cell-cell contacts, leading to rearrangement of actin cytoskeleton and acquisition of migratory and invasive abilities. Also, as a reversible process, MET could generate epithelial cells. AJ, adherens junctions; EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition; TJ, tight junctions. The image was produced using BioRender.com.

claudin-1, -3, and -7 proteins, is frequently observed during EMT (15). Low levels of claudin-1 in CRC promote EMT through NF- κ B activation and correlate with aggressive tumor behavior (16). Moreover, downregulation of claudin-3 induces IL6/gp130/Stat3 signaling by hyperactivation of Wnt/ β -catenin in CRC cells (17). In addition, loss of claudin-7 expression promotes EMT, invasion, and metastasis in CRC (18). It is almost certain that the downregulation of E-cadherin and the increased expression of vimentin are classic markers of EMT, which increases cell motility and facilitates invasiveness and metastasis. However, it has been showed that loss of E-cadherin is not an essential step for tumor invasion and different studies have reported that epithelial cells can collectively invade and colonize distant tissues and organs (19). Furthermore, E-cadherin is required for epithelial dissemination, and many metastases continue to express this protein (20). In fact, analysis of circulating tumor cells (CTCs) isolated from cancer patients showed simultaneous expression of epithelial and mesenchymal markers, indicating that cell dissemination appears to be a multi-stage process (21). Recently, it was shown that E-cadherin is internalized by annexin A2 protein, which contributes to the development of EMT in CRC cells (22). All the alterations described above, such as loss of cell polarity and cell-cell adhesion disruptions, are simultaneously connected to actin cytoskeleton rearrangement in the early steps of EMT.

Actin cytoskeleton reorganization

Cells undergoing EMT acquire increased migration and invasion potential, which is accompanied by actin cytoskeletal rearrangements. Actin organization is a dynamic process regulated by GTPases of the Rho family, such as Rho, Rac, and Cdc42, which are responsible for the formation of stress fibers, lamellipodia, and filopodia, respectively (23). The actin cytoskeleton also regulates many cellular events including cell migration, invasion, membrane trafficking, survival, proliferation, and polarity, which are crucial events in EMT. During growth factor-induced EMT, Rac1 activation regulates the formation, extension, and stabilization of lamellipodia at the front of cells. EMT-associated signaling pathways such as PAKs, NF- κ B, MAPKs, Wnt/ β -catenin/TCF, and STAT3 can also be triggered by Rac1 activation (24). The non-Smad TGF- β signaling pathway is involved in EMT, downregulating RhoA levels by ubiquitylation and modulating TJ and cell polarity assemblies. Additionally, at the leading edge of the cell, Cdc42 activates the Arp2/3 complex, contributing to the lamellipodium extension and directional sensing of migrating cells by regulating filopodial protrusion formation (25). Filopodia are finger-like actin-rich membrane protrusions composed of an actin binding protein called fascin-1 and cross-linked actin fibers. Fascin-1 is an actin turnover regulator and an important target in the EMT context, particularly associated with metastatic potential (26).

RhoA activates ROCK/LIMK/cofilin-1 signaling, which induces actin stress fiber formation, a contractile actin filament composed of α -actinin and myosin, impacting cell-cell adhesion disassembly and the turnover of focal adhesion, as well as the traction forces needed for cell motility (27). ROCK1 protein regulates the phosphorylation of myosin light chain (MLC), and its overexpression is associated with tumor progression and low five-year survival in CRC patients (28). LIMKs and SSH1 are proteins that regulate cofilin-1 by phosphorylation, modulate actin dynamics, and contribute to tumor aggressiveness

and metastasis of CRC cells. Recently, it was shown that during EMT, cofilin-1 promotes actin rearrangement, leading to cell-cell adhesion disruption and, consequently, cell migration and invasion are stimulated (15). Moreover, LIMK1 and SSH1 are upregulated in the mesenchymal consensus molecular subtype (CMS4) of CRC, and patients with high levels of LIMK1 exhibit low overall survival rates in canonical (CMS2) and metabolic (CMS3) subtypes (29). RhoA activation induces focal adhesion autophosphorylation and increases cell migration (30). In addition, the association of α -actinin 4 with increased motility and downregulation of junctional proteins supports its role in regulating the E-cadherin/ β -catenin complex disassembly of CRC cells in EMT (31). In contrast, cortactin is frequently used as an EMT marker because it regulates protease activity in actin-rich protrusions that are essential for matrix degradation and invasion of tumor cells (32). The role and potential clinical value of these actin regulator proteins during cell migration and invasion during EMT are still limited.

EMT GENETIC ALTERATIONS

The EMT program is activated by autocrine and paracrine signals from the tumor microenvironment, which include a variety of cytokines, interleukins, and growth factors that stimulate signaling pathways in tumor cells, converging in the activation of a group of transcription factors. In addition to EMT-TF regulation of genes associated with epithelial and mesenchymal states, EMT is also regulated by non-transcriptional mechanisms through microRNAs, alternative splicing, epigenetic modifications, and post-translational modifications, which affect protein stability and localization (33). Two topics will be assessed in this section: transcription factors (EMT-TFs) and post-translational modification involved in EMT.

Transcription factors driving the EMT

Three major groups of transcription factors regulate the EMT program: the SNAIL family of zinc-finger, the zinc finger E-box binding homeobox (ZEB) family, and the TWIST family of basic helix-loop-helix (bHLH). The expression of these three groups promotes the regulation of early events in the EMT program through the repression of the epithelial phenotype and activation of the mesenchymal phenotype. Other transcription factors have been shown to influence EMT, such as, family of prospero homeobox 1 (PROX1) and forkhead box (FOX) transcription factors (34). These factors often regulate the expression of each other and functionally cooperate to regulate EMT. Their activities are localized in the nucleus, where they have access to the DNA to coordinate a cascade of signaling, leading to disruption of cell adhesions, loss of cell polarity, and manifestation of a mesenchymal motile phenotype (33). Moreover, EMT-TFs are linked to the induction of many other events, particularly stemness, survival, and changes in cell metabolism (35). The contribution of EMT-TFs in activating the EMT program depends on the cell or tissue type involved and the induction of signaling pathways present in the tumor microenvironment.

The role of these transcription factors in EMT has been well established in various cancers, including CRC. Aberrant activation of Wnt/ β -catenin signaling is

common in patients, and nuclear accumulation of β -catenin results in the upregulation of EMT-TFs, mainly Snail, a core regulator of EMT that represses E-cadherin and promotes metastasis and invasion (36). The regulation of EMT-TFs is clinically relevant in metastasis because their expression is associated with an increased rate of cancer recurrence and decreased survival in CRC patients (37). Therefore, EMT-TFs are critical factors in regulating EMT, which lead to cancer metastasis. The Snail family consists of three transcription factors, Snail (also referred to as Snail1), Slug (Snail2), and the less characterized Smuc (Snail3), all of which repress epithelial genes by binding to E-box DNA sequences through their carboxy-terminal zinc-finger domain. Snail promotes EMT by repressing E-cadherin binding to the E-box sequence in the promoter region and recruits the polycomb repressive complex 2 (PRC2), which cooperates with reduced expression by epigenetic regulation (38). Furthermore, Snail increases the expression of genes related to the mesenchymal phenotype, such as fibronectin, vimentin, and N-cadherin, in addition to upregulating other EMT-TFs, including Slug, Zeb1, and Twist (39). Many signaling pathways, such as, TGF- β , PI3K/Akt and NOTCH, initiate the progression of EMT via the expression of higher levels of SNAIL (8). In CRC, Wnt/ β -catenin signaling and Snail cooperate to induce EMT by upregulating LEF1, leading to increased interaction with β -catenin to promote invasion (40).

Twist1 and Twist2 belong to a group of factors that share a basic helix (loop) protein structure. Twist1 represses E-cadherin and induces N-cadherin expression, probably through its association with methyltransferase SET8 (also known as SETD8), which mediates monomethylation of histone marks (41). The ZEB family consists of two members, Zeb1 and Zeb2, which bind to regulatory gene sequences in E-boxes. Structurally, Zeb proteins contain two zinc finger clusters localized at the N- and C-terminal that bind to E-boxes (42). Zeb1 and Zeb2 are two of the leading EMT regulators that directly bind to the E-box sequence in the E-cadherin promoter region or indirectly form a repressor complex with epigenetic proteins. Therefore, Zeb1 recruits histone deacetylase or methyltransferase to decrease E-cadherin expression and activate the EMT program (43). In addition, Zeb1 triggers the loss of basal-apical polarity by directly suppressing the activation of polarity factor genes as a consequence of increased metastasis (44). These interrelationships lead to numerous permutations that need to be resolved for a better understanding of EMT in colorectal cancer.

Post-translational modifications

Post-translational modifications (PTMs) drastically increase the diversity of protein structures and play a fundamental regulatory role in cell physiology. PTMs occur in amino acid side chains or peptide bonds and are usually mediated by enzymes. It is estimated that approximately 5% of the genome encodes enzymes that are responsible for PTMs (45). There are more than 400 types of PTMs, and the most common include phosphorylation, ubiquitylation, glycosylation, and acetylation (46). Transcription factors (TFs) that regulate EMT are regulated by phosphorylation (47). For instance, the main mechanism involved in the control of Snail stability is phosphorylation at Ser residues promoted by glycogen synthase kinase 3 beta (GSK-3 β), which induces its nuclear export and generates a binding site for E3 ubiquitin ligase, known as beta-transducin-repeat-containing protein

(β -TrCP). In contrast, SCP family phosphatases remove these phosphorylations, promoting Snail stabilization (48). Another protein that can phosphorylate Snail is protein kinase D1 (PKD1); in this case, after phosphorylation at Ser11, Snail is recognized by SCF-FBXO11 ubiquitin ligase, thus being targeted for proteasomal degradation (49). These data indicate that phosphorylation and ubiquitylation act in an interconnected manner to regulate EMT.

Glycosylation is the enzymatic addition of sugars to proteins and lipids. The two types of glycosylation that are most often found in proteins are *N*-glycosylation and *O*-glycosylation. *N*-linked glycans are attached to nitrogen atoms present in the side chain of asparagine (Asn) residues that constitute the Asn-X-Ser/Thr consensus sequence, where X can be any amino acid, except proline. In turn, *O*-linked glycans are attached to the oxygen atoms of the Ser or Thr residues. During EMT, the levels of several glycans are altered, which permit regulation of the mechanisms involved in this event. In TGF- β -induced EMT in mouse mammary gland cells, decreased levels of bisecting *N*-acetylglucosamine (GlcNAc) structures, a modification characterized by the attachment of GlcNAc to the core mannose of *N*-glycans in a β 1,4-linkage catalyzed by the MGAT3 (or GnT-III) enzyme, was observed (50). The overexpression of MGAT3 resulted in the inhibition of hypoxia-induced EMT in MCF7 breast cancer cells. While bisecting GlcNAc structures have been associated with reduced malignancy and EMT suppression, the β 1,6-GlcNAc branched *N*-glycans (structure formed by the transfer of a GlcNAc residue to the α 1,6-mannose via the β 1,6-linkage), which are catalyzed by MGAT5 (or GnT-V), have been associated with malignancy and EMT promotion (Figure 2) (51).

Key proteins that control EMT are also regulated by acetylation, with lysine (Lys) residues being frequently observed. In breast cancer cells, CREB-binding protein (CBP or CREBBP), a histone acetyltransferase (HAT), acetylates Slug at Lys211 and Lys166, thus contributing to the stabilization of this TF and consequently promoting EMT (52). CBP acetylates Snail at Lys146 and Lys187, which prevents Snail from interacting with a repressor complex that includes HDAC1, HDAC2, and Sin3A proteins, leading to an increase in pro-EMT gene expression (53).

These results of the studies detailed above show that the functionality of EMT master regulators is tightly regulated by PTMs. However, further research in this area is needed to understand this complex cellular process.

EMT AND THERAPY RESISTANCE

Complete loss of epithelial features concomitant with the gain of a fully mesenchymal phenotype during EMT is rare in human carcinomas. Normally, cells with high plasticity exhibiting both epithelial and mesenchymal features between different intermediate phenotypic states of EMT are observed. This plasticity promotes increased tumor heterogeneity and interaction with tumor-associated stromal cells, which exhibit pro-tumorigenic properties, and seem to be responsible for the mechanism of resistance to therapeutic treatments (e.g., radioresistance and chemoresistance); and the development and maintenance of a tumor stem phenotype (54).

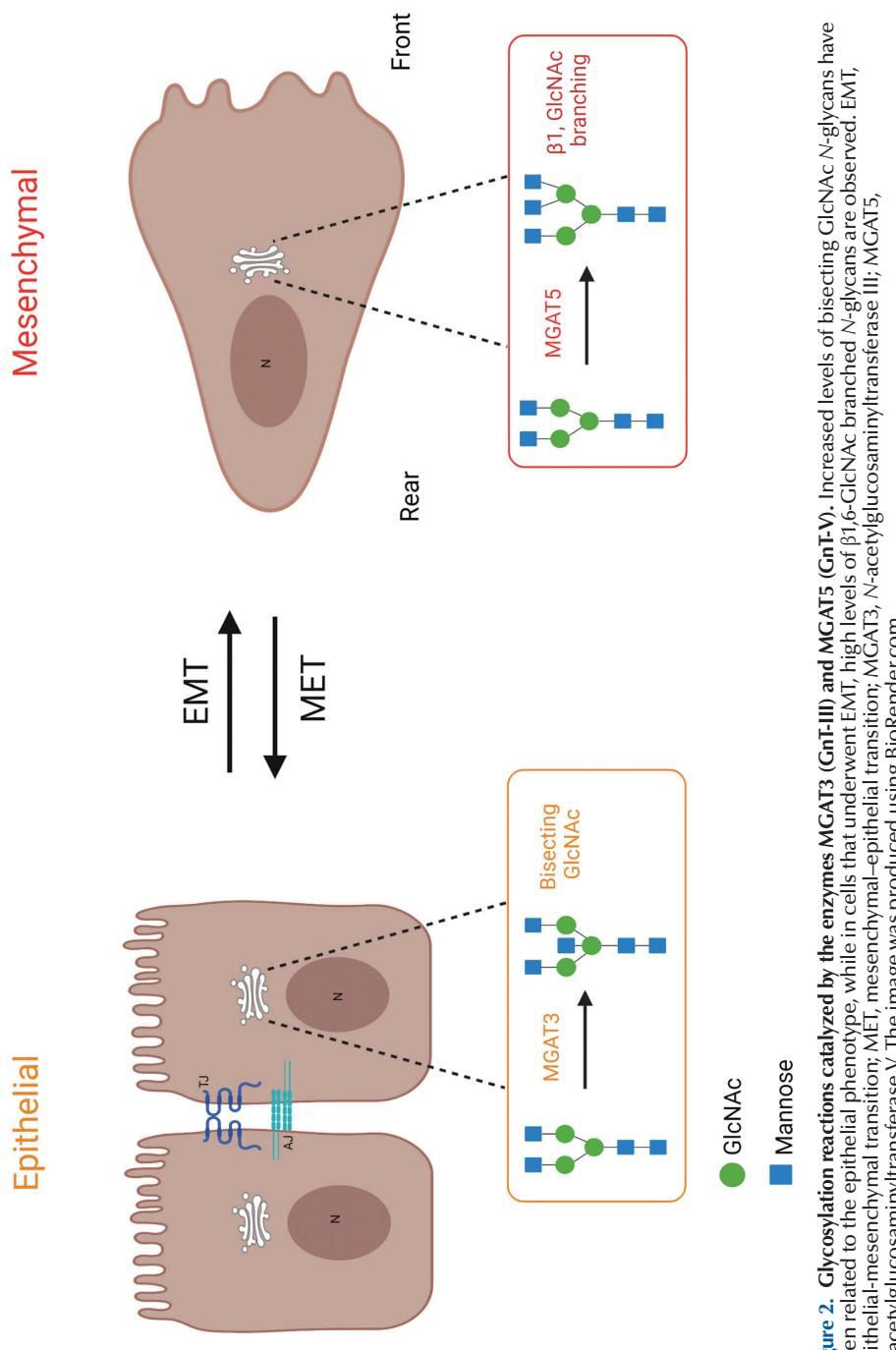


Figure 2. Glycosylation reactions catalyzed by the enzymes MGAT3 (**GnT-III**) and MGAT5 (**GnT-V**). Increased levels of bisecting GlcNAc N-glycans have been related to the epithelial phenotype, while in cells that underwent EMT, high levels of β 1,6-GlcNAc branched N-glycans are observed. EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition; MGAT3, N-acetylglucosaminyltransferase III; MGAT5, N-acetylglucosaminyltransferase V. The image was produced using BioRender.com.

Radioresistance

Radiotherapy (RT) is used for treatment with curative or palliative intent; however, resistance to this treatment is the main reason for therapeutic failure, which can lead to tumor recurrence and metastasis. In this context, some studies have shown that RT can induce EMT, thus leading to the acquisition of a resistant phenotype with decreased activation of cell death pathways by apoptosis (55). In CRC cells, it was shown that the progeny derived from radioresistant cells developed an EMT-like phenotype characterized by reduced E-cadherin expression, overexpression of β -catenin and vimentin, and increased cell migration and invasion (56). In a subsequent study using the same cellular progeny, EPHA4 receptor activation was found to be upstream of the PI3K/AKT, Wnt/ β -catenin, and ERK1/2 pathways, playing an important role in the regulation of events related to EMT (57). Another study using CRC cells showed RT induced EMT by increasing ROS and activating AKT/Src/ERK signaling, resulting in increased Snail and decreased E-cadherin expression (58). These results indicate that these events may be associated with therapeutic failure in CRC after RT.

Reactive Oxygen Species (ROS), cellular components of the tumor microenvironment, and a hypoxic condition, mainly through hypoxia-inducible factor (HIFs), play an important role in the activation of signaling pathways that induce EMT and tumor resistance. Some of these pathways include TGF- β , Wnt/ β -catenin, NOTCH, EGFR, NF- κ B, IL-6/STAT3, PI3K-AKT, and ERK. Likewise, specific miRNAs, lncRNAs, and circRNAs also contribute to the induction and maintenance of EMT-associated resistant phenotypes (59). In addition, some of these converge with the cancer stem cells (CSCs) activation pathways, where the induction of EMT in non-stem cells can promote radioresistance through the gain of stem-like characteristics. The mechanism linking these two events has not been fully elucidated. However, it is possible that proteins secreted by cells during EMT act in an autocrine manner, leading to the induction and maintenance of the CSC phenotype. The resistance associated with these cells has been attributed to mechanisms such as the decreased expression of pro-apoptotic proteins, increased expression of anti-apoptotic proteins, low proliferation rate, and evasion of the immune system (60). Some studies have shown that EMT negatively regulates senescence and vice versa. Thus, cyclin-dependent kinase inhibitors (CDKIs) inhibit EMT-TFs, whereas those in EMT show decreased expression of classic senescence markers, such as p21/p27 and p14/p16, through repression of the transcription of these proteins mediated by EMT-TFs (61). In fact, EMT induction or senescence in CRC cells after RT treatment did not describe the simultaneous occurrence of both phenotypes (62).

EMT is also related to other cellular events that contribute to radioresistance, such as the formation of polyplloid giant cancer cells (PGCCs). These cell types display both EMT and CSC characteristics and can generate daughter cells with these properties (63). Autophagy is another known RT resistance mechanism. Autophagy and EMT share signaling pathways such as RAS/RAF/MEK/ERK, beclin-1, and TGF- β , in which their mediators positively activate both mechanisms (64). It is possible that the events mentioned above may be responsible for therapeutic failure after RT in patients with CRC; however, the post-irradiation behavior and phenotype of radioresistant cancer cells remain largely unknown. Thus, the identification of biomarkers and altered pathways in RT-resistant cells may help improve clinical efficacy.

Chemoresistance

Many tumors present innate resistance before drug treatment (intrinsic resistance) or can become resistant after long-term treatment with these drugs (acquired resistance). This phenomenon of resistance by tumor cells to different anticancer drugs is termed multidrug resistance (MDR) and is a major challenge in modern cancer treatment. MDR results in simultaneous cross-resistance to multiple unrelated chemotherapeutic agents, and is associated with treatment failure, poor survival, and disease recurrence. One of the most important mechanisms underlying MDR is the overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) transporters that efflux anticancer drugs against a concentration gradient using ATP-driven energy. These pumps alter enzymes responsible for drug metabolism, enhance DNA repair response, alter the microenvironment and survival of CSCs, and activate EMT (65).

A link between EMT and chemoresistance was proposed in the early 1990s (66). Subsequently, numerous cancers have been reported to overexpress EMT markers after treatment with various chemotherapeutic agents (67). Using an EMT lineage-tracing system in transgenic mice with breast cancer, cyclophosphamide resistance was associated with Zeb1 and Zeb2 expression, reduced proliferation, and apoptotic tolerance (68). Snail or Twist suppression in mouse models of pancreatic ductal adenocarcinoma resulted in higher levels of nucleoside transporters in tumors, contributing to increased sensitivity to gemcitabine (69). In lung cancer cell lines, cisplatin resistance is mediated by Slug, which inhibits apoptosis by suppressing the proapoptotic protein PUMA (70). In addition, Snail overexpression has been associated with evasion of apoptosis, MDR phenotype, enhanced P-glycoprotein levels, and increased cell stemness in breast cancer cell lines (71). Patients displaying the mesenchymal subtype of CRC (CMS4) exhibited chemoresistance profiles in response to Hsp90 inhibitors in preclinical models (72) and unresponsiveness to adjuvant 5-fluorouracil (5FU) therapy in a clinical cohort (73).

The molecular mechanisms involved in chemoresistance are complex, as multiple signaling pathways can contribute in different ways and are tissue specific. TGF- β , Hedgehog, Notch, Wnt/ β -catenin signaling, and FOX transcription factor superfamily members are related to chemoresistance associated with EMT in diverse cancers. For instance, blocking TGF- β signaling by inhibiting Smad4 abolished doxorubicin resistance in a CRC cell line (74). Increased nuclear β -catenin was detected in the mesenchymal tissues of CRC patients resistant to doxorubicin, and was associated with low levels of E-cadherin, high levels of Snail, and expression of CSC-related markers (75). Indeed, Wnt/ β -catenin signaling has been shown to regulate the transcription of *MDR1* (P-glycoprotein) via TCF/LEF, pro-survival signaling, expression of CSCs marker genes, and normal stem cell differentiation and proliferation in intestinal crypts (76). CSCs properties are also associated with chemoresistance and EMT, sharing gene expression signatures and key signaling pathways. CSCs possess mechanisms of adaptation that mediate their growth, survival, and chemoresistance. These mechanisms include slow proliferation and increased levels of efflux pumps that permit the elimination of cytotoxic drugs and, consequently, exhibit a high ability to perpetuate the tumor after this therapy (77).

Different microenvironmental tumor factors, such as immune cells, cancer-associated fibroblasts (CAFs), hypoxia, matrix extracellular compounds, interleukins, and growth factors also play key roles in driving EMT, chemoresistance, and CSCs, and these components interact to form a permissive niche for tumor progression. A study using CRC patient-derived models showed that under hypoxia, CAFs secrete TGF- β 2 and induce the expression of hedgehog transcription factor (GLI2) via HIF-1 α in CSCs, promoting 5FU and oxaliplatin chemoresistance (78). Overall, these studies demonstrated that CRC chemotherapy is also intimately linked to the EMT molecular signature and an enriched CSCs population, contributing not only to relapse but also to resistance and metastasis.

EMT COMPONENTS AS TARGETS TO INHIBIT METASTASIS

Owing to their crucial role in processes such as tumor progression, migration, invasion, and therapy resistance, EMT components have become a target for researchers looking for ways to stop or at least hinder the metastatic cascade. However, there are three main complications associated with targeting EMT to inhibit metastasis. First, while targeting EMT-TFs or mesenchymal-specific proteins, another important cell population in the tumor, the fibroblasts, might be affected. These cells are active regulators of paracrine signaling and structural remodeling of the tumor microenvironment but are also essential for the maintenance of normal epithelial function across the digestive tract. Second, EMT plasticity with all its intermediate stages that are employed at each step of the metastatic cascade poses the persistent question of finding the correct stage to target (79). Finding a drug that inhibits the expression of mesenchymal characteristics might stop tumor cells from further invasion but may also promote metastatic colonization from early metastasized cells. Third, there is considerable redundancy in EMT-related pathways, with multiple EMT-TFs regulating specific genes and several pathways leading to this program (33). Therefore, a single-target strategy is most likely to be inefficient in a clinical setting. However, with multi-target therapies, unintended side effects from the interconnected pathways that participate in the EMT program are also crucial to other physiological processes in healthy cells.

A few targets such as natural compounds, small-molecule inhibitors, and monoclonal antibodies, have already been assessed in preclinical studies. TGF- β is an important EMT inducer and one of the main targets for EMT prevention. However, owing to its multiple receptors and canonical and non-canonical downstream signaling, inhibiting TGF-beta-induced EMT poses a serious challenge. Natural compounds such as resveratrol (80) and small-molecule inhibitors have been successful in preclinical studies, but none have reached human trials for CRC. Monoclonal targeting of TGF-beta receptors, fresolimumab, has been attempted for renal cell carcinoma and melanoma in a phase I trial, with no significant improvement in patient survival (81). It is important to highlight that TGF- β plays a dual role acting as tumor promoter or suppressor in CRC progression, depending on the activation status of its signaling pathways. As a tumor suppressor it acts in the early stages of tumorigenesis. However, it can also promote invasiveness and metastasis in advanced stages of tumor progression (82). Therefore, specific targeting might have unfavorable outcomes depending on the tumor stage. Multityrosine kinase inhibitors, such as cabozantinib and

regorafenib, have shown some success in clinical trials, with the latter being approved by the Food and Drug Administration (FDA) of the United States as a last resort for the treatment of metastatic CRC. Regorafenib abolishes EMT-induced invasion and metastasis through the activation of SHP-1 tyrosine phosphatase (83). Cabozantinib interferes with AXL signaling, downregulates EMT-TFs, and increases E-cadherin expression. However, in a combined treatment with the EGFR inhibitor panitumumab for KRAS wild-type CRC patients, cabozantinib showed a 16% response in terms of clinical parameters (84). Methotrexate successfully increased E-cadherin expression levels 10-fold in CRC cell lines, and it has been shown to be ineffective in clinical settings (85). The antibiotic salinomycin has also been shown to downregulate vimentin and prevent EMT induction by doxorubicin (86). However, targeting EMT effectors such as E-cadherin and vimentin, as previously discussed, may result in an increased number or metastatic size if we consider the reverse process of EMT, which is a crucial step in secondary tumor formation.

Considering all these characteristics, perhaps the most promising strategy for EMT targeting is an indirect approach. Repurposing already approved metabolic drugs has shown a significant EMT-inhibiting potential (87). The high potential of utilizing the increased cell plasticity inherent to invasive cancer cell instead of avoiding EMT represent an innovative technique of transdifferentiation therapy. For instance, Ishay-Ronen et al. have shown that, for breast cancer cells, cell proliferation and EMT plasticity can stop inducing terminal differentiation in cancer-derived adipocytes through a combined treatment with rosiglitazone (PPAR γ agonist) and trametinib (MEK inhibitor). This promising transdifferentiation strategy reduced invasion and metastasis of breast cancer in animal models (88). However, the available experimental evidence for colorectal cancer is still scarce compared with other tumor types.

CONCLUSION

EMT is a highly orchestrated program through a complex network of numerous regulatory factors and cell signaling pathways that interact with each other to regulate crucial processes involved in tumor progression. In recent years, a series of studies have facilitated the establishment of various EMT markers that are being used in preclinical models to inhibit the effect of EMT on invasiveness, metastasis, and drug resistance. However, the complex heterogeneity of tumors and the reversible plasticity of EMT, which is fundamental for tumor progression and drug resistance, are principal obstacles in the reproduction of clinical cancer progression. Therefore, further research to establish new biomarkers for EMT, with special emphasis on partial EMT as well as a better understanding of how EMT and therapy resistance are interconnected, is imperative for the development of new therapeutic approaches for CRC.

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Clinicopathological Features and Surgical Management of Gastrointestinal Stromal Tumors: State-of-the-Art

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Abstract: Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors, thought to arise from the interstitial cells of Cajal. Almost all GISTs have mutations in the oncogenic tyrosine protein kinase KIT or platelet-derived growth factor receptor-alfa. GISTs are mostly formed in the stomach and the small intestine.

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GISTs are often asymptomatic, but when symptoms occur, they most commonly include gastrointestinal bleeding, early satiety, and abdominal pain. These tumors do not have specific endoscopic or radiological features. The treatment for confirmed GISTs is surgery if the lesion is resectable with no metastases, or therapy with tyrosine kinase inhibitors if the lesion is unresectable, metastatic, or recurrent. The prognostic factors are tumor location, tumor size, mitotic index, and type of mutation. All surgical techniques can be performed laparoscopically using five trocars for wedge resection, subtotal gastrectomy or total gastrectomy based on tumor location. In case of intragastric resection with a single port under laparoscopic control, intraoperative endoscopy is used to identify the exact location of the lesion, and to guide single port device placement inside the stomach after gastrotomy. During subtotal and total gastrectomy, indocyanine green fluorescence angiography is performed to assess the vascular supply. This chapter discusses the clinicopathological features of gastric GISTs and describes the standard minimally invasive management techniques.

Keywords: gastrointestinal stromal tumor; minimally invasive surgery for GIST; prognostic factors of gastrointestinal stromal tumor; risk classification of gastrointestinal stromal tumor; surgical management of gastric GISTs

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors with a variable clinical behavior, arising from the interstitial cells of Cajal (1). GISTs belong to soft tissue sarcomas family (STSs) but are considered separately due to their peculiar histogenesis, clinical behavior, and specific therapy (2). The estimated global incidence of GISTs is 1–1.5 per 100,000 persons per year, and the prevalence is 13 per 100,000 persons per year (3). The median age at diagnosis is 62.5 years (3). GISTs in children and adolescents are very rare, but represent a distinct, often syndromic, subset (4). The etiology of GISTs is unknown in most cases, and the co-existence of another cancer is more common in GISTs patients than in the general population (5). The most common sites of GISTs are the stomach (50–60%) and small intestine (20–30%), but they may also be located in the colon, rectum, esophagus, mesentery, omentum, and retroperitoneum (6).

Almost all GISTs contain mutations in the oncogenic tyrosine kinase (KIT) or platelet-derived growth factor receptor-alpha (PDGFRA) genes (7, 8); however, KIT/PDGFR α mutations are mostly absent in children and young patients (4). Other mutations in GISTs patients may include loss of function of neurofibromatosis type 1 (NF1) or gain of function in the proto-oncogene BRAF (9). Several genetic syndromes that are linked to GISTs has been reported:

- *The Carney triad syndrome:* Gastric GISTs, paraganglioma, and pulmonary chondroma can occur at any age (10).
- *Familial GIST:* This syndrome is rare, occurs in families with autosomal dominant mutation of KIT and shows multiple GISTs in pediatric age (11).
- *Carney–Stratakis syndrome:* Germ-line mutations of one of the succinate dehydrogenases (SDH) subunits with development of GISTs and paragangliomas (12, 13).

- *Type-1 neurofibromatosis:* Localization is in the small bowel, often multi-focal, loss of function of NF1 and absence of mutations in KIT and PDGFRA (14).

The diagnosis and treatment of GISTs have rapidly improved after the discovery of mutations in KIT and PDGFRA genes and the subsequent introduction of tyrosine kinase inhibitors (TKIs) (15).

CLINICAL PRESENTATION

The most common symptoms of GISTs are gastrointestinal bleeding, anemia, early satiety, abdominal distension, pain or discomfort, and a palpable mass (16). Based on location, the clinical presentation may change: small bowel GISTs may present with acute events like hemorrhage or rupture, after a long silent period; colorectal GISTs can occur with abdominal pain, obstruction or bleeding; and dysphagia can appear in esophageal and gastro-esophageal junction GISTs. Gastrointestinal bleeding is more frequently observed in gastric GISTs than in other locations. Symptoms can change from chronic microcytic anemia to acute hematemesis or melena, and at diagnostic endoscopy they may be wrongly diagnosed as peptic ulcer. Non-specific systemic symptoms, which are common in some patients, like weight loss and night sweating, may be misleading and may delay the diagnosis. Often GISTs are asymptomatic until advanced stages but may be found incidentally during endoscopy, especially in the stomach, or at postmortem autopsy (17, 18). Metastasis to lymph nodes or extra-abdominal spread of GISTs is rare, except for the succinate dehydrogenase (SDH)-deficient GIST (19). Recently, the observation of so-called mini GISTs (<1cm) as an incidental microscopic diagnosis in the stomach resected from patients with gastric cancer, and at autopsy, has been reported (20–22).

The natural history of GISTs remains mostly unknown, and microscopic sub-clinical gastric GISTs have an unexpectedly high incidence in clinicopathologic studies (20, 23). In patients over 50 years of age, small GISTs (up to 10mm in diameter) are commonly found, especially in the proximal stomach (22). However, these mini/small GISTs are often biologically inert at medium- and long-term follow-up, except in cases with high-risk features (like irregular margins or ulceration) (24). Even though the gold standard treatment for symptomatic GISTs is surgical resection, the indication of surgery for incidentally discovered mini GISTs is still debated (25).

DIAGNOSIS

GISTs do not have specific endoscopic or endoscopic ultrasound (EUS) features, and often they are identified during endoscopy like a submucosal tumor (SMT), with pathological diagnosis only after surgery. In SMTs that are smaller than 2 cm with no high-risk features, and also in cases where histological diagnosis of GISTs was made after biopsy, only endoscopic follow up can be carried out until the patient becomes symptomatic or the tumor grows in size (24).

The decision-making process may also include a histological diagnosis by EUS-guided fine-needle aspiration (EUS-FNA) biopsy. The optimal follow-up timing for these lesions is still debated in the literature, so an initial short-term follow-up at six months with EUS is recommended, which may then be deferred in time if high-risk features do not appear. This prolonged follow-up does not worsen the prognosis of patients with gastric GIST, as confirmed by a recent retrospective study (26). Endoscopic resection of SMTs is not considered oncologically safe, due to the risk of positive margins and high risk of cells dissemination. EUS-FNA biopsy can provide a histological diagnosis before surgery and set the indications for neoadjuvant therapy on the basis of the histological characteristics. EUS-FNA biopsy is to be preferred instead of conventional endoscopic forceps biopsy, because standard biopsy forceps do not reach the lesion beyond the normal mucosa and submucosa (27). However, EUS-FNA biopsy is not necessary for tumors measuring less than 2 cm, for undoubtedly benign tumors and for tumors which have already been planned for surgical resection (28). Instead, contrast enhanced computed tomography (CT) is recommended for initial diagnosis of tumors larger than 2 cm in diameter (Figure 1), to evaluate for the presence of high-risk features (25, 26). Furthermore, the Japanese guidelines recommend surgical resection for all GISTs that are larger than 5 cm in diameter (29). Finally, in metastatic disease, a biopsy of an easily accessible metastatic site can be performed, followed by a local and/or systemic treatment.

PATHOLOGY

Immunohistochemistry plays a central role in the pathologic assessment of GISTs, with CD117 (KIT) immunopositivity (30) and more recently with the inclusion of



Figure 1. CT Scans. Abdominal CT scans showing an intraluminal and extraluminal gastric GIST. On the left, the extraluminal portion of the lesion is in close contact with the tail of the pancreas with no signs of infiltration. On the right, the two portions of the lesion, in continuity with each other, are shown by the arrows.

DOG1 (Discovered on GIST-1) protein as tumor marker (31). However, a small number of GISTs are immunonegative: 5% are negative for CD117, 5% are negative for DOG1 and about 1% for both (32). In case of diagnostic doubt in strongly suspected GISTs, but with CD 117 and DOG1 negativity, the analysis for activating mutations in KIT or PDGFRA may be of help (32). Another prognostic factor for risk stratification is the mitotic count (33): an index of less than 5 mitoses per 50 high-power fields classifies the GIST as at low risk; an index of mitoses between 6 and 10 per 50 high-power fields classifies the GIST as at intermediate risk; an index of mitoses more than 10 per 50 high-power fields classifies the GIST as at high risk (33).

Analysis of the mutational state is fundamental to predict the sensitivity to molecular-targeted therapy, especially for treatment with imatinib, and for overall prognostic value. In the diagnostic process of GISTs, the analysis of the mutational state should be carried out routinely as standard practice in all patients. Furthermore, in KIT or PDGFRA negative GISTs the recommendation is to perform immunohistochemistry for succinate dehydrogenase B (SDH-B), and, if negative, for SDH-A (32). In case of KIT or PDGFRA negativity, mutations in BRAF should also be searched, since BRAF inhibitors (Dabrafenib) may be included in the therapeutic strategy in these patients (33). A special focus is for patients with neurofibromatosis and a germline mutation in NF1, because of the increased risk of GISTs' development and recurrence (33).

STAGING

For staging and follow-up of GISTs, the main investigation is contrast enhanced abdominal and pelvic CT scan, because disease recurrence is almost exclusively located in the liver and/or in the peritoneum. Magnetic resonance imaging may be considered as an alternative investigation only in very selected young patients in order to limit exposure to radiation. The chest should be investigated with CT scan only in the staging process, but this is not considered a routine exam during follow-up. The 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET) scan can be used in patients treated with neo-adjuvant imatinib therapy in order to evaluate the response.

TREATMENT

For small GISTs measuring less than 2 cm in diameter, when the diagnosis by endoscopic biopsy may be difficult, the only way to make a histological diagnosis is by surgical excision, though most of these nodules are at very low risk. In these patients, a standard approach would include diagnosis by EUS-FNA biopsy and annual follow-up. Surgical excision is recommended only for patients who become symptomatic or for tumors increasing in size. In GISTs that are larger than 2 cm in diameter, the gold standard treatment is surgical excision. Only in selected cases, like low-risk GISTs and in patients with major morbidity at high risk for surgery, a follow-up strategy without surgery may be an option after in-depth discussion with the patient. A multidisciplinary approach is always mandatory,

including oncologist, surgeon, radiologist, and histopathologist. The optimal strategy would be to refer the patient to a high-volume center, like highly specialized centers for the management of sarcomas and GISTs with highly experienced surgeons.

When the surgical indication is set by the multidisciplinary team, in case of localized GISTs, the gold standard is complete surgical excision without lymphadenectomy if lymph nodes are clinically negative, but always respecting the principles of oncological surgery. In case of involvement of adjacent organs, in bloc resection is required. When the risk of tumor rupture is high, as in the case of large tumors, the laparoscopic approach should be avoided because of the high risk of cell dissemination.

The aim of surgery should be an R0 excision, meaning absence of residual tumor. If this is envisioned to be not possible, because of major functional sequelae, a neoadjuvant therapy is indicated (34, 35). After cyto-reduction therapy, lasting between 6 and 12 months, surgical excision is performed. Prior analysis of KIT or PDGFRA mutations is mandatory in order not to delay surgery, in case of non-responding tumors. An early radiological re-evaluation after a few weeks is possible to study tumor response to imatinib. In case of failure of neoadjuvant therapy, an R1 resection with microscopically positive margins may be proposed by the multidisciplinary team, especially for low-risk tumors (36). In case of histologically unexpected R1 margins, a surgical strategy with re-excision should be considered.

Imatinib is the gold standard treatment in patients with metastatic disease or with inoperable tumors because a surgical approach as primary treatment is not recommended in these patients. The MetaGIST group (37) reported that a standard dose of imatinib (400 mg daily) should be doubled in patients with KIT exon 9 mutations for a better progression-free survival rate.

In case of progressive disease during imatinib treatment, surgical treatment is not recommended. In case of limited local or distant recurrence, the indication for surgery may be discussed with the patient, to possibly achieve better progression-free interval as compared to a second-line treatment with sunitinib. In case of liver metastases, interventional radiology procedures may be considered. In case of disease progression after therapy, or in case of intolerance to imatinib, the approved second line treatment is tyrosine kinase inhibitor (TKI) sunitinib (38) with benefits in progression-free survival, possibly also with a continuous lower daily dose (39). However, a small number of patients do not respond even to sunitinib, and this is suggestive of special mutations in loop domain of KIT or in exon 18 of PDGFRA. Figure 2 shows a proposed algorithm for the management of SMT.

PROGNOSTIC FACTORS AND RISK CLASSIFICATION

Prognostic factors for GISTs are tumor location, tumor size, mitotic index, type of mutation, and the presence of tumor rupture (29). However, discrimination between benign and malignant GISTs is difficult. The occurrence of postoperative metastases is possible in case of small tumors with low mitotic index. The Miettinen and Lasota (32), and the modified Fletcher classification (33), are the two most utilized risk-classification methods; both incorporate tumor size, mitotic index, and tumor site.

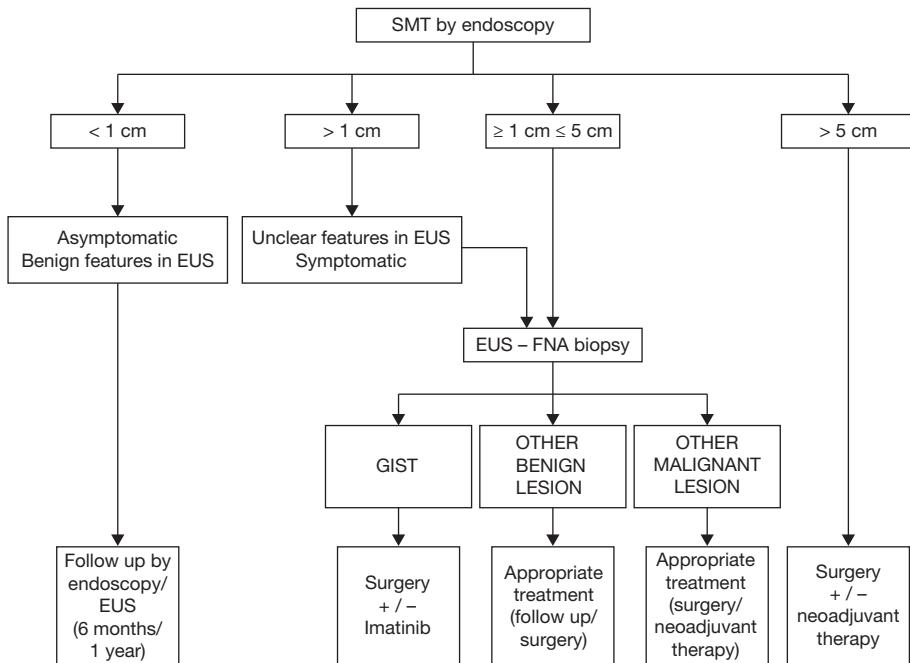


Figure 2. Proposed strategies for management of submucosal tumor (SMT).

SURGICAL TECHNIQUES

GIST resection may be performed by open, laparoscopic, or robotic surgery (40–43) but the surgical approach that is most frequently reported in the literature is the laparoscopic one (40–43). In fact, minimally invasive surgery is associated with better intra- and post-operative outcomes as compared to open surgery in term of intraoperative blood loss, hospital stay, postoperative pain and return to daily activities, with the same oncological results of open surgery (40–43). However, the open approach still has a rationale, in case of large masses infiltrating adjacent structures, which may be technically difficult to remove by minimally invasive surgery (40–43). On the other hand, the role of the robotic approach in comparison to laparoscopy has not been clarified yet, due to the lack of comparative studies between the two approaches (41). The laparoscopic approach for the management of GIST is briefly described below.

The patient is under general anesthesia, placed in supine position with abducted legs and the surgeon stands between the patient's legs. The operating table is placed in anti-Trendelenburg position. Pneumoperitoneum at 14mmHg is established with a Veress needle in the left hypochondrium (Palmer's point), and a 30°, 10 mm optic is used. During subtotal and total gastrectomy, indocyanine green (ICG) fluorescence angiography (FA) may be employed to assess the vascular supply. ICG powder 25 mg (Verdyte, Diagnostic Green, Aschheim-Dornach,

Germany) is diluted in 5 cc of sterile water and 3 cc of the solution are intravenously administered to evaluate the vascular organ perfusion by FA during surgery (total of 15 mg per patient). The camera is positioned approximately 5 cm away from the tissue in zooming modality and fluorescence evaluation is performed in real time.

Laparoscopic gastric wedge resection

Five trocars are used: one 11 mm trocar is placed in the supraumbilical position; two 12 mm trocars are placed along the right and left midclavicular lines, two cm above the transverse umbilical line; and two 5 mm trocars are placed, one in subxiphoid position and the other one along the right anterior axillary line. The first step of the procedure is to identify the lesion. It can be located on the anterior or on the posterior wall of the stomach. In case of posterior location, it is necessary to gain access to the lesser sac. Gastric mobilization is performed using an advanced energy device (LigaSure™, Medtronic, Minneapolis, Minnesota, USA or Ultracision, Harmonic Scalpel, Ethicon Endo Surgery, Cincinnati, Ohio, USA). Next, wedge resection of the tumor is performed using a linear stapler with two or three 60 mm gold or blue cartridges (Echelon Flex Powered Endopath, Ethicon Endo-Surgery, Johnson & Johnson, Cincinnati, Ohio, USA), based on the gastric tumor location and on surgeon's preference, buttressed with absorbable material (polyglycolic acid and trimethylene carbonate, Seam-guard® Gore & Associates, Inc. Newark, Delaware, USA). Intraoperative endoscopic control is recommended in order to ensure that tumor margins are free (R0 resection) and to avoid stenosis. The specimen is removed by an endobag through a Pfannenstiel incision or by enlarging one of the 12 mm trocar incisions.

Intragastric resection with single port under laparoscopic control

After establishing pneumoperitoneum, one 11 mm trocar is placed in supraumbilical position, and two 5 mm trocars are placed laterally to the previous one along the midclavicular line on the left and on the right. Another 5 mm trocar is placed in a subxiphoid position to retract the liver. Intraoperative endoscopy is used to identify the exact location of the lesion and to define the best position to insert the single port device. Next, a single port (TriPort Plus, Advanced Surgical Concepts, Bray, Ireland) replaces one of the 5 mm trocars and it is placed inside the stomach after creation of a 12–15 mm gastrotomy. Full thickness resection of the gastric wall harbouring the tumor is performed by using the ultrasonic device (Ultracision, Harmonic Scalpel, Ethicon Endo Surgery, Cincinnati, Ohio, USA), and then closing the residual defect by means of running 3–0 absorbable barbed suture (V-Loc™, Medtronic, Minneapolis, Minnesota, USA). The specimen is removed using an endobag. The single port is then removed from the stomach, but it is left in the abdominal cavity to be used as a trocar. The gastrotomy is closed by using a linear stapler with 60 mm blue cartridges (Echelon Flex Powered Endopath, Ethicon Endo-Surgery, Johnson & Johnson, Cincinnati, Ohio, USA) and the stomach is then insufflated by the endoscopist in order to evaluate the presence of leakage by performing an air leak test (44).

Laparoscopic subtotal gastrectomy

Five trocars are used. One 11 mm trocar is placed in supraumbilical position, two 12 mm trocars are placed along the right and left midclavicular lines two cm above the transverse umbilical line, and two 5 mm trocars are placed, one in a subxiphoid position and the other one along the right anterior axillary line. The liver is retracted with a grasper introduced from the subxiphoid trocar. Gastric mobilization is performed by using an advanced energy device (LigaSure™, Medtronic, Minneapolis, Minnesota, USA or Ultracision, Harmonic Scalpel, Ethicon Endo Surgery, Cincinnati, Ohio, USA).

The first step of the procedure is to mobilize the greater omentum. The lesser sac is opened by dividing the short gastric vessels along the greater curvature and right gastroepiploic artery, taking down any retrogastric adhesions (Figure 3). The duodenum is prepared 2 cm beyond the pylorus, paying attention not to injure the gastroduodenal artery, and it is divided by linear stapler with a 60 mm blue cartridge (Echelon Flex Powered Endopath, Ethicon Endo-Surgery, Johnson & Johnson, Cincinnati, Ohio, USA) buttressed with absorbable material (polyglycolic acid and trimethylene carbonate, Seam-guard® Gore & Associates, Inc. Newark, Delaware, USA). After opening the *pars flaccida* of the lesser omentum along the lesser curvature of the stomach, the right gastric artery near the antrum, the descending branch of the left gastric artery and the coronary vein are divided using the energy device. The stomach is then divided above the tumor using the linear stapler with 60 mm blue cartridges. Before the anastomosis is created, ICG-FA is performed in order to assess the vascular supply of the gastric stump and jejunum.

A double-loop Roux-en-Y reconstruction technique is used to restore bowel continuity, as reported for gastric bypass in bariatric surgery. The greater omentum is divided and a side-to-side mechanical antecolic gastro-jejunal anastomosis is created on the posterior wall of the stomach (biliary limb, measured at 60 cm from the Treitz ligament). The enterotomy is closed using a running suture with a 3–0 reabsorbable barbed suture (V-Loc™, Medtronic, Minneapolis, Minnesota, USA). A mechanical side-to-side jejuno-jejunal anastomosis is then created (alimentary limb, measured 80–100 cm from the first anastomosis), with a similar technique as previously described, by using a linear stapler with white cartridge, followed by enterotomy closure with a running suture. The two anastomoses are checked by the methylene blue test. After that, the small bowel between the gastro-jejunal and the jejuno-jejunal anastomosis is divided by a linear stapler with 60 mm white cartridge. The specimen is removed using an endobag through a Pfannesteil incision.

Laparoscopic total gastrectomy

Five trocars are used. One 11 mm trocar is placed in supraumbilical position, two 12 mm trocars are placed along the right and left midclavicular line two cm above the transverse umbilical line, and two 5 mm trocars are placed, one in a subxiphoid position and the other one along the right anterior axillary line. The liver is retracted with a grasper introduced from the subxiphoid trocar. Gastric mobilization is performed using an advanced energy device (LigaSure™, Medtronic,

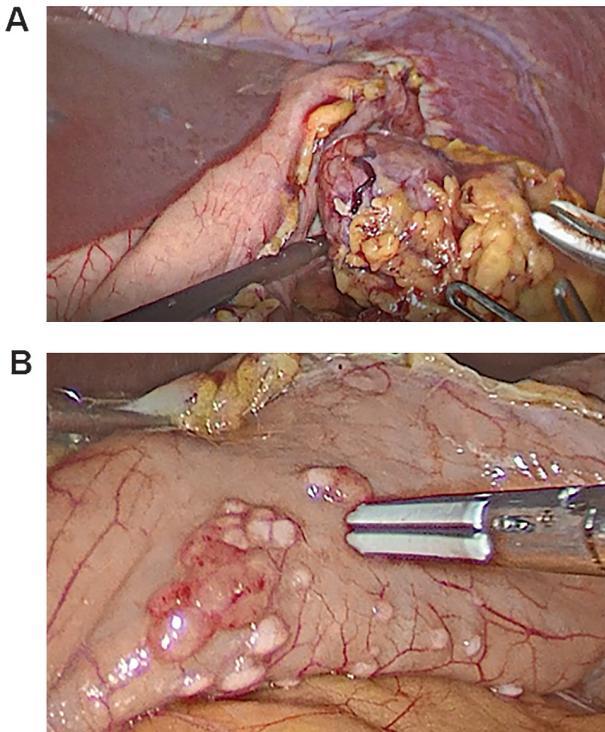


Figure 3. Extraluminal portion of gastric GIST. **A**, Laparoscopic resection. After division of the short gastric vessels on the greater curvature and along the right gastroepiploic artery, the lesser sac is opened showing a large extraluminal portion of a gastric GIST in continuity with the intraluminal portion, both located along the posterior wall of the gastric fundus and body. **B**, After division of the short gastric vessels on the greater curvature and along the right gastroepiploic artery, the lesser sac is opened showing multiple synchronous satellite nodules of gastric GIST located in the antrum of the same patient displayed in Figure A.

Minneapolis, Minnesota, USA or Ultracision, Harmonic Scalpel, Ethicon Endo Surgery, Cincinnati, Ohio, USA). The first step of the procedure is to mobilize the greater omentum. The lesser sac is opened by dividing the short gastric vessels on the greater curvature and along the right gastroepiploic artery (Figure 3). The dissection proceeds cranially along the greater curvature up to the angle of His and with exposure of the left crus of the esophageal hiatus.

The duodenum is prepared beyond the pylorus, and it is divided by a linear stapler with 60 mm blue cartridge (Echelon Flex Powered Endopath, Ethicon Endo-Surgery, Johnson & Johnson, Cincinnati, Ohio, USA) buttressed with absorbable material (polyglycolic acid and trimethylene carbonate, Seam-guard® Gore & Associates, Inc. Newark, Delaware, USA). The lesser omentum is opened, and the right and left gastric arteries are divided, as well as the left gastric vein.

Esophageal hiatus dissection continues from the left crus towards the right one and posteriorly until the aorta is visualized. Next, the abdominal esophagus is divided by using a linear stapler with 60 mm blue cartridge. Before creation of the anastomosis, ICG-FA is performed in order to assess the vascular supply of the esophageal stump and jejunum. A double-loop Roux-en-Y reconstruction

technique is used, as reported for gastric bypass in bariatric surgery. In this case, the jejunal loop runs posteriorly to the colon after creating a retrocolic window through the transverse mesocolon on the left of the middle colic vessels. The greater epiploic is divided and a side-to-side mechanical esophago-jejunal anastomosis on the posterior wall of the esophagus is performed (biliary limb, measured 60 cm from the Treitz ligament), followed by closure of the residual enterotomy with a 3-0 absorbable barbed suture (V-Loc™, Medtronic, Minneapolis, Minnesota, USA). A mechanical side-to-side jejunoo-jejunal anastomosis is created (alimentary limb, measured 80–100 cm from the first anastomosis), following a similar technique as previously described, using a linear stapler with white cartridge. Both anastomoses are checked by the methylene blue test to detect any leakage. The small bowel is then divided between the esophago-jejunal and the jejunoo-jejunal anastomosis using the linear stapler with 60 mm white cartridge. The specimen is then removed by endobag through a Pfannenstiel incision.

OPEN vs LAPAROSCOPIC SURGERY

Inaba et al. reported data obtained from the National Cancer Database (NCDB) of 5096 patients who underwent open and laparoscopic surgery for GIST between 2010 and 2014 (42). The study included 2910 (57%) stage I, 954 (19%) stage II, and 1232 (24%) stage III patients. Patients' characteristics were similar between the two groups, with no statistically significant differences (42). Laparoscopy, in comparison to the open approach, showed decreased 90-day mortality and 30-day readmission rates, in all stages, even though a statistically significant difference was observed only in stage I (42). Moreover, laparoscopy was associated with shorter hospital stay in comparison to open surgery in all stages (42). Regarding follow-up, the Kaplan-Meier long-term survival curves showed better results for the laparoscopic approach in stages I and II, with no significant differences in stage III (42).

In the meta-analysis by Chen et al., 19 observational studies comparing laparoscopic and open surgery were included (45). They reported significantly lower intraoperative blood loss, shorter time of first flatus and first oral intake days in the laparoscopic group, indicating quicker recovery of the bowel function (45). Furthermore, a lower dose of postoperative analgesics consumption and a shorter hospital stay in the laparoscopic group were observed (45). The postoperative complication rate was also statistically significantly lower in the laparoscopic group (45). During follow up, the recurrence rate in the laparoscopic group was lower and the difference was statistically significant ($p = 0.03$). However, it should be considered that in the open surgery group the tumors were larger and had a higher mitoses rate, both being negative prognostic risk factors (45).

CONCLUSION

GISTs are mesenchymal tumors marked by differentiation towards the interstitial cells of Cajal, and almost all contain mutations in oncogenic KIT or PDGFRA. The most common localizations of GISTs are the stomach and the small intestine.

The treatment for confirmed GISTs is surgery if the lesion is resectable with no metastases, or therapy with tyrosine kinase inhibitors if the lesion is unresectable, metastatic, or in case of recurrent disease. The prognostic factors are tumor location, tumor size, mitotic index, and type of mutation. When feasible, laparoscopic surgery is the recommended option for management of GISTs because it is associated with more favorable outcomes in terms of complications, length of hospital stay, postoperative pain and recurrence rates, as compared to the open approach.

Conflict of Interest: The authors declare no potential conflict of interest with respect to research, authorship and/or publication of this chapter.

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The Role of Macrophage-Derived Extracellular Vesicles in Gastrointestinal Cancers

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Abstract: Extracellular vesicles are lipid-bound vesicles derived from cells that can interact with other cells, participate in cell signaling, and transfer biologically active molecules. The pro-tumorigenic role of extracellular vesicles has been extensively investigated. The production of these vesicles occurs in both physiological and pathological processes by many cell types, including the macrophages. Macrophages have differential role in tumor biology: the M1 macrophages are cytotoxic (anti-tumor activity), and the M2 macrophages are pro-tumorigenic. A subpopulation of these macrophages is described as tumor-associated macrophages and several studies have described the importance of extracellular vesicles

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derived from tumor-associated macrophages in the advancement and progression of gastrointestinal cancers. This chapter highlights the role of macrophage-derived extracellular vesicles in gastric, hepatic, pancreatic, and colorectal tumors. It also discusses the importance of molecules and cell signaling pathways involved in this context and emphasize the relevant role of these extracellular vesicles in tumor development.

Keywords: extracellular vesicles in gastrointestinal cancers; gastrointestinal cancer; macrophage; macrophage-derived extracellular vesicles; microRNA.

INTRODUCTION

Extracellular vesicles (EVs) are lipid-bound small vesicles derived from the plasma membranes or interior of the cells. EVs are divided into two main groups: larger EVs or microparticles (100 nm to 1000 nm in diameter), and smaller EVs or exosomes (10 to 150 nm in diameter). Apart from these, apoptotic bodies, formed during the final stages of cellular apoptosis, are also considered a type of EV (1, 2). EVs are crucial for cell signaling and transferring biologically active molecules such as lipids, proteins, and nucleic acids. EVs can interact with other cells through the delivery of compartmentalized material or can be taken up by target cells. The production of EVs occur both in physiological and pathological conditions such as cancer (3). Studies associate the presence of EVs with tumor development, invasion, angiogenesis, and metastasis (3). Studies on the interaction of tumor-derived-EVs with the immune system cells have recently increased considerably. Moreover, the role of EVs derived from immune cells as modulators of cellular responses has gained attention; in this context, we highlight the role of macrophage-derived EVs in tumor progression (4).

Macrophages are classified into two different subpopulations based their polarization status: M1 (classically activated) and M2 (alternatively activated). These states represent only a portion of the existing subpopulations of macrophages. M1 macrophages are known for their cytotoxic activities against cancer cells whereas M2 macrophages play a role in the elimination of pathogens, angiogenesis, and extracellular matrix remodeling with subsequent tissue repair (5). Since 1970, macrophages have been described within the tumor microenvironment; these cells, known as tumor-associated macrophages (TAM), assemble M2 macrophages, and are capable of promoting tumor growth (6). Recently, many studies have described the importance of TAM-derived EVs (TAM-EVs) in the advancement and progression of gastric, liver, pancreas, and colorectal tumors. This chapter highlights the interaction of EVs released by macrophages in gastrointestinal cancer, as can be observed in Figure 1 and summarized in Table 1.

GASTRIC CANCER

Gastric cancer (GC) is the fifth most common malignant tumor and the fourth cause of cancer-related deaths worldwide (7). The major cause of GC is *Helicobacter pylori* infection, leading to approximately 870,000 new GC cases annually

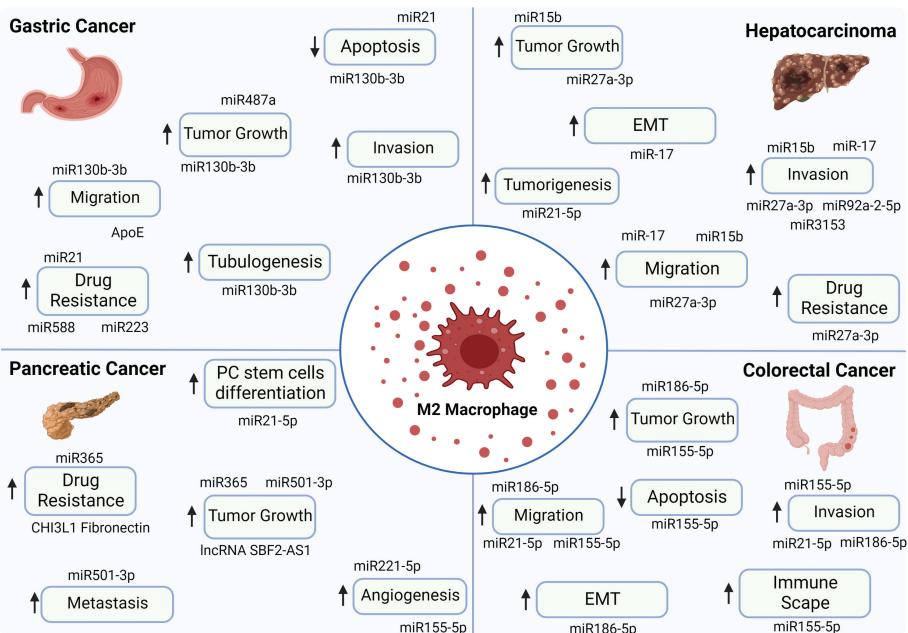


Figure 1. Extracellular vesicles released by macrophages and their effects on gastrointestinal cancers. Created with BioRender.com. EMT, epithelial mesenchymal transition; miR, microRNA, PC, pancreatic cancer.

TABLE 1

Extracellular vesicles released by macrophages and their effects on gastrointestinal cancers

Gastric Cancer

| Vesicle Origin | Content | Effect | Mechanisms | Reference |
|-------------------------------|------------|--|---------------------------|-----------|
| M2 macrophages from THP-1 | miR130b-3b | Apoptosis protection; Induces migration, invasion, tubulogenesis, and tumor growth | Suppresses MML3 and GRHL2 | (17) |
| M2 macrophages from RAW 264.7 | N.D | Increases TFF2 and GSII Lectin | N.D. | (13) |
| M2-EV | miR487a | Induces cell proliferation, tumor growth | Inhibits TIA1 | (16) |

Table continued on following page

TABLE 1

Extracellular vesicles released by macrophages and their effects on gastrointestinal cancers (Continued)

| | | | | |
|--|----------|---|--------------------------|------|
| M2 macrophages from human and murine (C57BL/6) bone marrow monocytes | ApoE | Induces migration | PI3K – AKT | (14) |
| M2 macrophages | miR21 | Decreases gastric cancer cell apoptosis; Drug resistance against Cisplatin | Downregulates PTEN | (21) |
| M2 macrophages from THP-1 | miR223 | Drug resistance against doxorubicin | Inhibits FBXW7 | (22) |
| M2 macrophages from murine bone marrow monocytes | miR588 | Drug resistance against Cisplatin | Inhibits Cylindromatosis | (23) |
| M1 macrophages | miR16-5p | Activates T Cell Reduces tumor growth | Downregulates PD-L1 | (24) |

Pancreatic Cancer

| Vesicle Origin | Content | Effect | Mechanism | Reference |
|--|-------------------------|--|----------------------------|-----------|
| M2 macrophages from murine (C57BL/6) bone marrow monocytes | miR155-5p; miR221-5p | Induces angiogenesis | N.D. | (30) |
| M2 macrophages from THP-1 | miR365 | Induces tumor growth | Activates BTG2/FAK/ AKT | (29) |
| M2 macrophages from THP-1 | miR501-3p | Induces tumor growth and metastasis | Activates TGFB (TGFBR3) | (27) |
| M2 macrophages from THP-1 | miR21-5p | Promotes the differentiation and activity of PC stem cells | Activates KLF3 | (31) |
| M2 macrophages from THP-1 | lncRNA SBF2-AS1 | Induces tumor growth | Enhances XIAP | (27) |

Table continued on following page

TABLE 1
**Extracellular vesicles released by macrophages and their effects on gastrointestinal cancers
(Continued)**

| M2 macrophages from THP-1 cells | miR365 | Drug resistance against Gemcitabine | N.D. | (26) |
|--|------------------------|---|---------------------------------------|-----------|
| M2 macrophages | CHI3L1 and fibronectin | Drug resistance against Gemcitabine | Activates ERK | (28) |
| M1 macrophages from THP-1 | N.D. | Reduces Drug resistance against Gemcitabine and Deferasirox | N.D. | (32) |
| Hepatocarcinoma | | | | |
| Vesicle origin | Contente | Effect | Mechanism | Reference |
| M2 macrophages from THP-1 | miR27a-3p | Induces stemness, proliferation, invasion, migration, tumor growth, and drug resistance in HCC cells; | Downregulates TXNIP | (34) |
| M2 macrophages | miR17 | Increases expression of EMT- related proteins and stemness-related genes; Migration and Invasion | Impairment on TGF- β 1/BMP-7 | (35) |
| M2 macrophages from murine bone marrow monocytes | miR21-5p | Increases levels of PD1 and TIM3 on CD8 $^{+}$ T cells and higher malignant degree of tumorigenesis in C57/BL6 mice | Activates YOD1/ YAP/ β -catenin | (44) |

Table continued on following page

TABLE 1

Extracellular vesicles released by macrophages and their effects on gastrointestinal cancers (Continued)

| | | | | |
|--|--------------------------|---|---|------|
| M2 macrophages from THP-1 polarized in contact to arsenic | miR15b | Increases proliferation, migration, and invasion | Downregulates LATS1 and inhibits Hippo pathway, and growth of HCC xenografts on nude mice | (45) |
| M2 macrophages from THP-1 polarized in contact with hepatic cancer cells | miR92a-2-5p and miR 3153 | Increases invasion | Suppresses AR and PHLPP and increases AKT phosphorylation | (38) |
| M1 macrophages from THP-1 | miR326 | Decreases proliferation and colony formation ability and suppression of migration and invasion; Reduces tumor growth. | Inhibits NF-kB | (46) |
| TAM treated with propofol | miR142-3p | Inhibits invasion | Downregulates RAC1 | (47) |

Colorectal Cancer

| Vesicle origin | Contente | Effect | Mechanism | Reference |
|---|-----------------------|---|-------------------------------|-----------|
| M2 macrophages from THP-1 | miR186-5p | Induces migration, invasion, and tumor growth, increasing EMT | Downregulates DLC1; Activates | (48) |
| M2 macrophages from colorectal cancer tissues | miR155-5p miR21-5p | Induces migration and invasion | Downregulates BRG1 | (49) |
| M2 macrophages from colon cancer tissues | miR155-5p | Induces proliferation and Immune escape; Inhibits apoptosis | Downregulates ZC3H12B | (50) |

Table continued on following page

TABLE 1
Extracellular vesicles released by macrophages and their effects on gastrointestinal cancers (Continued)

| | | | | |
|--|------------------|---|------|------|
| M1 macrophages from MC38 subcutaneous tumor models | TAM-EVs proteins | Induces T cells proliferation and activation of proteins related to immune response, inflammation, cell migration and adhesion, signal transduction, as well as proteins associated with lipidic metabolism and transport | N.D. | (51) |
| M1 macrophages from RAW 264.7 | N.D. | Decreases tumor cell viability | N.D. | (52) |

ApoE, apolipoprotein E; AKT, protein kinase B; EMT, epithelial mesenchymal transition; FBXW7, WD repeat domain-containing 7; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GRHL2, grainyhead-like 2; LATS1, large tumor suppressor kinase 1; miR, microRNA; N.D., not defined; NF- κ B, nuclear factor κ B; PI3K, phosphoinositide 3-kinases; PHLPP, PH domain leucine-rich repeat-containing protein phosphatase; TAM, tumor-associated macrophage; TGF, tumor growth factor; TIA, T-cell intracellular antigen-1; TXNIP, Thioredoxin-interacting protein; YAP, yes-associated protein 1; XIAP, X-linked inhibitor of apoptosis protein.

worldwide (8, 9). Other risk factors include age, obesity, smoking, and dietary habits (10). Because early stage of GC is asymptomatic, most cases are diagnosed at advanced stages, compromising effective treatment (11). The crosstalk between GC and macrophages occurs via extracellular vesicles, produced from gastric tumor cells and macrophages (12). Macrophages treated with deoxycholic acid release EVs, which in turn increase spasmolytic polypeptide-expressing metaplasia markers (TFF2 and GSII lectin) in gastric organoids, a significant risk factor for GC (13). M2-EVs could be internalized by gastric cancer cells, transferring ApoE, an apolipoprotein expressed in TAM within the tumor microenvironment. Once ApoE is internalized, the PI3K-AKT signaling pathway is activated, promoting tumor cell migration (14).

Several studies have shown the involvement of microRNA (miR)-enriched EV in the initiation, progression, angiogenesis, metastasis, and chemoresistance of GC (15). Yang and colleagues observed that M2 macrophages could induce gastric tumor cell proliferation in an EV-dependent manner (16). The authors observed that the presence of miR487a in M2-EVs targeted the T-cell intracellular antigen-1 (TIA), leading to gastric cancer cell proliferation and tumor growth (16). M2-EVs containing miR130b-3b have the same ability to promote GC growth. Zhang and colleagues showed an increase of miR130b-3b in GC and

gastric tumor cells (17). EVs derived from THP1-differentiated M2 macrophages were rich in miR130b-3b, leading to apoptosis protection, migration, invasion, tubulogenesis, and tumor growth (17). M2-EVs also downregulated MML3 (myeloid/lymphoid or mixed-lineage leukemia 3), which in turn downregulated Graniyhead-like 2 (GRHL2) (17), a tumor suppressor (18). Low expression of MML3 has been implicated in low survival of GC patients (19).

Macrophage-derived EVs, obtained by differentiating U937 cells to macrophages, loaded with miR21 inhibitor induced apoptosis and inhibited migration of gastric tumor cell (20). Corroborating these findings, Zheng and colleagues observed, in vitro and in vivo, that M2-EVs can deliver miR21 to gastric tumor cells (20). The authors also observed that miR21 can downregulate PTEN, leading to suppression of cell apoptosis and PI3K/AKT signaling activation, conferring cisplatin resistance to gastric cancer (21). Additionally, macrophage-derived EVs containing miR223 can confer doxorubicin resistance in GC cells through inhibition of F-box and WD repeat domain-containing 7 (FBXW7). On the other hand, when miR223 were knocked down in macrophages, their M-EVs could not confer doxorubicin resistance in GC (22). Cui and colleagues also observed the role of miR588 in chemoresistance (23). The authors observed that EVs containing miR588 derived from M2 macrophages induced resistance to cisplatin in vitro and in vivo. Furthermore, miR588 targeted cylindromatosis, a deubiquitinating enzyme that counteracts the E3 ubiquitin ligases-mediated protein ubiquitination, which is essential to tumorigenesis (23).

While M2-EVs have a role favoring GC, M1-EVs can act in a counter way. Li and colleagues observed that M1-EVs carrying miR16-5p downregulate PD-L1, allowing T cell immune response, culminating in a reduction of GC progression (24). Thus, M1-EVs could represent an attractive cell-based therapy for GC treatment.

PANCREATIC CANCER

The pancreas is considered an essential organ of the digestive system and has relevant endocrine functions. Malignant tumors that affect this organ have been increasingly reported and studied. Pancreatic tumor types described as malignant can be related to endocrine or non-endocrine functions. Malignant tumors related to non-endocrine functions in the pancreas are the seventh cause of cancer mortality worldwide and are divided into two main classes: pancreatic ductal adenocarcinoma (PDAC), and cystadenocarcinoma. PDAC constitutes about 85% of pancreatic cancers (PC), with approximately 50,000 cases diagnosed yearly (7). Older age, obesity, diabetes mellitus, smoke, and alcohol are some risk factors that may be closely associated with a higher incidence of PC. Conditions such as chronic pancreatitis and family history can also be considered risks for developing this type of cancer. Treatment for patients diagnosed with PDAC are limited, and the survival rate of these patients is extremely low, and more therapeutic interventions need to be studied and described to improve patient survival (25).

The interaction of immune system cells with PC cells is widely described in the literature and is considered a key factor for tumor development and progression. TAM-EVs are already described as critical players in PC progression (26–29). As a

form of communication, immune system cells release EVs, which can affect the metabolism of target tumor cells. It was observed by Yang and colleagues that EVs from PC cells could polarize macrophages to the M2 phenotype, increasing the expression of specific markers for TAM, such as Arginase-1 and CD206 and, in turn, M2 macrophages-derived EVs (M2-EVs) were able to induce angiogenesis in PDAC (30). M2-EVs facilitated neovascularization in murine aortic endothelial cells, and these pro-angiogenic effects were attenuated when exosomes were removed from the conditioned medium of M2 macrophages. EVs also mediated increased migration, proliferation, and invasion by PC cells from macrophages (27, 29, 30).

It is well established that EVs are crucial elements that support communication between biologically active cells, exchanging nucleic acids (circRNA, mRNA, miR), lipids, and proteins, and act on intercellular signaling carrying important information to target cells (2). Several miR such as miR (27), miR365 (26, 29), and miR21-5p (31) have been described as present in M-EVs in PC, as crucial for sustaining PC development (27, 29, 31). Long non-coding RNAs (lncRNAs) present in M-EVs have also been described as a promoter of PC development (PANC-1 cell line). lncRNA SBF2-AS1 downregulation can promote miR122-5p expression and reduce XIAP (X-linked inhibitor of apoptosis protein), limiting PC progression (27). Specific proteins have also been identified by proteomics in PC TAM-EVs, such as chitinase 3-like-1 (CHI3L1) and fibronectin (FN1), which are crucial for chemotherapeutics resistance mechanisms (28). M2-EVs were also able to promote angiogenesis *in vivo* in nude mice by carrying specific microRNAs to endothelial cells, such as miR155-5p, miR221-5p (30), and miR501-3p (27).

miR365 expression in M-EVs promotes the expression of the same miR in PANC-1 and BxPC3 (tumor cell lines) compared to non-tumor cells and induces resistance to specific drugs used in chemotherapy, such as gemcitabine in a murine model (26) through ERK pathway activation (28); Proliferation, migration, and invasion were increased in tumor cells treated with M2-EVs. Treatment with an exosome generation inhibitor (GW4869) mitigated these effects, suggesting the importance of exosomes for PC development. Specific proteins involved in epithelial-mesenchymal transition (EMT) have been observed in PC cell lines, with increased mesenchymal marker expression such as vimentin and SNAIL and decreased epithelial marker e-cadherin expression; this suggests plasticity, mobility, and invasion induction. When miR365 was silenced in M2-EVs, PC cells malignant behaviors such as proliferation, migration, invasion, expression of apoptotic genes, and EMT markers were attenuated. miR365 suppression has been shown to limit tumor growth in mice and negatively regulate BTG2/FAK/AKT pathway. Thus, overexpression of BTG2 could reverse the pro-tumorigenic effect of M2-EVs in PC (29). Yin and colleagues noted that TAM recruitment in PDAC is highly associated with PC metastasis once TAM infiltrates were observed in tissues of metastatic patients other than healthy patients (27). TAM infiltrate also correlated with increased expression of miR501-3p. M2-EVs also increased PDAC cells migration and invasion in nude mice, promoting tumor formation and metastasis with a significant increase in tumor weight and volume through the TGF β pathway (TGF β R3 downregulation) (27).

Chang and colleagues observed M2-EVs ability to promote PC stem cell differentiation (via miR21-5p high expression) and sustain cancer development through migration, invasion, and protection from apoptosis by M2-EVs (31). High levels of miR21-5p are considered a critical factor for poor prognosis in PC

patients, so M2-EVs with miR21-5p downregulation were able to decrease tumor stem cells differentiation in vitro and in vivo, decreasing tumors volume and size in an animal model, via KLF3 pathway activation that acts as an important tumor suppressor gene (31).

On the other hand, M1-EVs improved chemotherapeutic response to gemcitabine and deferasirox when delivered to tumor cells inside M1-EVs, decreasing proliferation, migration, and chemoresistance in PC (32). In this way, we highlight the importance of M2-EVs for the development and tumor progression of PC (26–31) and M1-EVs as a possible therapeutic strategy for PC treatment improving chemotherapeutic agents' efficiency as a front line to combat the disease (32).

HEPATOCELLULAR CARCINOMA

Since 1980, liver cancer incidence and death rates have tripled and doubled, respectively (33). This disease was predicted to be the sixth most diagnosed cancer worldwide in 2020, and liver cancer was the third cause of death among cancers, with 830,000 deaths (7). Among primary liver cancers, hepatocellular carcinoma (HCC) is the main pathological type and the most common, accounting for about 80% of the cases (34, 35). HCC has a poor prognosis due to its heterogeneity (35) and limited therapeutic methods, which are often only available for the early stages of cancer (36). Interactions between tumor cells and their microenvironment play a role in its heterogeneity and pathogenesis. More precisely, the interaction between cancer cells and the growth factors and cytokines released by the microenvironment cells can affect their plasticity (37) and promote liver fibrosis, initiation, progression, and metastasis (34, 38).

Even though immune cells have a tumor suppressive role, they can be affected by cancer cells, thus contributing to tumor development. Macrophages, which in the liver are called Kupfer cells, are the most abundant immune cells in this tissue and establish a direct relationship with hepatocytes to maintain homeostasis (39). TAM within the HCC tumor microenvironment promotes cell proliferation, invasion, migration, angiogenesis, and immunosuppression and is often correlated with poor prognoses (40). Besides their capacity to directly transfer cellular lipids and proteins to other cells, macrophages secrete extracellular vesicles affecting neighboring cells.

Li and colleagues have demonstrated that EVs derived from THP-1 cells driven into M2 macrophages increased miR27a-3p in SMMC-7221 HCC cell lines, increasing their proliferation, migration, and invasive capacities (34). In addition, M2-EVs further enriched in miR27a-3p promoted stemness, proliferation, invasion, migration, drug resistance in HCC cells, and accelerated tumor growth in vivo in a nude mice model. According to the authors, these effects on HCC cells were promoted by miR27a-3p-induced TXNIP downregulation (34). Accordingly, it has been demonstrated that TAM-EVs deliver MIR17HG and miR17 to HepG2 HCC cells, leading to an impairment on TGF- β 1/BMP-7 pathways, an increased expression of EMT-related proteins and stemness-related genes, and both migratory and invasive capacities. Corroborating, in vivo experiments conducted in mice have demonstrated that the treatment with TAM-derived EVs increased the

expression of ACVR1, ID1, and vimentin and decreased the expression of E-cadherin in M2-scarce tumor xenografts (35).

CD8⁺T cells-mediated immune response is critical for inhibition of HCC progression. Their dysfunction is one of the main reasons for tumor escape from the immune system, together with the presence of excessive T suppressor cells. When in the condition of prolonged antigen exposure, CD8⁺T cells undergo exhaustion, weakening their anti-tumoral effect (41–43). Pu and colleagues demonstrated that the injection of HCC cells together with EVs derived from murine bone marrow monocytes differentiated into M2 macrophages promoted a higher malignant degree of tumorigenesis in C57/BL6 mice together with a decrease in the number of CD8⁺T cells; these were accompanied by increased levels of PD1 and TIM3 on CD8⁺T cells, and impairment in both their proliferative and killing capacity (44). Straightforward, they have demonstrated the role of the miR21-5p content within EVs on CD8⁺T cells exhaustion once the inhibition of miR21-5p in EVs partially reverted this effect. The authors have concluded that these effects were mediated by miR21-5p-dependent YOD1 targeting, thus favoring the YAP/β-catenin pathway (44).

It is known that arsenic, an environmental toxicant, causes HCC; recently, it has been demonstrated that when in contact with arsenic, monocytes from THP-1 lineage were polarized towards M2 macrophages and released EVs enriched in miR15b.

When those EVs were transferred to HCC cells, they promoted downregulation of large tumor suppressor kinase 1 (LATS1), thus, inhibiting the Hippo pathway, which is involved in inhibiting the occurrence of metastasis on HCC. Therefore, HCC cells underwent increased proliferation, migration, and invasion. Corroborating those data, EVs from M2-THP-1 macrophages inhibited Hippo signaling, promoting the growth of HCC xenografts on nude mice (45).

When in co-culture with hepatic cancer cells, THP-1 macrophages presented increased expression of the M2 markers: arginase-1, CD163, CD206, and TGF-β; in addition, M-EVs transferred both miR92a-2-5p and miR3153 to HCC cells, resulting in the suppression of both androgen receptor (AR) and PHLPP expression, culminating in AKT phosphorylation and increased invasiveness (38).

On the other hand, studies have demonstrated that M-EVs can suppress HCC cell progression. Bai and colleagues have demonstrated that M1 macrophages derived from THP-1 cells deliver miR326 to HCC cells, reducing their proliferation and colony formation ability and suppressing both migratory and invasive capacities (46). Furthermore, those EVs also promoted HCC cells apoptosis. Authors have concluded that the suppressive roles of miR326 were performed through the inhibition of NF-κB in HCC cells. It is worth mentioning that these results were also observed in vivo; in mice transplanted with HCC cells, the treatment with M1-EVs overexpressing miR326 reduced both tumor volume and weight (46). EVs released by TAM treated with propofol have also demonstrated anti-tumor effects via delivery of miR142-3p to HCC, culminating in RAC1 down-regulation, which inhibited cell invasion both in vitro and in vivo (47).

Conversely, it is essential to highlight that transfer of miR from macrophages to hepatic cancer cells is not exclusively dependent on EVs. Aucher and colleagues have demonstrated that the transfer of miR425 and miR122 from human macrophages to hepatic HuH7 HCC cells was only triggered by direct contact, dampening cell proliferation (39).

COLORECTAL CANCER

Colon is the lower region of the intestinal tract, ranging from the caecum to the rectum, having water and salts absorption function from non-digested foods, and elimination of waste products with mass movements (33). Estimates of the GLOBOCAN 2020 showed that colorectal cancer is the third in incidence and the second in the cause of death considering both sexes worldwide (7). The tumor stage strongly influences patients' survival at the time of diagnosis. Data show that colorectal cancer correlate is more common in developed countries (33).

To highlight the importance of macrophages-derived extracellular vesicles in colorectal cancer, Guo and colleagues conducted a study using human colorectal adenocarcinoma cell lines, SW480 and HCT-8, and a human monocyte cell line, THP-1 differentiated to M2 phenotype (48). The study showed that M2-EVs could deliver miR186-5p to cancer cells, being the most abundant microRNA in M2-EVs. miR186-5p performs a pro-tumor function, increasing migration, invasion, and growth of cancer cells, besides negatively regulating the mRNA and protein expression of DLC1, a tumor suppressor gene. This microRNA also induces the β -catenin signaling pathway, associated with the EMT process. Altogether, these results demonstrate that miR186-5p is responsible for promoting the effects of M2-EVs and paves the way for the investigation of other microRNAs delivered to cancer cells within M2-EVs and display a similar pro-tumor effect (48).

Lan and colleagues, using human colon cancer cell lines SW48, SW480 e CO-115, demonstrated that M2-EVs extracted from samples of colorectal cancer patients co-cultivated with colorectal cancer cells could influence tumor biology, allowing the transfer of functional molecules (49). Among these molecules, miR21-5p and miR155-5p are overexpressed in M2-EVs and have protumorigenic action that directly influences migration and invasion of tumor cells. One of these microRNAs targets is BRG1, reducing its expression and involving in tumor metastasis (49). Similarly, Ma and colleagues have shown that M2-EVs from colorectal cancer patients have anti-apoptotic action and demonstrated a new target, ZC3H12B, which is regulated negatively by promoting immune escape through IL6 upregulation in human colon cancer cell lines SW48 e HT29 (50).

Using a subcutaneous tumor model with MC38 cell inoculation in mice, Cianciaruso and colleagues have shown that TAM-EVs derived from this model have an M1 profile, correlated with the increase in survival (51). It was also observed that M1-EVs appear to be able to stimulate T cells. Through proteomic analysis, the study showed that TAM-EVs express 62 proteins related to immune response, inflammation, cell migration, adhesion, and signal transduction, as well as proteins associated with lipid metabolism and transport (51).

The importance of EVs in the biology of colorectal tumors has been proven in recent years, as shown above. However, could EVs be used in the therapeutic field? Veld and colleagues evaluated M1-Like and M2-Like macrophages as photosensitizers carriers of Zinc Phthalocyanine (ZnPc) for photodynamic therapy for cancer treatment (52). The study used the MC38 colon cancer cell line and RAW264.7 mouse monocyte/macrophages. The results showed that EVs could function as drug carriers, with M1 containing ZnPc being capable of reducing MC38 viability showing efficiency for the photodynamic therapy (52).

CONCLUSION

We have compiled how extracellular vesicles released by M2/TAM macrophages are associated with the progression of gastrointestinal cancers, while those from M1 macrophages exert anti-tumoral effects. Furthermore, we highlight two possible strategies for cancer management: blocking the release of M2-EVs or inducing M1-EVs within the tumor microenvironment.

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Multigene Expression Biomarkers and Score Systems for Predicting Therapeutic Benefit in Gastrointestinal Cancers

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Abstract: Gastrointestinal cancer is a leading cause of death among cancer patients worldwide. For both gastric and colorectal cancers, the 5-year overall survival for advanced stages remain low. Their polygenic and heterogeneous nature is characterized by alterations in multiple molecular pathways throughout its development, which is a big challenge for patient risk stratification and for treatment options. In this chapter, we describe the development of prognostic and predictive multigene signatures in gastrointestinal cancer patients for clinical use. We identified and validated a novel 53-gene prognostic signature and score system that robustly and reliably predicts overall survival in gastric cancer patients. We also discovered that the predictive potential of the 53-gene signature that can

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identify gastric cancer patients who may benefit from adjuvant FOLFOX chemotherapy. In addition, we developed a 15-gene signature with robust prognostic function in colorectal cancers. Both signatures are independent of molecular subtypes and clinical outcomes. The predicting capability of these signatures supersedes previously published prognostic signatures in the same types of cancers. For clinical application, we developed a nucleic acid hybridization-based gene expression assay for the signatures. Future prospective studies are warranted to test the clinical value of these multigene signatures and fully deploy them into patient use.

Keywords: colorectal cancer; gastric cancer; multigene expression assay for gastrointestinal cancers; multigene expression biomarkers for gastrointestinal cancers; overall survival

INTRODUCTION

Worldwide, gastrointestinal cancers represent more than one-quarter of the cancer incidence and over one-third of all cancer-related deaths (1). Currently, curative surgery with adjuvant chemotherapy is the most common treatment design for stage II–III gastric cancer and stage III colorectal cancer. Despite some improvements in recent years, the 5-year overall survival for advanced stages of gastrointestinal cancers remains low (below 30% for gastric cancer and about 14% for stage IV colorectal cancer). This may be contributed by many factors including genetic, histopathological, and clinical variations among patients. Therefore, it is a big task to identify those factors with critical and independent value for predicting patient clinical outcome for a more accurate personalized risk assessment for treatment decisions.

Both gastric cancer and colorectal cancer are polygenic disorders with variable responsiveness to treatment such as chemotherapy and immunotherapy, as observed in clinical practice. Recent comprehensive omics studies with microarray technology and next generation sequencing/genome wide association studies have unveiled vast genomic information and many heterogeneous features of the two diseases (2–5). For example, four molecular subtypes have been identified in gastric cancer (Epstein-Barr virus (EBV)-positive, microsatellite unstable, genetically stable and chromosomal instability) (5) and colorectal cancer (microsatellite instability, genome stable, chromosomal instability, and hypermutated-single nucleotide variant) (6) through comprehensive molecular profiling using The Cancer Genome Atlas (TCGA). Such classification reflects both background genetics and molecular pathogenetic features. However, new biomarkers are needed to identify gastrointestinal cancer patients for susceptibility toward the clinical therapies.

To bridge this gap, genomic biomarkers have increasingly been developed and utilized in recent years, to stratify patients and predict clinical outcome, for instance, being used as prognostic and predictive biomarkers in various types of cancers (7–10). Such genomic assays on predicting clinical outcome may aid physicians in determining a most suitable clinical therapy for the patient, as effectively shown in the breast cancer with FDA-approved Oncotype DX (8) and MammaPrint (7) tests.

In gastrointestinal cancers, numerous reports on gene expression patterns have been published to predict patient outcomes such as recurrence, metastasis, and benefit from adjuvant therapies (11–18). However, to the best of our knowledge, extended validation of bioinformatics findings is rare with such biomarker signatures, and these have not yet been clinically implemented, except for the 7-gene *Oncotype DX* colon cancer test for the prediction of recurrence risk for stage II and III colorectal cancers (19, 20).

In this chapter, we describe the development of prognostic and predictive multigene signatures in gastrointestinal cancer patients for clinical use. We first identified a novel 53-gene prognostic signature and score system that robustly and reliably predicts overall survival in gastric cancer patients (12), which were validated in multiple centers (16). We also discovered the predictive potential of the 53-gene signature that can identify gastric cancer patients who may benefit from adjuvant FOLFOX (leucovorin calcium, fluorouracil, and oxaliplatin) chemotherapy (16). Later, we developed a 15-gene signature with robust prognostic function in colorectal cancer (21). Both signatures are independent of molecular subtypes and clinical outcomes. The predicting power of these two signatures supersedes previously published prognostic signatures in the same types of cancers. For clinical application, we developed a nucleic acid hybridization-based gene expression assay for the signatures and successfully employed it in a multiple hospital-based retrospective cohort study (16). Effective translation of laboratory findings into medical practice depends on both their clinical implications and assay development.

PROGNOSTIC/PREDICTIVE MULTIGENE EXPRESSION SIGNATURE DEVELOPMENT IN GASTROINTESTINAL CANCERS

The research interest in our laboratories has been in identifying distinct subsets of cancer patients with prognostic and/or predictive outcomes for precision medicine. We developed a novel multi-step bioinformatic analytic strategy to identify robust multi-gene expression prognostic/predictive signatures and to build related scoring systems for clinical use. Figure 1 shows how to mine publicly available omics data and associated clinical information, e.g., TCGA (RNA-sequencing data) and Gene Expression Omnibus (NCBI-GEO) (microarray-based), followed by a canonical discriminant analysis to establish a 53-gene expression signature. In the process, the Kaplan-Meier method together with Cox regression analysis was used to evaluate association of the gene expression levels with patient overall survival in gastric cancer (12, 16).

In addition to the gastrointestinal cancers, we have also successfully established a 27-gene panel for lung adenocarcinoma (22), and a 11-gene panel for ovarian cancer (23). In such cases, we first demonstrated that these expression-based signatures were able to better predict prognosis in comparison with other already published multigene signatures using the same datasets, as described in detail below, which clearly indicate that our strategy has its own advantage. As a matter of fact, this is the most important step, otherwise there is no meaning to

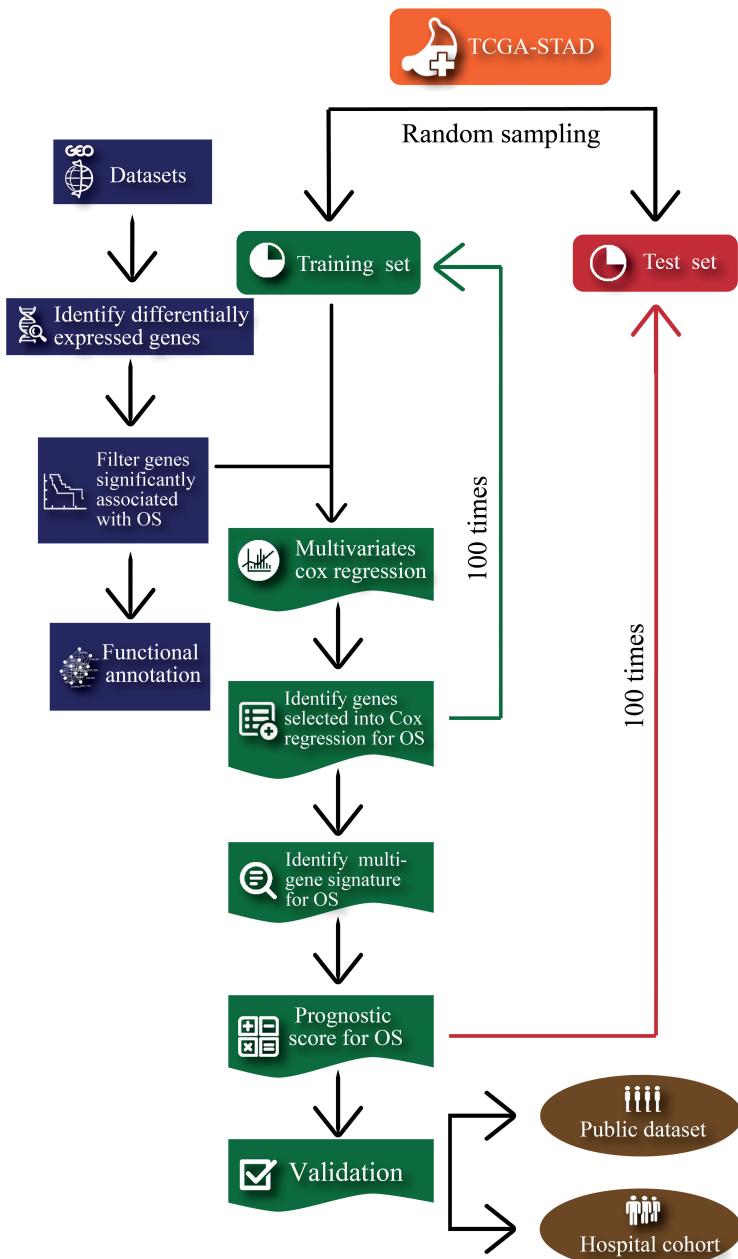


Figure 1. A working flowchart for the identification, development, and validation of a prognostic multigene signature (using gastric cancer as an example). Left panel: screening of consistently unregulated genes in gastric cancer tissue using meta-analysis and identification of overall survival-associated genes with Kaplan–Meier analysis using log-rank testing. Right panel: the steps to develop a 53-gene expression based prognostic signature and score system using TCGA-STAD.

develop a multigene biomarker that is inferior in predicting ability to other already published ones.

Next, to check whether the prognostic impact of a signature is independent of any potential confounding factors, we usually perform univariate and multivariate Cox regression analysis on all available clinicopathological parameters that may affect the prognostic capability. In addition, we examine and rule out any significant correlation of our signature with molecular subtypes of the same cancer type. For example, we investigated whether the prognostic value of the 53-gene and 15-gene signatures would be enhanced in certain molecular subtypes of gastric cancer or colorectal cancer, as such subtypes are associated with different survival outcomes and treatment benefits.

The validation of a newly established signation is usually carried out using two different sets of data: (i) transcriptome data from publicly available independent datasets; and (ii) collection of patient samples to perform gene expression assay in a cohort study, ideally a multicenter based one.

For a successful clinical application, it is essential to develop a reliable, sensitive, and high-throughput gene expression assay in suitable patient samples. RT-qPCR, as a mature technology, is routinely used to quantify mRNA levels of prognostic genes in clinical settings, as best demonstrated in the 21-gene Oncotype DX assay (7, 8). We recently developed a modified RNA hybridization assay using routinely prepared formalin fixed paraffin-embedded (FFPE) specimen, for the quantitative measurement of mRNA (16), which offers a 96-well high-throughput platform. This technique, as reported before, could be more reliable than RT-PCR to detect RNA or DNA signal in archived FFPE samples (24–26).

Another type of multigene prognostic/predictive biomarkers is based on a group of genes with similar functions. One example is using a panel of DNA repair genes to predict therapeutic responses in cancer patients. For example, as recent evidence revealed, DNA repair landscape is a significant factor in driving response to immune checkpoint blockade therapy (27). We developed a novel 15-DNA repair gene signature (DRGS) and scoring system to evaluate its efficiency in discriminating different molecular and immune characteristics and therapeutic outcomes of patients with gastric cancer (28). Multi-omics data analysis demonstrated that the patients with high DRGS score were characteristic of high levels of anti-tumor lymphocytes infiltration, tumor mutation burden (TMB) and PD-L1 expression, and such patients exhibited a longer overall survival and may benefit more from immune checkpoint blockade therapy, as compared to the low-score patients (28). Therefore, the DRGS and its scoring system may have implications in tailoring immunotherapy in gastric cancer.

53-GENE PROGNOSTIC ASSAY IN GASTRIC CANCER

In 2016, to test the hypothesis that tumor-specific genetic features of gastric cancer are a key driver of tumor outcome, which can be utilized to establish prognostic scoring to improve prediction of overall survival of gastric cancer patients, we analyzed differential gene expressions in gastric cancer using publicly available databases. We first identified 276 genes that were robustly differentially expressed between normal and gastric cancer tissues in TCGA gastric adenocarcinoma

cohort (TCGA-STAD) and NCBI- GEO (GSE30727), of which, 249 genes were discovered to be significantly associated with overall survival by univariate Cox regression analysis (12). Functional annotation studies showed that significant enrichment of these genes in cell cycle, RNA/ncRNA process, acetylation and extracellular matrix organization. Finally, a 53-gene signature was established, and a prognostic scoring system developed based on a canonical discriminant function of 53 genes and successfully applied it to predict overall survival of gastric cancer patients in the TCGA gastric adenocarcinoma cohort (TCGA-STAD) as well as in the GSE15459 dataset (Figure 2) (12).

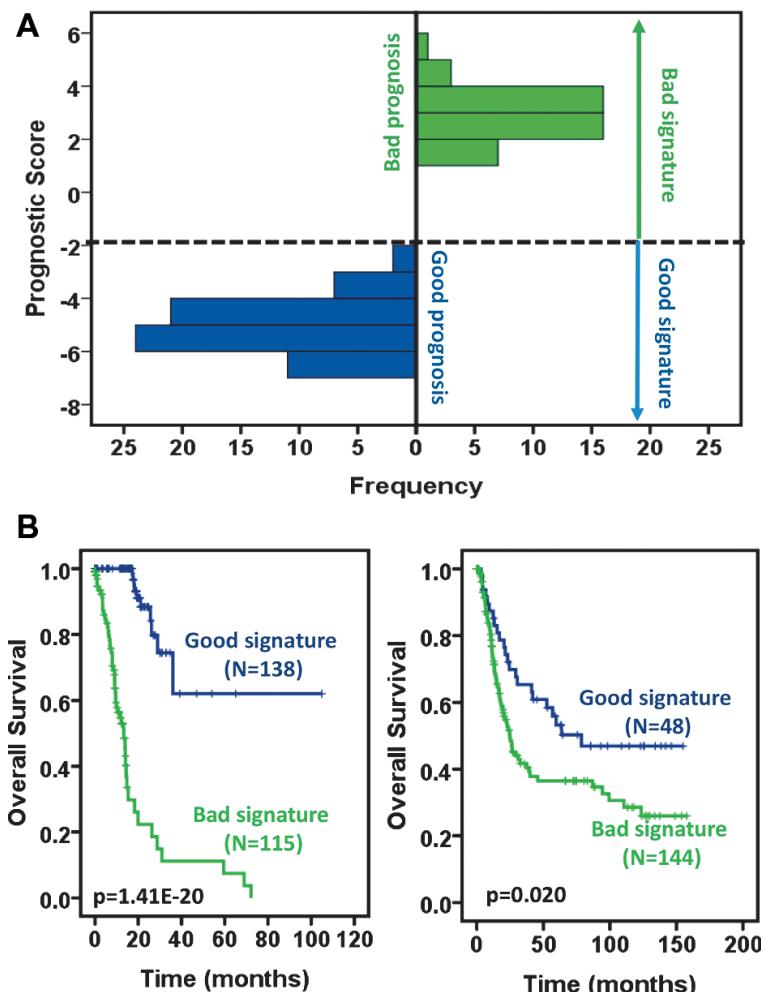


Figure 2. Development of a 53-gene prognostic scoring system for gastric cancer patients.
A. Distribution of prognostic score between patients with good and bad prognosis in the TCGA data. **B-C.** Prognostic scores are significantly associated with overall survival of gastric cancer patients in TCGA (B) and GSE15459 (C) as demonstrated by Kaplan-Meier survival curves. The p values were obtained from a log-rank test between two groups. This figure is taken from reference 12 with permission.

Using cross-validation approach combined with a multivariate Cox regression analysis, we evaluated and compared the performance of our 53-gene signature with three other published multigene models (13–15) in TCGA-STAD. We discovered that the differences between our signature and any of the other three signatures were significant for both the “intermediate vs. good” and “poor vs. good” groups ($p<0.0001$) (Figure 3A), indicating that the 53-gene score

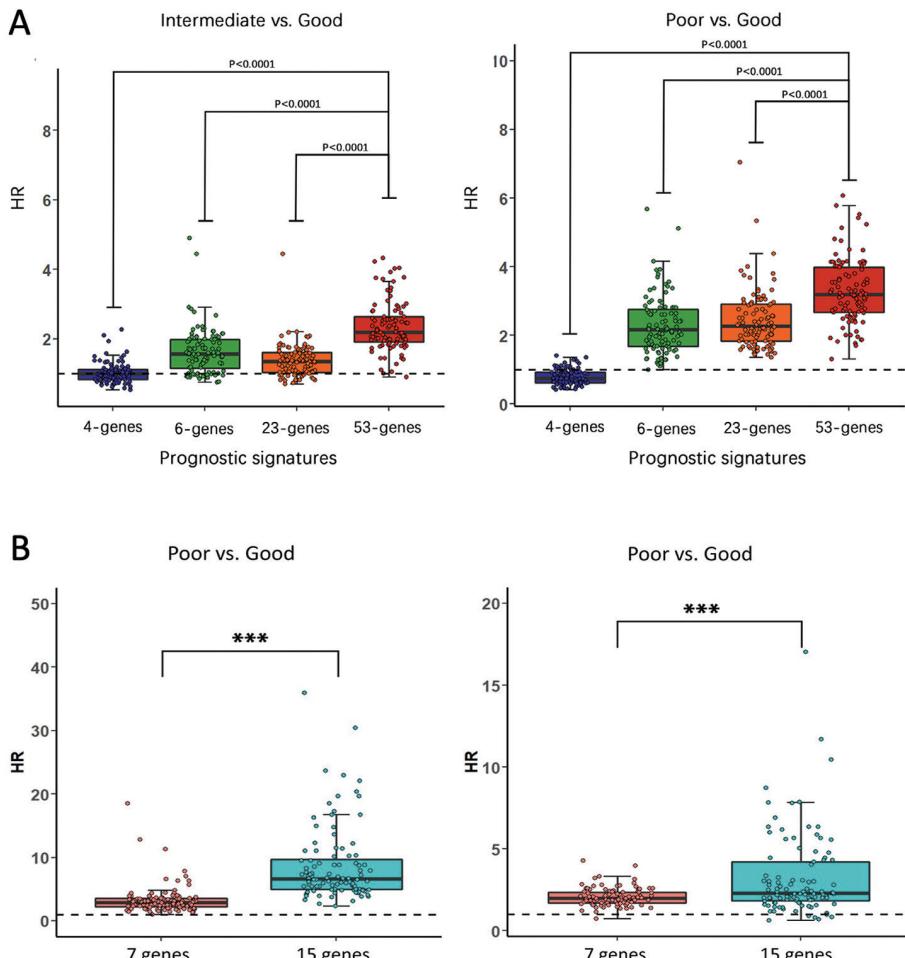


Figure 3. Comparison of the prognostic performance of our signatures with existing prognostic signatures in gastric cancer and colorectal cancer patients. For both signatures, the hazard ratio values of all the 100 test sets were calculated using a Cox model based on the prognostic score between groups. A. The differences between the 53-gene signature and other three signatures (13–15) were significant for both the intermediate vs. good and poor vs. good groups ($p<0.0001$, Mann-Whitney U test). This figure is taken from reference 16 with permission. B. Comparison of the 15-gene signature with the 7-gene Oncotype DX colon cancer signature. The comparison was made using the hazard ratio values obtained from 100 test sets between our 15-gene signature and the 7-gene panel in GSE17536 (A) and GSE28722 (B). For each of these datasets, the hazard ratio values calculated for poor vs. good were plotted. *** indicates $P < 0.001$ in Wicoxon test. This figure is taken from reference 21 with permission.

significantly performs better than other signatures in discriminatively determining overall survival of patients with gastric cancer (16).

Following establishing the 53-gene signature, we carried out a retrospective multi-center study and successfully validated the prognostic power of the 53-gene prognostic assay in 540 patients from three hospitals (enrolled between 2008–2013) using a reliable high-throughput mRNA hybridization-based assay (16). To the best of our knowledge, this is the first multi-center clinical study for validating a multi-gene expression signature in a relatively large-sized gastric cancer patient cohort (16). In this study, 180 patients from two hospitals were randomly selected to build a prognostic prediction model based on the 53-gene signature using leave-p-out (one-third out) cross-validation method together with Cox hazard regression and Kaplan-Meier analysis, and then the model was tested in the independent cohorts, a total of 360 patients with stage I–IV gastric cancers.

Multivariate Cox regression analysis demonstrated that the 53-gene signature predicts prognosis in gastric cancer patients independent of clinicopathologic information including age, gender, TNM staging, WHO histologic types and differentiation (16). Therefore, the 53-gene prognostic score is an independent prognostic factor.

One key discovery of this work is that this prognostic signature is also predictive of drug response in gastric cancer patients, when the effect of adjuvant FOLFOX (leucovorin, fluorouracil, and oxaliplatin) chemotherapy and other first-line chemotherapies were compared for patient overall survival in different prognostic score groups (16). The former is the most commonly used chemotherapy for gastric cancer after surgery in the patients enrolled in our study. We found that patients with good score had a significantly better 5-year overall survival rate from FOLFOX regime than those from other chemotherapy plans. As shown in Figure 4, for patients treated with FOLFOX, the 5-year overall survival rate can reach more than 80% in the group with good prognostic scores, which is significantly higher than ~60% in patients underwent other first-line chemotherapies ($P = 0.028$). However, we did not notice significant difference in intermediate and poor score groups between the FOLFOX and other treatment groups. These data indicate that patients with a good score may experience much better benefit

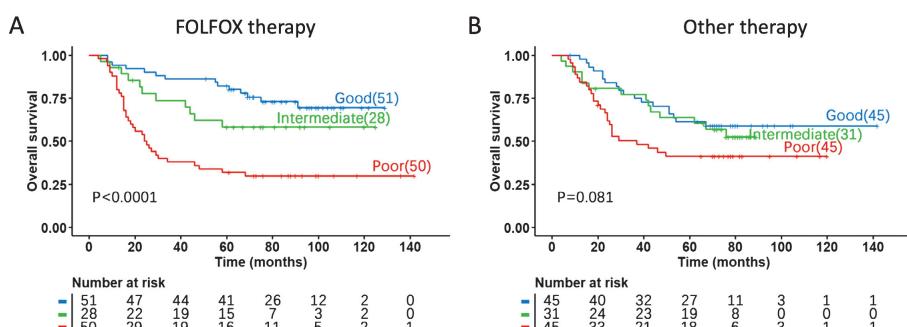


Figure 4. The predictive value of the 53-gene prognostic score in gastric cancer chemotherapy. The Kaplan-Meier curves and P values of overall survival in two different chemotherapy groups were plotted. A, Patients with FOLFOX; B, Patients with other first-line drugs/regimes. The P-values were obtained by log-rank test. This figure is taken from reference 16 with permission.

from FOLFOX chemotherapy after gastrectomy as compared with other chemotherapies. Therefore, the 53-gene prognostic signature could be a promising predictive biomarker for FOLFOX regimen.

15-GENE PROGNOSTIC SIGNATURE IN COLORECTAL CANCER

For our gene expression signature development in colorectal cancer, we first identified 738 genes that were consistently deregulated in colorectal cancer versus normal colon tissue in six transcriptome datasets (21). Of them, 78 genes were significantly associated with overall survival of colorectal cancer patients. Next, we utilized the concordance statistics for Cox modeling (29) to further refine the gene set with respect to their goodness-of-fit in survival models and to determine the optimal number of genes in the prognostic signature. The final set of 15 genes demonstrated clear discriminative capability to stratify colorectal cancer patients based on good versus poor prognosis (21).

With Cox regression analysis in two datasets, we compared the prognostic power of the 15-gene signature with the 7-gene panel in the *Oncotype DX Colon Test*. As shown in Figure 3, in the two GEO colon cancer datasets used, the median hazard ratio of our signature for poor versus good outcomes was 2.32- and 1.58-fold higher, respectively, as compared to the 7-gene signature, indicating that the 15-gene signature outperforms the 7-gene panel in predicting the overall survival of colorectal cancer patients.

To validate the 15-gene signature in different datasets, we used two datasets, GSE28722 and GSE39582, for Cox regression analysis. We found that high prognostic score patients had a significantly shortened overall survival compared to low score patients. Moreover, the efficacy of this signature was assessed in a retrospective cohort of 203 patients from Nanjing Drum Tower Hospital with stage I or II colorectal cancer. Overall survival analysis demonstrated significantly different survival rates ($P < .0001$ by log-rank test) among the three prognostic score groups in the above Chinese patient cohort (21) with early-stage colorectal cancer. Similar to gastric cancer, we examined whether the prognostic power of this signature is independent of clinicopathological factors potentially associated with patient outcomes in both The Cancer Genome Atlas Colon Adenocarcinoma (TCGA-COAD) and our Nanjing Drum Tower Hospital cohort. Data support that the prognostic effectiveness of the 15-gene signature was independent of all the clinical parameters tested, including molecular subtypes ($P < .05$) (21).

CONCLUSION

This chapter is focused on our recent study on developing prognostic and predictive multigene signatures/score systems in gastrointestinal cancer patients in clinical use. In conclusion, we identified and validated in both publicly available databases as well as multi-hospital cohorts a novel prognostic/predictive 53-gene signature that robustly and reliably predicts overall survival in patients with

gastric cancer. We also observed that the predictive potential of 53-gene signature-based score towards the benefit of FOLFOX chemotherapy. We also developed a 15-gene signature with similar functions in colorectal cancer, which was also validated in two independent public datasets and in one hospital. These signatures are independent of molecular classifiers and clinical variables that are associated with patient outcomes. Very critically, our data showed that both the 53-gene and 15-gene signatures supersede previously published prognostic signatures for gastric cancer and colorectal cancer, respectively. The nucleic acid hybridization-based gene expression assay developed is now applicable clinically to assess the overall survival for gastrointestinal cancer patients. For future directions, clinical prospective cohort studies with large patient sizes are warranted to fully deploy these multigene signatures and score systems into clinical use.

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Biomarkers for the Early Detection of Pancreatic Ductal Adenocarcinoma

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Abstract: Pancreatic ductal adenocarcinoma has one of the worst survival rates among adult cancers, with only 11% in the United States surviving five years after diagnosis. The majority of patients are diagnosed with late-stage disease, since early-stage pancreatic ductal adenocarcinoma is typically either asymptomatic or presents with non-specific symptoms. Pancreatic ductal adenocarcinoma thus remains a highly fatal disease. Today, surgical resection (removal of the pancreas) is the only potentially curative modality of treatment available. Detecting pancreatic cancer lesions early enough to perform surgery is, however, beset with difficulties. Nevertheless, the timeline of progression from low-grade precursor lesions to invasive cancer does offer a window of opportunity to detect the disease earlier than is currently possible. By providing physicians with actionable information early enough for the cancer to be removed surgically, the overall 5-year pancreatic ductal adenocarcinoma survival rate could increase from 11% to over 50%. In this chapter, we describe the development and clinical implementation of a proteomic, multi-biomarker blood test for the early detection of pancreatic ductal adenocarcinoma.

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Keywords: biomarkers for pancreatic adenocarcinoma; blood test for pancreatic cancer; CA19-9 in pancreatic cancer; early detection of pancreatic cancer; pancreatic ductal adenocarcinoma

INTRODUCTION

With an increasing incidence and a 5-year survival rate in the United States of just 11%, pancreatic ductal adenocarcinoma is one of the deadliest cancers (1, 2). Projections estimate that pancreatic cancer will surpass colon cancer by 2030 as the second leading cause of cancer death in the United States (3). As with any cancer, improving survival relies on detecting it at a potentially curable stage, which is particularly challenging with pancreatic ductal adenocarcinoma, given that early-stage cancers are typically asymptomatic or present with only non-specific symptoms (4). As a result, most patients are diagnosed with advanced non-resectable disease, and only 20% of sporadic pancreatic cancers are diagnosed during a potentially resectable stage (1, 2). A recent study of high-risk, asymptomatic individuals with germline cyclin dependent kinase inhibitor 2A (CDKN2A) mutations undergoing pancreatic carcinoma surveillance reported that 75% of tumors detected were resectable, resulting in a 5-year survival rate of 24%, a substantial increase over the typical 5-year survival rate (5). These results suggest that survival in pancreatic ductal adenocarcinoma patients can be significantly increased with earlier diagnosis (6–9), and that active surveillance of high-risk individuals can significantly improve their survival (6, 10, 11). Unfortunately, only a minority of individuals who qualify for high-risk surveillance (21% in a recent study) are enrolled in such programs (10).

Diagnosing pancreatic ductal adenocarcinoma, particularly in early stages, remains challenging and there is no gold standard. Reliance rests on imaging modalities such as magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS), but neither is very sensitive or specific. Small lesions require precise targeting for successful fine needle aspiration or needle biopsy, and this may not be possible if lesions are hard to visualize by imaging (9). CA19-9, a blood biomarker used to monitor pancreatic ductal adenocarcinoma disease progression, has limited specificity in diagnosing or detecting pancreatic ductal adenocarcinoma, since other cancers and conditions (such as cirrhosis) can cause elevated CA19-9 levels (12, 13). Additionally, individuals who are genotypically Lewis antigen null (i.e., le/le with inactivating mutations in both copies of the FUT3 gene) (12–16) have low or no expression of CA19-9. Different ethnic groups have varying frequency of the Lewis null phenotype, ranging from 6% to more than 20% (17), further reducing the ability to rely on CA19-9 as an accurate biomarker for pancreatic ductal adenocarcinoma detection. Although CA19-9 is not currently recommended for surveillance (12, 13, 18), it has been recently suggested to have value as “an anchor marker” for detection of pancreatic cancer (16, 19).

Identifying minimally invasive, reliable, and effective methods for detecting pancreatic ductal adenocarcinoma at an early stage is an important unmet clinical need. In this chapter, the development and validation of the IMMray™ PanCan-d assay, a multi-biomarker signature for pancreatic ductal adenocarcinoma, is described. Intended for individuals at high risk for developing pancreatic ductal

adenocarcinoma, IMMray™ PanCan-d encompasses both immunoregulatory and cancer-associated biomarkers (20–31).

PANCREATIC DUCTAL ADENOCARCINOMA PRECURSOR LESIONS (EARLY PANCREATIC NEOPLASIA)

Three types of precursor lesions are thought to give rise to pancreatic ductal adenocarcinoma, with varying rates and probabilities of progression. Two of these lesions are associated with cyst formation in the pancreas: intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). The third, and perhaps the most common type of precursor lesions, are pancreatic intraepithelial neoplasia (PanINs): flat epithelial dysplasia of pancreatic ducts similar to dysplasia in other sites such as the esophagus or uterine cervix. Higher grades of dysplasia are associated with a greater risk of pancreatic ductal adenocarcinoma. PanINs are usually not detectable by diagnostic imaging and may be multifocal. Effective evaluation of all these precursor lesions requires a multidisciplinary approach (imaging and cytological sampling) that could be greatly improved by the availability of sensitive and specific serum biomarkers. As the NCCN guidelines state: “Decisions about diagnostic management and resectability should involve multidisciplinary consultation at a high-volume center with use of appropriate imaging studies” (32).

Identifying individuals at high risk for pancreatic ductal adenocarcinoma

The known non-genetic risk factors for pancreatic ductal adenocarcinoma are listed in the top panel of Table 1 (4, 13). Although in combination these risk factors may produce a substantial increase in pancreatic ductal adenocarcinoma risk, individually they are modest in magnitude and are not sufficient to warrant active surveillance. Genetic risk factors for pancreatic ductal adenocarcinoma (4, 33) are listed in the bottom panel of Table 1 and many of these are now considered sufficient to offer active surveillance. An individual with multiple close relatives diagnosed with pancreatic ductal adenocarcinoma (familial pancreatic ductal adenocarcinoma) has a 10-fold increased lifetime risk of developing pancreatic ductal adenocarcinoma compared with the general population.

While heritable single gene mutations are responsible for some familial cases, in many cases the genetic causes are unknown. Individuals with hereditary pancreatitis as well as individuals with a history of acute and/or chronic pancreatitis are also at markedly elevated lifetime risk of pancreatic ductal adenocarcinoma. Developing type 2 diabetes after 50 years of age is also associated with an 8-fold risk for pancreatic ductal adenocarcinoma in the first 3 years after diagnosis. Individuals with pancreatic abnormalities detected by diagnostic imaging are also at increased risk. Like many other cancers, an individual’s risk for developing pancreatic ductal adenocarcinoma increases with age, with the majority of cases occurring after age 60 (9). Many of these groups at higher risk may benefit from annual surveillance using a sensitive and specific test that can detect early pancreatic neoplasia.

TABLE 1**Identified Risk Factors Associated with Developing Pancreatic Ductal Adenocarcinoma**

| Non-Genetic Risk Factors (4, 13) | Relative Risk |
|---|----------------------|
| Current cigarette use | 1.7–2.2 |
| Current pipe or cigar use | 1.5 |
| > 3 alcoholic drinks per day | 1.2–1.4 |
| Chronic pancreatitis | 13.3 |
| Body Mass Index > 40 kg/m ² , male | 1.5 |
| Body Mass Index > 40 kg/m ² , female | 2.8 |
| Diabetes mellitus, type 1 | 2 |
| Diabetes mellitus, type 2 | 1.8 |
| Cholecystectomy | 1.2 |
| Gastrectomy | 1.5 |
| Helicobacter pylori infection | 1.4 |
| Genetic Risk Factors (4, 33) | Relative Risk |
| Hereditary breast and ovarian cancer syndrome: BRCA1, BRCA2, PALB2 | 2–3.5 |
| Lynch syndrome (hereditary non-polyposis colorectal cancer): MLH1, MSH2, MSH6, PMS2, EPCAM | 8.6 |
| Familial adenomatous polyposis: APC | 4.5–6 |
| Peutz-Jeghers syndrome: STK11/LKB1 | 132 |
| Familial atypical multiple mole melanoma (FAMMM): P16INK4A/CDKN2A | 47 |
| Hereditary pancreatitis: PRSS1, SPINK1 | 69 |
| Ataxia-telangiectasia: ATM | Increased |
| Familial pancreatic cancer (2 or more first-degree relatives with pancreatic cancer): gene(s) unknown | 9–32 |
| First-degree relative of person with sporadic pancreatic cancer: gene(s) unknown | 2–4 |

Shortfalls of current pancreatic ductal adenocarcinoma diagnostic tools

Although diagnostic imaging is useful in assessing pancreatic abnormalities, it often cannot differentiate between benign conditions (e.g., chronic pancreatitis) and pancreatic neoplasia with certainty (34). In addition, diagnostic imaging fails to detect many (perhaps most) early pancreatic ductal adenocarcinomas when they are of a stage amenable to surgical cure (9, 34). The serum tumor marker CA19-9 (reference range < 37 U/ml) has been useful for monitoring patients with advanced pancreatic ductal adenocarcinoma, but its clinical utility in early detection has not been demonstrated. Additionally, it's been found to be non-specific as it can also be elevated in unrelated conditions. Unfortunately, no other single serum tumor biomarker has shown performance even as good as CA19-9. A different approach to biomarker development has been undertaken to create a more

sensitive and specific assay for pancreatic ductal adenocarcinoma by combining the results of multiplex immunoassays for specific serum proteins.

BIMARKER ASSAY DEVELOPMENT FOR THE EARLY DETECTION OF PANCREATIC DUCTAL ADENOCARCINOMA: PROTEIN MICROARRAY TECHNOLOGY

Human blood serum contains a very large amount of potentially useful diagnostic information. Affinity proteomics has now been developed as an accurate approach that can generate actionable information that can result in more precise and evidence-based options to manage cancer (29). To achieve this, there is clearly a need to move from a single biomarker to multiplex biomarkers, a so-called signature, that can provide significantly increased diagnostic accuracy. Protein biomarker discovery has been driven more by technology rather than focusing on specific clinical needs. Proteomic technology platforms have developed rapidly with substantially increased resolution in terms of depth of proteome coverage and speed. Multiplexed enzyme-linked immunosorbent assays (ELISA) have also demonstrated clinical applicability and have paved the way for next-generation multiparametric diagnostics, in particular high-density antibody microarrays (35). Such protein or antibody microarrays can theoretically display almost unlimited resolution of the most complex proteomes. However, in contrast to the more mature transcriptional profiling technologies, the proteome coverage of antibody microarrays is limited by the number of available well-characterized antibodies. Improved accuracy has been achieved using antibody microarrays, reverse phase protein arrays and bead-based arrays, demonstrating the feasibility of multiparametric proteomic analysis. Although novel technologies open new avenues for clinical proteomics by introducing substantially improved proteome coverage, the quality of available samples can also be problematic in terms of their analytical quality.

Sample acquisition procedures must be strictly standardized to achieve accurate and reproducible proteomic data. However, in most retrospective studies, standard operating procedures for sample collection did not exist or were highly variable. The introduction of such pre-analytical variables can not only affect sample integrity but also introduce bias, due to differences in the acquisition of different sample cohorts. Furthermore, comprehensive information about patient demographics, such as gender, age, tumor stage and treatment schemes, as well as lifestyle factors, such as smoking and alcohol habits, is important to design the proper case-control studies. Each sample subgroup should be clearly defined, and enough samples must be collected from each cohort, since differences in treatment modalities can have a major impact on the results of a proteomic analysis. Therefore, sample quality, as well as their clinical documentation is essential for high quality clinical proteomics (36), which has become more evident in comparison to the more robust genomic approaches.

During biomarker analysis, the main challenge is to define biomarker combinations that deliver optimal clinical accuracy. This cannot be based simply on the *p* values for each biomarker, since this approach discards information about synergistic contributions among the biomarkers that could improve classification accuracy.

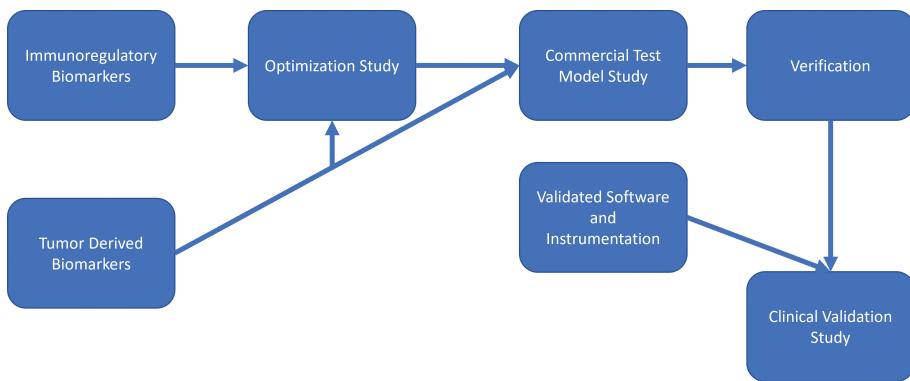


Figure 1. Biomarker development pathway. Development pathway for a biomarker signature for the early detection of pancreatic ductal adenocarcinoma.

A combination of ‘orthogonal biomarkers’ that do not depend on each other is optimal. In this case, the information contributed by each biomarker provides independent information about the disease process. To achieve this, an ordered approach is needed to select the biomarkers with the largest impact on accuracy, and to eliminate biomarkers with the lowest impact on the accuracy. This can be achieved by combining the leave-one-out cross-validation procedure with a backward elimination algorithm or using feature selection based on binary classification algorithms, such as Random Forest. For example, eliminating biomarkers one by one and identifying those that contributed the least to a correct sample classification produces a ranking of the biomarkers. This enables the selection of a biomarker signature displaying optimal accuracy for each application.

Advanced cancer diagnostics based entirely on proteomics has only recently delivered biomarker signatures with the required clinical accuracy (20). Both technological difficulties in decoding complex proteomes, as well as lack of rigorous validation, have been barriers to realize this potential. Recently, antibody microarrays have reached the point at which clinically relevant information related to risk classification and/or prognosis can be routinely generated (35). Here we describe the iterative approach that was used to develop and validate a multiplex assay for the early detection of pancreatic ductal adenocarcinoma using serum samples, as outlined in Figure 1.

IMMUNOREGULATORY PROTEINS AS BIOMARKERS FOR PANCREATIC DUCTAL ADENOCARCINOMA

Blood samples from patients diagnosed with a lesion in the pancreas were collected and processed before resection or start of chemotherapy. Pancreatic cancer staging was performed according to the American Joint Committee on Cancer guidelines (37). Blood samples from controls were collected, using the same standard operating procedure. 5 µL of the serum samples was used for the analysis, with a recombinant antibody microarray composed of 349 human recombinant single-chain variable

fragments (scFvs) that were directed against 156 antigens. The concept was to utilize the body's response to disease rather than the tumor secretome. Consequently, the selected biomarkers were involved mainly in immunoregulation (30).

In order to develop a final biomarker signature, data were divided into a training set including two thirds of the samples (~1,000 samples) and a test set including one third of the samples (~340 samples). The same ratio of case versus control samples was preserved within the randomly generated data sets. Four unique test and training sets were generated using this approach. A backward elimination algorithm was applied to each training set in R (open-source statistical software from The R Foundation), excluding one antibody at a time. This approach allows an unbiased selection of biomarkers contributing orthogonal information, compared with other biomarkers.

The generated biomarker signature was used to build a model by support vector machine in R, utilizing only data from the training set. The model was tested on the corresponding test set (to avoid overfitting) and its performance was measured using area under the curve (AUC) values from receiver operating characteristic (ROC) curves. Analyte detection was based on the recombinant antibody microarray platform as described above. Because the focus was to interrogate the body's response to pancreatic ductal adenocarcinoma, the selected antibodies targeted predominantly immunoregulatory proteins. The obtained AUC value for differentiating stage I and II pancreatic ductal adenocarcinoma versus normal controls was 0.96. The corresponding value for normal controls versus stage III and IV pancreatic ductal adenocarcinoma was 0.99.

Although some of the biomarkers included in the final signature had been previously reported as potential pancreatic ductal adenocarcinoma biomarkers, no obvious mechanistic model explains the composition or possible interaction of these biomarkers with pancreatic ductal adenocarcinoma development. Perhaps gradual changes in the tumor microenvironment can be reflected in the biomarker content in blood. The result of this study was identification and validation of a biomarker signature, based on two large, case-control studies of patients with pancreatic ductal adenocarcinoma, which was able to detect stage I and II cancer samples with high accuracy.

ADDITION OF A TUMOR-DERIVED BIOMARKER (CA19-9) TO THE ASSAY (OPTIMIZATION STUDY)

The Optimization Study aimed to evaluate how a biomarker signature could separate patients with pancreatic ductal adenocarcinoma (stages I–IV) from individuals with various symptomatic conditions not caused by pancreatic ductal adenocarcinoma. These controls were selected to mirror the relevant clinical settings (benign biliary obstructions, cirrhosis, etc.) encountered by healthcare professionals. In addition, it evaluated the utility of adding the established pancreatic ductal adenocarcinoma biomarker CA19-9 to a biomarker signature. The IMMray™ platform is designed to detect protein biomarkers so that CA19-9, which is an oligosaccharide, was measured separately using an electrochemiluminescence immunoassay (Roche Cobas®). In total, 923 serum samples were analyzed with the IMMray™ discovery set up and a CA19-9 ELISA. Patient samples from

136 pancreatic ductal adenocarcinoma (stage I–IV), 570 symptomatic individuals and 217 healthy controls were tested in a randomized manner. All pancreatic ductal adenocarcinomas were histologically confirmed. Based on one year follow-up data, none of the symptomatic controls (back pain, unexplained weight loss, etc.) developed pancreatic cancer. To minimize confounding and pre-analytical variables, all patient samples were collected and processed using the same standard operating procedures, stored at –80°C and tested within a year of collection. Data analysis for each group was performed using the support vector machine algorithm. Data was divided into a training and test set, and test performance given as ROC AUC values were then evaluated for the test set.

The biomarker signature together with CA19-9 had the capacity to differentiate pancreatic ductal adenocarcinoma (stages I–IV) from symptomatic individuals without cancer, including individuals with type 2 diabetes. Importantly, early-stage pancreatic ductal adenocarcinoma (stages I & II) was discriminated from controls with an unprecedented accuracy of 0.984. These findings needed to be further validated but have significant clinical implications for individuals in primary and secondary care settings with non-specific but concerning symptoms where pancreatic ductal adenocarcinoma may be suspected. This study paved the way for the IMMray™ PanCan-d Commercial Test Model Study, in which the final biomarker signature was selected, and the commercial test model built.

COMMERCIAL TEST MODEL STUDY (CTMS)

The IMMray™ PanCan-d Commercial Test Model Study (CTMS) aimed to select and lock the IMMray™ PanCan-d biomarker signature and evaluate its performance in differentiating pancreatic ductal adenocarcinoma (stages I–IV) vs. controls that simulate clinical test situations. Serum samples obtained from patients in Europe and the United States with non-specific but concerning symptoms, including diabetics, as well as samples from healthy individuals were analyzed. In total, 1113 patient serum samples were analyzed with a focused IMMray set up and CA19-9 assay. Patient samples from 315 pancreatic ductal adenocarcinoma (stage I–IV), 488 symptomatic individuals who did not have pancreatic cancer (including 79 with diabetes and 56 with chronic pancreatitis) and 310 healthy controls were tested. All these samples were freshly collected through eight reference sites in USA and Europe. Data analysis was performed using Immunovia's software algorithms and the data were divided into training and test sets. The test performance was evaluated using ROC AUC curves. In this CTMS study, we showed for the first time that the IMMray™ PanCan-d 9-plex signature, including CA19-9, had the capacity to differentiate between pancreatic ductal adenocarcinoma stage I & II and all controls, including diabetes, symptomatic, healthy individuals, with a ROC AUC of 0.950. We also locked and tested the model algorithms as part of this study, which were subsequently incorporated in the final IMMray™ PanCan-d test process.

CLINICAL VALIDATION OF IMMRAY™ PANCAN-D

IMMray™ PanCan-d is a multiplex micro-immunoassay that combines measurements of 9 serum biomarkers including CA19-9 using a mathematical algorithm (31).

After its development, this signature was created and locked during the Commercial Test Model Study, as described above. The algorithm can be expressed as a linear equation which includes the levels of 9 serum biomarkers (\log_2 transformed fluorescence intensity) multiplied by positive or negative real number coefficients:

$$A1^*(\log_2 \text{intensity } 1) + A2^*(\log_2 \text{intensity } 2) + \dots + A9^*(\log_2 \text{intensity } 9) + C = \text{Decision Value}$$

$A1-A9$ are real number coefficients determined from the support vector machine and C is the Y intercept for this linear equation. The IMMray PanCan-d single chain variable fragment (scFv) antibodies included in the IMMray™ PanCan-d microarray are listed in Figure 2.

The clinical validation study analyzed samples collected from multiple sites across Europe and the United States, including 57 early-stage (stage I and II) pancreatic ductal adenocarcinoma patients, 110 stage III and IV pancreatic ductal adenocarcinoma patients, 203 individuals at high risk for developing familial/hereditary pancreatic ductal adenocarcinoma enrolled in a surveillance program, and 216 healthy controls. All serum samples were collected in red top tubes and allowed to clot for 30–60 min before centrifugation for 10 minutes at 3,000 xg. Serum was then aliquoted and immediately frozen at -80°C . Samples were transported on dry ice and then thawed for analysis. All samples were analyzed within 2 years of their collection, and all were stored at -80°C until thawed for analysis. Pancreatic ductal adenocarcinoma staging was performed according to the American Joint Committee on Cancer Guidelines (37). Blood samples from patients with confirmed pancreatic ductal adenocarcinoma were collected and processed before treatment. Samples were blinded to laboratory staff and randomized using an Excel template designed to avoid an imbalance of any cohort in any assay batch (maximum batch size = 62 samples).

Sample cohort characteristics

Pancreatic ductal adenocarcinoma patients had a median age of 70 years, which is 11 years older than the high-risk population. Both cohorts were older than the healthy cohort with a median age of 49. Women were more frequent in the high-risk cohort, while the pancreatic ductal adenocarcinoma cohort had more men than women, as expected. 28% of the high-risk cohort had prior cancers and they were either cured or were in remission at the time that they were inducted into the study. This large number of prior neoplasms is not unexpected as many individuals in this cohort had documented germline mutations predisposing to pancreatic carcinoma as well as other tumor types. Together, the 203 high-risk subjects were receiving 619 prescription medications, some of which were adjuvant therapy for

| Tumor-Associated | Hormone Transport | Bone Metabolism | Protease Inhibitor | Coagulation | Complement |
|------------------|-------------------|-----------------|--------------------|-------------|-----------------------|
| • MUC16 (CA125) | • IGFBP3 | • OPG | • Cystatin C | • GSN | • C5 • CFB • C4 |

Figure 2. Antibodies included on microarray. Identities of IMMray™ PanCan-d single chain variable fragment antibodies included on the microarray.

prior cancers (e.g., aromatase inhibitors). All individuals in the high-risk cohort underwent active imaging surveillance. 25% of this cohort had presumptive IPMNs and 27% had other imaging abnormalities in their pancreas. Detected IPMNs ranged from 1 to 10 in number (median 2) and from 0.2 to 2.2 cm in size (median 0.6). None had main duct IPMNs and none had worrisome features.

The healthy cohort was recruited from multiple sites in Europe and North America. This group was more ethnically diverse than the other cohorts and had no history of cancer. The familial/genetic high-risk cohort was collected from 3 US sites (University of Pittsburgh Medical Center, Massachusetts General Hospital, and University of Pennsylvania) participating in the PanFAM prospective clinical trial (ClinicalTrials.gov Identifier: NCT03693378) and was made up of individuals with a strong family history of pancreatic cancer and/or individuals with known genetic mutations predisposing to pancreatic ductal adenocarcinoma who meet current criteria for active surveillance listed in Table 2. None of the individuals tested were known to have developed pancreatic ductal adenocarcinoma at the time of sample collection.

IMMray™ PanCan-d results (frozen signature and predefined classification cutoffs)

A histogram showing the distribution of decision values for the three sample cohorts is shown in Figure 3A. The decision values for the healthy and high-risk cohorts are clustered and are generally similar to one another (although they were

TABLE 2

Inclusion Criteria for the High-Risk Cohort Study

| | Age |
|--|--|
| Two or more relatives with pancreatic adenocarcinomas (PDAC) on the same side of the family, where two PDAC-affected individuals are first degree related (FDR) + at least one PDAC-affected individual is an FDR of the Participant | ≥50 years old OR 10 years before onset in family |
| Two affected FDRs with PDAC | ≥50 years old OR 10 years before onset in family |
| Any of <i>BRCA1</i> , <i>BRCA2</i> , <i>PALB2</i> , <i>ATM</i> mutations confirmed pathogenic or likely pathogenic + one FDR or secondary degree related (SDR) with PDAC | ≥50 years old OR 10 years before onset of an FDR and SDR |
| Familial atypical multiple mole-melanoma (FAMMM) with confirmed pathogenic or likely pathogenic mutation variants in: <i>p16</i> , <i>CDKN2A</i> | ≥50 years old |
| Known mutation carrier for <i>STK11</i> (Peutz Jeghers Syndrome) | ≥35 years old |
| Lynch syndrome (HNPCC) with confirmed pathogenic or likely pathogenic variants in: <i>MLH1</i> , <i>MSH2</i> , <i>MSH6</i> , <i>PMS2</i> , or <i>EPCAM</i> + one FDR or SDR with PDAC | ≥50 years old OR 10 years before onset of an FDR or SDR |
| Hereditary pancreatitis with confirmed <i>PRSS1</i> pathogenic or likely pathogenic history of pancreatitis | ≥40 years old |

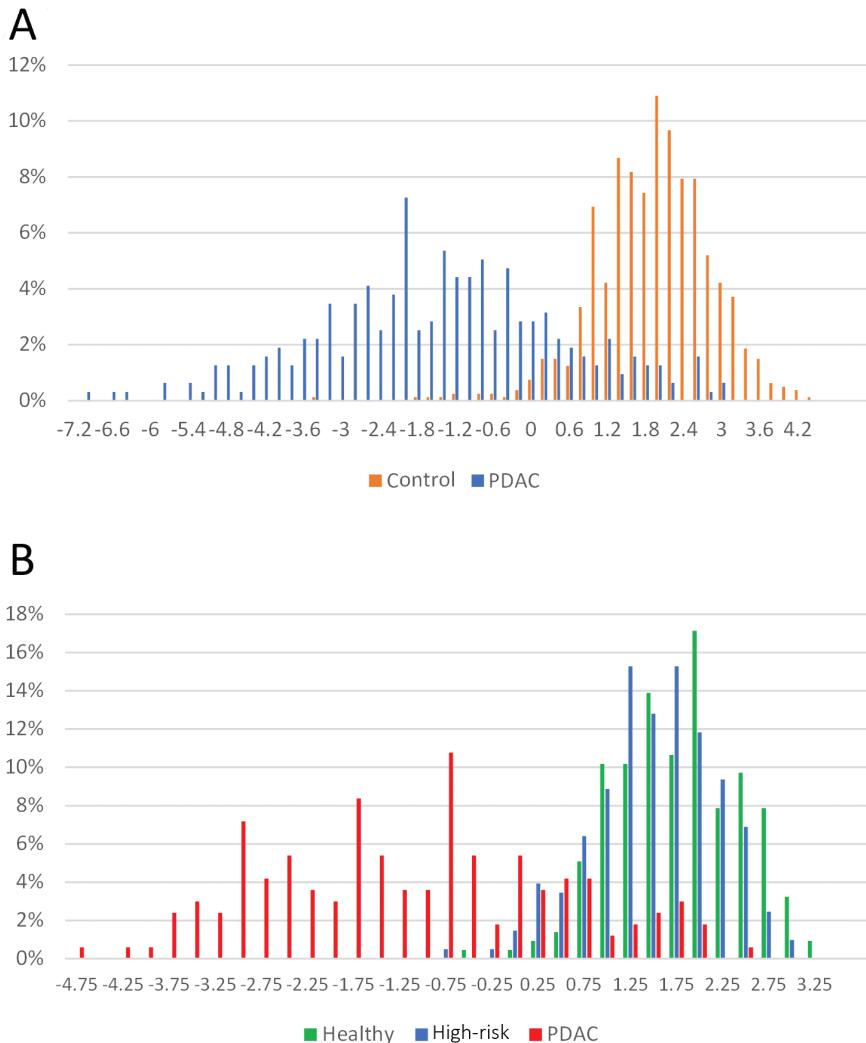


Figure 3. Decision values. Histograms showing the distribution of observed Decision Values for different subject cohorts from the Clinical Test Model Building Study (Figure 3A) and the Clinical Validation Study (Figure 3B).

statistically different by t test, $p < 0.001$). The mean decision values for the high-risk and healthy cohorts are 1.40 and 1.65, respectively. The corresponding standard deviations were 0.67 and 0.68. Both cohorts differed substantially from the pancreatic ductal adenocarcinoma cohort that showed a much greater variation in decision values (-4.75 to 2.5) and had a strong negative bias (mean value = -1.26 with a standard deviation of 1.58).

Excluding borderline results, these decision values correspond to a test sensitivity of 85% for early stage (stages I & II) pancreatic ductal adenocarcinoma and 87% for all-stage pancreatic ductal adenocarcinoma with a specificity of 98%

compared with the high-risk cohort and 99% compared with the healthy cohort. Using its clinical reference range cutoff, CA19-9 alone demonstrated 75.8% sensitivity and 97.6% specificity in these cohorts. 10% of samples were classified as borderline, and there was a higher percentage of borderline results among the pancreatic ductal adenocarcinoma cohort than in the control cohorts. Evaluation of the test classifications in the context of gender or smoking status did not demonstrate statistical significance by Chi Square ($p = 0.48$ and $p = 0.61$, respectively). The median age for individuals with negative and borderline classifications in the high-risk cohort were 59 and 60. The median age of individuals with negative, borderline, and positive classifications in the pancreatic ductal adenocarcinoma cohort were 68, 71, and 71 respectively. The distribution of decision values in this study is very similar to that obtained in the CTMS study in which the algorithm was developed and locked in 2019 (see Figure 3B).

CA19-9 and IMMray™ PanCan-d results

Prior publications have shown that individuals with very low baseline CA19-9 values are frequently deficient in Fucosyltransferase 3 (the enzyme FUT3) which normally adds the terminal sugar to form CA19-9. Based on these published findings and the fact that CA19-9 is a component of decision values for IMMray™ PanCan-d, we evaluated IMMray™ PanCan-d performance in the subsets of each study cohort that expressed CA19-9 levels greater than 2.5 U/ml. Excluding samples with CA19-9 values equal to or less than 2.5 U/ml excluded 55 samples from the analysis but improved assay sensitivity from 85% to 89% for early-stage pancreatic ductal adenocarcinoma and from 87% to 92% for all-stage pancreatic ductal adenocarcinoma (Figure 4).

Recent work has revived interest in CA19-9 as an important pancreatic ductal adenocarcinoma biomarker. CA19-9 has been largely relegated to a limited role in measuring tumor progression and/or response to therapy. One recent article brings forward the concept of CA19-9 as an “anchor” biomarker that can be combined with other biomarkers to achieve superior diagnostic performance (16). An important limitation of CA19-9 as a pancreatic ductal adenocarcinoma biomarker results from genetic variation in one of the enzymes that is required for its synthesis. FUT3 (also called the Lewis Antigen) catalyzes the addition of terminal sugar to DuPan2 to form CA19-9. The prevalence of FUT3 deficiency (both alleles nonfunctional) has been reported to vary in different ethnic groups and these findings were supported by this study. The following rates of CA19-9 values below 2.5 U/ml in the subjects from different ethnic backgrounds were observed: 8% of US Caucasians, 24% of US Hispanics, and 26% of African Americans were presumptively deficient. These observed frequencies are similar to those reported for FUT3 negative individuals in the US Caucasian and African American populations (17). Since the 8 biomarkers measured on the IMMray platform contribute significantly to discrimination between samples from individuals with pancreatic ductal adenocarcinoma and controls, we re-evaluated the decision values for samples with CA19-9 values less than 2.5 U/ml by removing the CA19-9 contribution from them and obtained a ROC AUC of 0.87.

The improvement in IMMray™ PanCan-d test sensitivity to 92% by excluding samples with very low CA19-9 values is clinically important. This exclusion also avoids the likelihood of under-diagnosing pancreatic ductal adenocarcinoma in

PDAC vs. Controls with CA19-9 ≤ 2.5 U/ml excluded

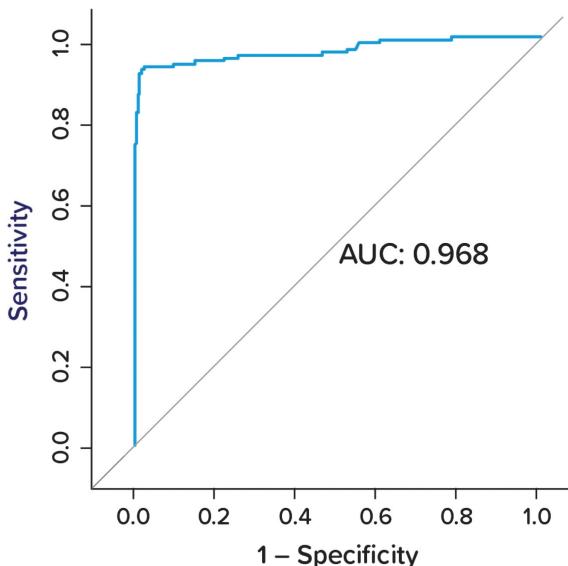


Figure 4. Test performance. Receiver operating characteristic (ROC) curves and area under the curve (AUC) for IMMray™ PanCan-d test performance in pancreatic ductal adenocarcinoma (PDAC) vs all controls, excluding samples with CA19-9 values of 2.5 U/ml or less.

ethnic groups with a higher prevalence of FUT3 (Lewis null) genotypes (e.g., Hispanics and African Americans in this study). The discrimination of the signature without CA19-9 in these samples is encouraging and provides a starting point for developing a companion assay to better address this population.

PAN-CANCER BIOMARKER ASSAYS

Although pan-cancer biomarker assays are a subject in and of themselves and a detailed discussion is beyond the scope of this chapter, a few comments are needed here since some of these tests can potentially detect pancreatic ductal adenocarcinoma as well as a variety of other tumors. Several pan-cancer assays are currently under development and one (Galleri™ from GRAIL, Inc.) is available clinically. Most of these assays, including Galleri™, depend heavily or exclusively on the detection of novel DNA methylation patterns from cell-free DNA. Since these tests depend predominantly on the release of DNA from tumor cells, their ability to detect small cancers is limited. The one pan-cancer commercial assay currently available, Galleri™, has reported a sensitivity for stage I and II pancreatic ductal adenocarcinoma of 61% compared with the IMMray PanCan-d sensitivity of 89%, most probably reflecting on the value of including nontumor cell derived biomarkers in the latter assay.

CONCLUSION

Antibody microarray technology displays great promise for protein expression profiling of complex proteomes. In some cases, the addition of biomarkers to an anchor biomarker, such as CA19-9, can improve the accuracy of that biomarker sufficiently to substantially alter its clinical utility. Microarray technology has now evolved from proof-of-concept designs to established high-performing technology platforms capable of evaluating non-fractionated complex proteomes from human samples. A variety of platforms, displaying a wide range of performances, based on monoclonal, polyclonal, and recombinant antibodies, are available from both academic laboratories and commercial vendors. To date, the technology has been used to detect disease-associated (biomarker) signatures for bladder cancer, colorectal cancer, gastric adenocarcinoma, lung cancer, breast cancer, pancreatic adenocarcinoma, prostate cancer. We feel that the most important clinical uses of these antibody microarrays will be in disease diagnosis, prognosis, and therapy selection for complex polygenic diseases. Efforts have now been launched to extend the technology beyond the current state-of-the-art, to be able to perform true global proteome analysis, setting a new standard for disease proteomics. This chapter has attempted to describe the clinical landscape for developing a multiplex proteomic test for early cancer detection and provide a paradigm for the iterative selection of an optimal biomarker signature that may include not only tumor cell products but also components of the tumor microenvironment and the host immune and inflammatory response to a tumor. In this context, we feel that IMMray™ PanCan-d can now have a significant clinical impact on individuals at risk for pancreatic ductal adenocarcinoma and perhaps ultimately in managing pancreatic ductal adenocarcinoma patients' care through optimal selection of therapeutic modalities and detection of tumor recurrence. The WHO has proposed that millions of patients with cancer could be saved from premature death if diagnosed and treated earlier. To achieve this, more advanced diagnostic approaches must be developed for multiple tumor types and applied to detect lethal cancers, such as pancreatic cancer, earlier in their clinical course.

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The Epidemiology of Stomach Cancer

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Abstract: The aim of this chapter is to examine stomach cancer incidence and mortality rates worldwide, focusing on numbers in Brazil. Data on new cases and deaths due to stomach cancer for 2020 and projections for 2040 were evaluated. A total of 1,089,103 new stomach cancer cases were estimated globally for 2020, with the highest incidence rates noted for Mongolia, Japan, and the Republic of Korea. Concerning mortality, a total of 768,793 deaths were reported for the same year, with the highest rates observed in Mongolia, Tajikistan, and China. In Brazil, a total of 13,360 new stomach cancer cases among men and 7,870 among women were estimated for 2020, with 13,850 deaths reported for both sexes. Although both incidence and mortality rates currently exhibit a downward trend in most countries, including Brazil, the number of new cases and deaths each year is not negligible, indicating the need for continued actions to reduce exposure to stomach cancer risk factors and the expansion of early diagnoses with timely treatment.

Keywords: Brazilian stomach cancer incidence; epidemiology of stomach cancer; stomach cancer mortality rates in Brazil; worldwide stomach cancer incidence rates; worldwide stomach cancer mortality rates

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INTRODUCTION

Stomach cancer remains one of the major health challenges worldwide, despite recent declines in incidence and mortality rates (1). According to the Global Cancer Observatory (2), stomach cancer was ranked as the 5th most frequent tumor and the 4th cancer with the highest mortality rates for both men and women in 2020. Incidence and mortality rates are twice as high in men compared to women (3) and about 75% of all cases and deaths occur in Asia (2). In Brazil, the country with the highest number of new cases and deaths due to stomach cancer in Latin America, this condition was ranked as the 5th most incident tumor and the 5th most deadly for both men and women in the same year (3). These variations can, at least in part, be explained by an uneven stomach cancer risk factor exposure distribution (4).

About 95% of all stomach cancers comprise adenocarcinomas, which, according to their topographical location, can be categorized into two main anatomical sites, namely cardia and distal (non-cardia) tumors. Most stomach cancers occur in distal regions and exhibit chronic *Helicobacter pylori* infection as their most notable cause. Cardia cancers have been associated with gastroesophageal reflux and obesity. Smoking, excessive alcohol consumption, salt intake, salt-preserved foods, smoked foods, pickled vegetables, coffee, low fruit and vegetable intakes and exposure to ionizing radiation (X-radiation, gamma-radiation), as well as being male and over 50 or 60 years old have been identified as risk factors for both anatomical sites, implying a predominant occurrence in low socioeconomic status areas (4–7).

In addition, other factors, such as occupational exposures to dust, coal, metals, talc, asbestos and rubber, heredity/genetic mutations, such as a history of stomach cancer in first-degree relatives, the presence of hereditary syndromes such as familial adenomatous polyposis (FAP), hereditary diffuse gastric cancer (HDGC) and Peutz-Jeghers syndrome, mutations in *GSTM1* or *CDH1* genes and interleukin IL-17 and IL-10 polymorphisms, previous gastric surgery, pernicious anemia, *Epstein-Barr virus* infection and being blood type A, as well as some races/ethnicities (Hispanics, Asians, Alaska natives, African Americans and American Indians) have also been associated with a high risk of developing this neoplasm (1, 4, 5, 8).

Primary and secondary prevention strategies should comprise the cornerstones of stomach cancer prevention (1). Primary prevention strategies include healthy behaviors such as ceasing smoking, reducing salt intake, limiting alcohol intake, and increasing fruit and vegetable consumption. On the other hand, inadequate evidence that *Helicobacter pylori* eradication may reduce the risk of stomach cancer is available, as barium-meal gastric photofluorography and serum pepsinogen or gastric endoscopy may also reduce stomach cancer mortality rates.

In this context, this chapter aims to assess the global numbers of new cases and deaths due to stomach cancer in 2020 and evaluate temporal incidence and mortality trends, focusing on data from Brazil.

WORLDWIDE STOMACH CANCER INCIDENCE RATES

World cancer incidence data for 2020 were estimated by the Global Cancer Observatory (2), an interactive web-based platform that offers global cancer statistics.

Projections were based on GLOBOCAN incidence estimates from 185 countries or territories for 36 types of cancer, categorized by sex and age group. National incidence rates and estimates derived from national mortality data employing the mortality-incidence ratio were obtained. In the absence of local data, rates derived from cancer registries in neighboring countries were used.

Stomach cancer was responsible for 6% of all cancer cases in 2020, excluding non-melanoma skin cancer cases. Estimates indicate a total of 1,089,103 new stomach cancer cases, 719,523 among men and 369,580 among women, corresponding to population-adjusted incidence rates of 15.5 and 7.0/100,000, respectively. The highest incidence rates for both sexes were observed in Mongolia (32.5/100,000), Japan (31.6/100,000) and the Republic of Korea (27.9/100,000), followed by some Central and South American countries, Russia, and China, while the lowest rates were noted in Mozambique (0.75/100,000), Indonesia (1.3/100,000) and Comoros (1.3/100,000) (2) (Figures 1A and 1B).

For the same year, stomach cancer ranked the 4th most common tumor in men and the 7th in women (2), excluding non-melanoma skin cancer (Figure 2). About ¾ of all stomach cancer cases were reported for Asia (74.5%), followed by Europe (12.9%), Latin America and the Caribbean (6.5%), Africa (3.0%), North America North (2.8%) and Oceania (0.3%) (2).

Brazilian stomach cancer incidence rates

In Brazil, cancer incidence data is based on data available in population-based cancer registries (PBCR). Currently, about 30 PBCR comprising at least 2 years of information are available, covering between 10 and 22% of the Brazilian population, depending on the year (9, 10). The National Cancer Institute (*Instituto Nacional de Cáncer - INCA*) has computed annual estimates on the number of new cancer cases and their respective incidence rates since 1995 (11).

A total of 13,360 new stomach cancer cases were estimated for men and 7,870 for women for each year of the 2020–2022 triennium in Brazil, corresponding to incidence rates of 12.81 per 100,000 men and 7.34 per 100,000 women. Since the first national estimates in 1995 (12) the total number of new stomach cancer cases per year has increased among both men and women, from 11,100 to 21,230 in 2020, totaling a 91.3% increase. During the same period, the Brazilian population increased by 34.3% (13). Furthermore, excluding non-melanoma skin tumors, INCA data (11) indicates that stomach cancer among men ranks 2nd in frequency in Northern Brazil (11.75/100,000), and 3rd in the Northeast (10.63/100,000). Concerning other Brazilian regions, it is the 4th most frequent cancer (South 16.02/100,000, Southeast 13.99/100,000 and Midwest 9.38/100,000). Among women, stomach cancer is the 5th most frequent in Southern (9.15/100,000) and Northern (6.03/100,000) Brazil, occupying the 6th and 7th position in the other Brazilian regions (Central-West 6.71/100,000 and Northeast 7.03/100,000, Southeast 7.30/100,000, respectively). These rates place practically all Brazilian regions among the two highest global incidence stomach cancer rate quintiles according to the Global Cancer Observatory presented in Figure 1 (rates of over than 9.4 and 4.8/100,000 among men and women, respectively).

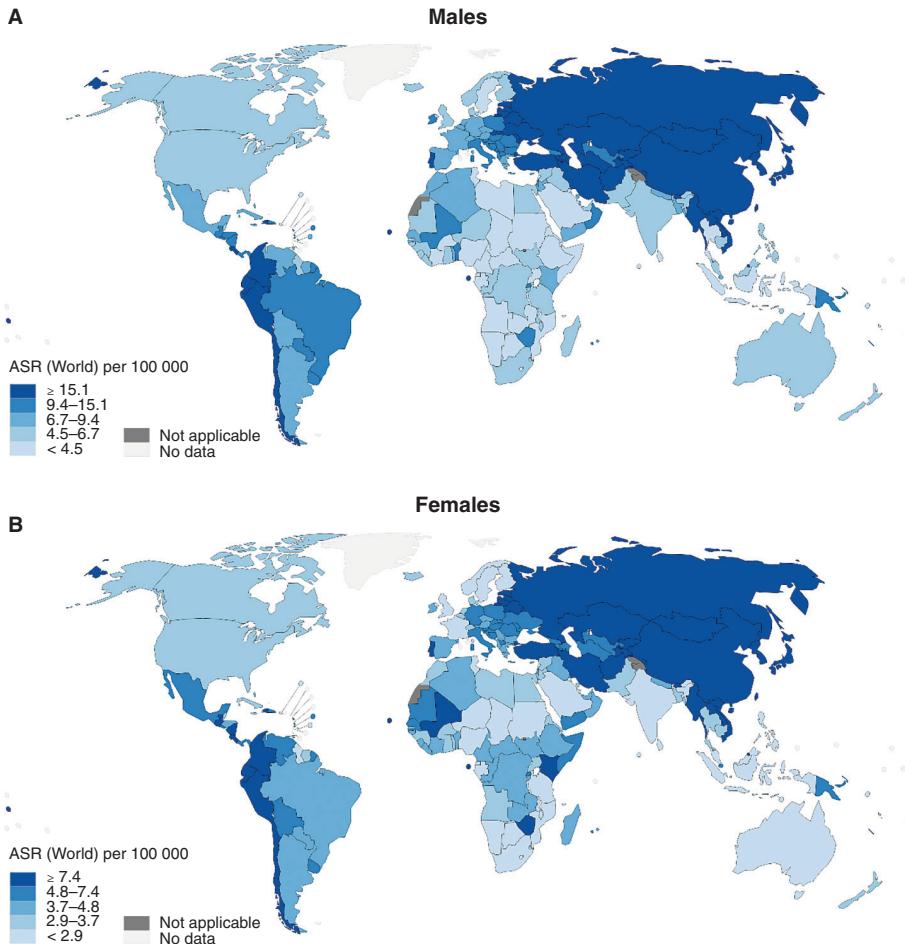


Figure 1. Estimated 2020 stomach cancer incidence rates per 100,000 men (A) and women (B), standardized by age (world population). Reproduced from the Global Cancer Observatory (2).

WORLDWIDE STOMACH CANCER MORTALITY RATES

Worldwide cancer mortality rates are also estimated by the Global Cancer Observatory (2). Mortality rates by sex and age groups for 2020 are based on national estimates from observed mortality rates and, when not available, on the incidence-mortality ratios reported in cancer registries from neighboring countries. Excluding non-melanoma skin cancer, a total of 768,793 deaths due to stomach cancer were reported worldwide in 2020, 502,788 among men and 266,005 among women. The estimated world population-adjusted mortality rates were of 11.0 and 4.9/100,000 for men and women, respectively. The highest mortality rates for both sexes were observed in Mongolia (24.6/100,000), Tajikistan (19.7/100,000) and China (15.9/100,000), while the lowest were

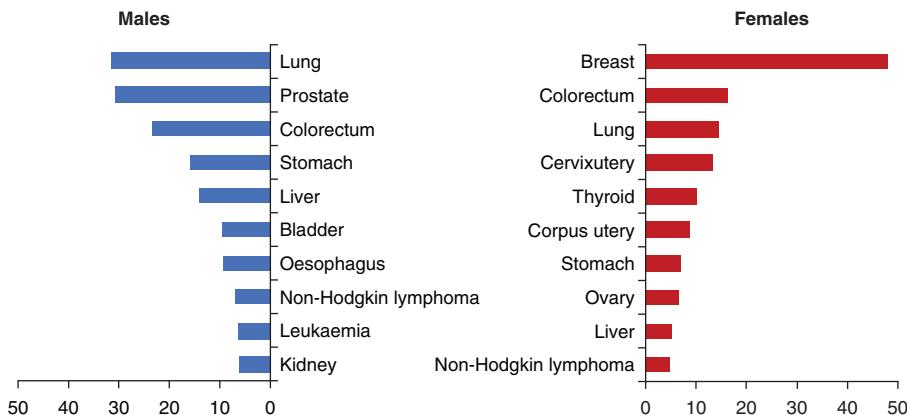


Figure 2. Estimated 2020 incidence rates per 100,000 men and women, standardized by age (world population). Reproduced from the Global Cancer Observatory (2).

observed in Mozambique. (0.69/100,000), Indonesia (1.1/100,000) and Comoros (1.2/100,000) (Figures 3A and 3B) (2). For the same year, stomach cancer ranked 3rd as the deadliest among men and the 5th among women excluding non-melanoma skin cancer cases (Figure 4) (2), appearing as the main cause of death by cancer in several South Central and Asian countries, including Iran, Afghanistan, Turkmenistan and Kyrgyzstan (3). A scenario very similar to that observed in terms of incidence is noted for mortality rates, with most cases reported for Asia (74.7%), followed by Europe (13.1%), Latin America and the Caribbean (6.6%). Africa (3.7%), North America (1.7%) and finally, Oceania (0.3%) (2).

Stomach cancer mortality rates in Brazil

In Brazil, cancer mortality data are made available by the Ministry of Health's Mortality Information System (*Sistema de Informação sobre Mortalidade - SIM*). The SIM was implemented in 1975 and consists of online data on deaths from all causes in the country, including cancer, from 1979 to 2020 (14). Brazilian indicators and cancer mortality rates can also be found at the Online Atlas of Mortality available on the INCA website in the form of tables, graphs, and maps (15). In 2020, the last year with complete data available, 8,772 deaths from stomach cancer were recorded in the country among men and 5,078 among women, totaling 13,850 deaths, with age-adjusted mortality rates for the world population of 8.47/100,000 for men and 4.69/100,000 for women (15).

TEMPORAL TRENDS IN STOMACH CANCER INCIDENCE AND MORTALITY IN BRAZIL AND WORLDWIDE

A drop in incidence and mortality rates due to stomach cancer has been noted in most countries over the last few decades, mainly attributed to the consumption of

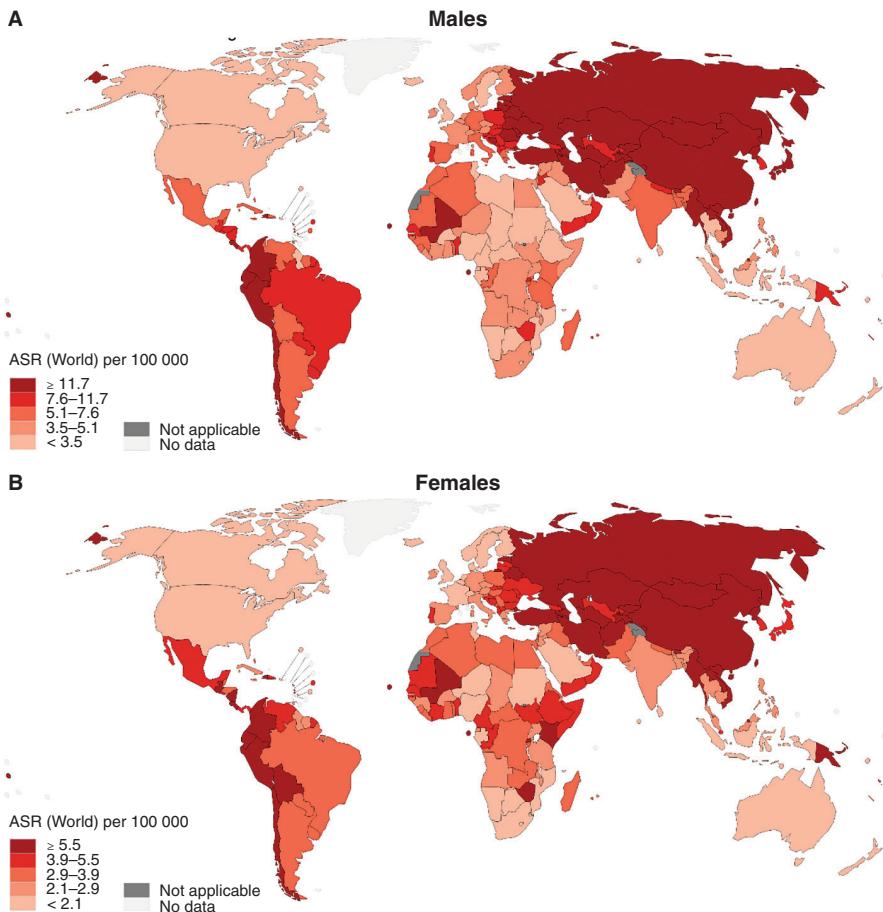


Figure 3. Estimated 2020 stomach cancer mortality rates per 100,000 men (A) and women (B), standardized by age (world population). Reproduced from the Global Cancer Observatory (2).

fresh food made possible by refrigeration technology and economic development (7, 16).

One study covering data from 1980 to 2018 from 48 countries indicates incidence rate decreases in 29 countries (60.4%) and mortality rate reductions in 41 countries (85.4%). The authors note that these declines were noted mainly in patients aged 40 and over (in 30 of 48 countries = 62.5%), although some rates in certain countries were also noted as increasing in populations under 40 years old (4).

In Brazil, age-adjusted mortality rates due to stomach cancer by the world population per 100,000 men or women between 1980 and 2020 significantly declined (−23.6% per year among men and −10.0% per year among women) (Table 1) (15).

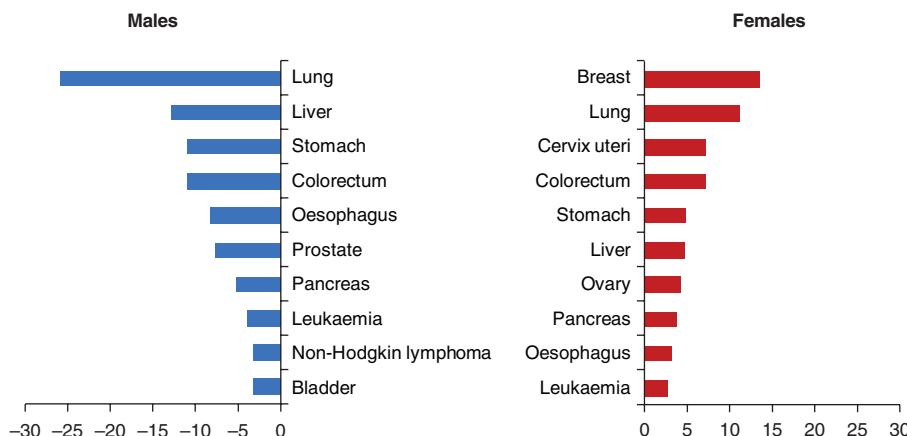


Figure 4. Estimated 2020 mortality rates per 100,000 men and women, standardized by age (world population). Reproduced from the Global Cancer Observatory (2).

TABLE 1

Crude and population-adjusted stomach cancer incidence rates per 100,000 for men and women

| Year | Males | | Females | |
|------|------------|---------------|------------|---------------|
| | Crude rate | Adjusted rate | Crude rate | Adjusted rate |
| 1980 | 9.89 | 16.98 | 4.87 | 7.71 |
| 1981 | 10.11 | 17.02 | 4.84 | 7.46 |
| 1982 | 9.88 | 16.38 | 4.87 | 7.33 |
| 1983 | 9.92 | 16.27 | 4.77 | 7.01 |
| 1984 | 9.72 | 15.68 | 4.67 | 6.74 |
| 1985 | 9.52 | 15.22 | 4.38 | 6.20 |
| 1986 | 9.30 | 14.65 | 4.49 | 6.26 |
| 1987 | 9.51 | 14.85 | 4.62 | 6.33 |
| 1988 | 9.28 | 14.35 | 4.56 | 6.16 |
| 1989 | 9.10 | 13.95 | 4.45 | 5.93 |
| 1990 | 9.04 | 13.71 | 4.32 | 5.68 |
| 1991 | 8.95 | 13.43 | 4.47 | 5.78 |
| 1992 | 9.32 | 13.58 | 4.56 | 5.71 |
| 1993 | 8.88 | 13.43 | 4.42 | 5.75 |
| 1994 | 8.65 | 13.06 | 4.35 | 5.65 |
| 1995 | 8.77 | 13.27 | 4.32 | 5.63 |

Table continued on following page

TABLE 1
Crude and population-adjusted stomach cancer incidence rates per 100,000 for men and women (Continued)

| Year | Males | | Females | |
|------|------------|---------------|------------|---------------|
| | Crude rate | Adjusted rate | Crude rate | Adjusted rate |
| 1996 | 8.94 | 12.40 | 4.50 | 5.30 |
| 1997 | 8.86 | 12.29 | 4.53 | 5.34 |
| 1998 | 8.94 | 12.44 | 4.35 | 5.13 |
| 1999 | 8.72 | 12.16 | 4.41 | 5.18 |
| 2000 | 8.59 | 11.07 | 4.37 | 4.65 |
| 2001 | 8.38 | 10.78 | 4.24 | 4.54 |
| 2002 | 8.63 | 10.85 | 4.25 | 4.41 |
| 2003 | 8.68 | 10.70 | 4.38 | 4.43 |
| 2004 | 8.85 | 10.63 | 4.44 | 4.38 |
| 2005 | 8.94 | 10.48 | 4.58 | 4.41 |
| 2006 | 8.86 | 10.14 | 4.73 | 4.42 |
| 2007 | 9.08 | 10.15 | 4.80 | 4.37 |
| 2008 | 8.86 | 9.68 | 4.65 | 4.14 |
| 2009 | 8.95 | 9.49 | 4.39 | 3.80 |
| 2010 | 9.24 | 9.44 | 4.90 | 4.11 |
| 2011 | 8.94 | 9.02 | 4.71 | 3.89 |
| 2012 | 8.98 | 8.83 | 4.93 | 3.98 |
| 2013 | 9.34 | 8.98 | 4.94 | 3.88 |
| 2014 | 9.14 | 8.59 | 4.86 | 3.73 |
| 2015 | 9.17 | 8.43 | 4.94 | 3.72 |
| 2016 | 9.39 | 8.43 | 4.99 | 3.70 |
| 2017 | 9.10 | 7.95 | 4.83 | 3.49 |
| 2018 | 9.21 | 7.84 | 5.04 | 3.56 |
| 2019 | 9.38 | 7.80 | 5.10 | 3.51 |
| 2020 | 8.47 | 6.88 | 4.69 | 3.16 |

Reproduced from Instituto Nacional de Câncer (15)

Time trends in long-term stomach cancer incidence rates have been scarcely studied under a global perspective. Recently, an analysis on data from 108 cancer registries from 43 countries located on five continents pointed towards a downward incidence rate trend until 2030 in 41 of the 43 analyzed countries (6). Other authors have warned of increased new stomach cancer cases in adults under 50 living in developed countries, mainly associated with *Helicobacter pylori* infection (17, 18).

The Global Cancer Observatory's projections regarding cancer incidence and mortality rates for 2040 assume that the rates estimated for 2020 will remain constant, incorporating only demographic changes. Thus, a 62.8% increase in the number of new cases is estimated for the next 20 years compared to the 1,089,103 estimated cases for 2020, totaling 1,773,179 cases worldwide. Regarding mortality rates, estimates indicate that, in the same period, the total number of deaths due to stomach cancer will increase from 768,793 to 1,274,582, representing a 65.8% increase (19).

Concerning Brazil, the same projections indicate a 78.8% increase for new cases, from 20,139 in 2020 to 36,017 in 2040, higher among men (81.8%; from 12,961 to 23,558 cases) compared to women (73.6%; from 7,178 to 12,459 cases). The proportional mortality increases are expected to be higher than incidence rates for both men and women, increasing from 15,783 to 28,830 deaths per year, comprising an 82.7 % increase (19). It should be noted that these projections only consider the demographic changes expected to occur in the country.

CONCLUSION

Although incidence and mortality rates due to stomach cancer have declined in most countries, including Brazil, the number of new cases and deaths is not negligible. Under this scenario, efforts are required to reduce exposure to stomach cancer risk factors and obtain early diagnoses with timely treatment.

Conflict of Interest: The author declares no potential conflict of interest with respect to research, authorship, and/or publication of this manuscript.

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Minimally Invasive Esophagectomy for Esophageal Cancer

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Abstract: Esophageal cancer is currently the eighth most common cancer, and the sixth leading cause of death from cancer in the world due to its highly aggressive nature. Better prognosis can be achieved with early diagnosis in early stages of the disease. The increasing incidence rate and the distribution of esophageal cancer varies with tumor type location and with geographical area. Multiple factors like ethnicity, genetic factors, and lifestyle play a role. Currently, Barrett’s esophagus is still the only known precursor. Due to its natural history, esophageal cancer is commonly diagnosed in more advanced stages. In tumors confined to the mucosa, local endoscopic treatment is considered curative whereas when the tumor invades the submucosa, surgical esophagectomy is the current standard treatment. In case of locally advanced disease, neoadjuvant chemo or chemo-radio

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therapy is now considered the gold standard treatment. The advent of minimally invasive surgical techniques has reduced morbidity and mortality of esophagectomy without compromising the oncological outcomes. In the chapter, the McKeown mini-invasive esophagectomy technique is described.

Keywords: Barrett's esophagus; esophageal adenocarcinoma; McKeown esophagectomy; minimally invasive esophagectomy; squamous cell carcinoma

INTRODUCTION

Esophageal cancer (EC) is currently the eighth most common cancer in the world and the sixth leading cause of cancer-related death, with a 15–25% five-year survival rate (1–3), due to its highly aggressive nature. Early diagnosis in the early stages of the disease offers better prognosis (2). The incidence of EC has increased by 50% in last two decades with 482,300 new cases diagnosed per year worldwide (4). The increasing incidence and distribution of EC varies with tumor type and with geographical area: squamous cell carcinoma (ESCC) has a higher prevalence in east Asia, southern Europe, eastern and southern Africa, but it has a lower prevalence in north America (4). On the other hand, adenocarcinoma (EAC) is the predominant histological type in the United States, northern Europe, and Australia (2). These geographical variations correlate with the multiple factors that play a role in the origin of EC, that is ethnicity, genetic factors, and lifestyle.

RISK FACTORS AND GENETIC IMPLICATIONS

Several studies have been performed to better understand the etiology and risk factors for EC, and currently Barrett's esophagus (BE) is the only known precursor. BE is a metaplastic transformation from the normal stratified squamous mucosa of the esophagus to a simple columnar epithelium, and its presence conveys a 30- to 40-fold increased risk of developing EAC (5). However, BE is present only in 5% of patients with diagnosed adenocarcinoma (6), so the major challenge is to identify other potential risk factors which include:

Gender and race: There is an increased risk of EAC in people older than 50 years, but no association trend risk per age has been found (7). In white versus black ethnicities the risk of developing EAC is doubled (8). There is also a difference in gender distribution, with a 2-4-fold higher prevalence among males compared to females (9).

Smoking: Smoking is a known risk factor, which is associated with BE and EAC, as well as with ESCC (10, 11), with a higher frequency in men than in women (12).

Gastroesophageal reflux disease (GERD): GERD is the most important risk factor associated both with BE and EAC (13): about 10% of patients with a diagnosis of GERD will develop BE (13, 14), and symptomatic patients have a 5-fold increased risk to progress to EAC as compared to asymptomatic patients (15).

Diet and Alcohol consumption: The reported protective dietary measures against BE (16, 17) are consumption of omega-3-fatty acids, fibers from fruits and vegetables, dietary vitamin C, beta-carotene, and vitamin E. Acetaldehyde derived from alcohol metabolism is the cause of gene mutations, so an alcohol intake exceeding 170 g per week significantly increases the risk of EC (10, 18).

Obesity: Both increased body mass index (BMI) and increased visceral obesity are associated with EC risk, and the risk increases with greater BMI values (19).

Genetics factors: There are genetic conditions related to the development of EC, like Tylosis (*hyperkeratosis palmaris et plantaris*), a genetic autosomal dominant disease associated with a very high lifetime risk of developing ESCC (20). Some genomic mutations are related with EC: a recent large-scale study revealed that more than 83% of ESCCs contained a somatic mutation in TP53 (21). Several other gene mutations, like Cyclin Dependent Kinase Inhibitor 2A (CDKN2A), Retinoblastoma-Associated Protein 1 (RB1), nuclear factor erythroid-derived 2-like 2 (NFE2L2), Checkpoint Kinase 1 (CHEK1), Checkpoint Kinase 2 (CHEK2), Notch homolog 1 translocation-associated (NOTCH1) and Neurogenic locus notch homolog protein 3 (NOTCH3), have been found in ESCC (21, 22). Overexpression of the epidermal growth factor receptor in 59.6–76% of ESCC patients is associated with a poorer prognosis (23, 24). Moreover, other epigenetic alterations, such as DNA methylation, histone modifications, and loss of genome imprinting are related to the development of EC (25).

DIAGNOSIS AND THERAPEUTIC STRATEGIES

Due to its natural history, EC is usually diagnosed at advanced stages. Thus, its early diagnosis would improve treatment outcomes. In patients with BE, annual surveillance by endoscopy should be a standard clinical practice, with random esophageal biopsies performed in all 4 quadrants, each 2 cm of columnar epithelium (26, 27). In the non-dysplastic BE population, the annual cancer risk is very low, ranging between 0.12% and 0.40% per year (28). However, dysplasia without BE increases the risk of cancer at 1% for lesions with low-grade dysplasia and more than 5% for lesions with high grade dysplasia (28), although dysplasia is hardly detectable in asymptomatic individuals. In some areas with high incidence of ESCC, such as northern China, chromoendoscopy techniques could improve the performance of endoscopy and may increase the early detection rate of dysplasia with a cost-effective benefit (29, 30).

The cases of EC detected during screening endoscopy for BE are more likely to have early-stage cancer with longer survival than patients with symptomatic EC (31). However, currently, less than 20% of EC are diagnosed in patients with BE or dysplasia, whereas 80–90% of the total EC are diagnosed in patients with healthy esophageal mucosa (26). When EC is detected at endoscopic biopsy, detailed clinical staging is performed with computed tomography (CT), endoscopic ultrasonography, and positron emission tomography (PET), and tumor staging is based on the 8th edition of AJCC (Tables 1 and 2). In patients with a surgical indication, preoperative workup also includes pulmonary function testing and cardiac evaluation in all cases.

TABLE 1**TNM classification of Esophageal Cancer from the 8th edition AJCC cancer staging manual (32)**

| Category | Criteria |
|-------------------|--|
| T category | |
| TX | Tumor cannot be assessed |
| T0 | No evidence of primary tumor |
| Tis | High-grade dysplasia, defined as malignant cells confined by the basement membrane |
| T1 | Tumor invades the lamina propria, muscularis mucosae, or submucosa |
| T1a | Tumor invades the lamina propria or muscularis mucosae |
| T1b | Tumor invades the submucosa |
| T2 | Tumor invades the muscularis propria |
| T3 | Tumor invades adventitia |
| T4 | Tumor invades adjacent structures |
| T4a | Tumor invades the pleura, pericardium, azygos vein, diaphragm, or peritoneum |
| T4b | Tumor invades other adjacent structures, such as aorta, vertebral body, or trachea |
| N category | |
| NX | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Metastasis in 1–2 regional lymph nodes |
| N2 | Metastasis in 3–6 regional lymph node |
| N3 | Metastasis in 7 or more regional lymph nodes |
| M category | |
| M0 | No distant metastasis |
| M1 | Distant metastasis |

Treatment of early esophageal carcinoma (T1a)

When the tumor does not exceed the submucosa and there is no nodal involvement (T1a, T1b), the tumor is defined as early EC (32). In tumors confined to the mucosa (T1a) the estimated risk of nodal metastases is 1–2%, so a local endoscopic treatment is considered as curative treatment, as well as in case of high-grade dysplasia (Tis) (33, 34). In these cases, the approach should include a combination of endoscopic mucosal resection (EMR) to remove the neoplastic lesion with an ablative technique – like radiofrequency – to manage any residual dysplastic tissue (35). This combined non-invasive treatment modality achieves up to 98% therapeutic efficacy with low morbidity (34, 35). In patients with T1a tumor, esophagectomy is considered as a second option with an outcome that is similar to endoscopic treatment, but it carries a risk of major morbidity, and it should therefore be considered only in patients with a high risk of recurrence, such as in case of multifocal lesions not liable for an ablative treatment (36).

TABLE 2**cTNM staging of Esophageal Cancer from the 8th edition AJCC cancer staging manual (32)**

| cStage group | cT | cN | cM |
|--------------------------------|-------|------|----|
| Squamous cell carcinoma | | | |
| 0 | Tis | N0 | M0 |
| I | T1 | N0-1 | M0 |
| II | T2 | N0-1 | M0 |
| | T3 | N0 | M0 |
| III | T3 | N1 | M0 |
| | T1-3 | N2 | M0 |
| IVA | T4 | N0-2 | M0 |
| | T1-4 | N3 | M0 |
| IVB | T1-4 | N0-3 | M1 |
| Adenocarcinoma | | | |
| 0 | Tis | N0 | M0 |
| I | T1 | N0 | M0 |
| IIA | T1 | N1 | M0 |
| IIB | T2 | N0 | M0 |
| III | T2 | N1 | M0 |
| | T3-4a | N0-1 | M0 |
| IVA | T1-4a | N2 | M0 |
| | T4b | N0-2 | M0 |
| | T1-4 | N3 | M0 |
| IVB | T1-4 | N0-3 | M1 |

Treatment of stage I esophageal carcinoma (T1b, N0/N1)

In patients with tumors invading the submucosa (T1b) the rate of nodal metastases exceeds 10% and endoscopic treatment with a curative intent is not feasible (34, 35). According to established guidelines (37–39), surgical esophagectomy is the standard treatment for stage I EC in all histologic subtypes (37–39). Definitive chemo-radiotherapy (CRT) may be considered only in patients who decline surgery or those who are not fit for major surgery (37–39).

Neoadjuvant treatment for locally advanced resectable ESCC/EAC (T2/T3)

In patients with locally advanced EC, the long-term outcomes of surgery alone are not satisfactory. Many studies have been conducted to evaluate the efficacy of adjuvant/neoadjuvant therapy, the results being that postoperative chemotherapy only extended the disease-free survival (40, 41), whereas preoperative

neoadjuvant chemotherapy significantly increased the overall survival (42, 43). Based on these trials, preoperative CRT is now considered the gold standard treatment for locally advanced resectable disease, both in Japan and in Western Countries, with recommendations of postoperative CRT in case of nodal metastases at pathologic examination (38, 44, 45). Again, definitive CRT alone may be considered as an alternative treatment in patients who decline surgery or who are unfit for major surgery with curative intent (46), and it is considered the standard treatment for locally advanced unresectable EC (47).

SURGICAL TREATMENT

Surgery has made enormous strides from Torek's first thoracic esophageal resection with extra-anatomic reconstruction with a rubber tube in 1913 (48). The evolution of surgical techniques to replace the esophagus has included three main techniques:

- Ivor Lewis esophagectomy (49): a two-field transthoracic esophagectomy performed through a right or left thoracotomy;
- McKeown esophagectomy (50): a three-field esophagectomy with cervical anastomosis, performed through a thoracotomy, a laparotomy and a cervicotomy;
- Esophagectomy without thoracotomy by Orringer and Sloan (51): a transhiatal esophagectomy without thoracotomy (THE).

Historically, these traditional open esophagectomy (OE) procedures are associated with high morbidity and mortality rates (52, 53) and with an in-hospital mortality rate ranging between 1.2% and 8.8% (53, 54). More accurate patient selection from improved imaging modalities, the advent of minimally invasive surgical techniques and progresses in thoracic anesthesia in the 1990s have drastically reduced the morbidity and mortality rates of esophagectomy without compromising the oncological outcomes. From the first esophagectomy through a right thoracoscopic approach reported by Sir Alfred Cuschieri in 1992 (55) to the current mini-invasive approaches (that include laparoscopic, thoracoscopic, and robotic-assisted techniques), minimally invasive esophagectomy (MIE) has currently become an excellent option for esophageal resection.

Minimally Invasive Ivor Lewis Esophagectomy

Advantages:

- Oncologic "en bloc" thoracic esophageal and gastric lymph node resection;
- Lower rate of anastomotic leak;
- Lower rate of recurrent laryngeal nerve injury.

Disadvantages:

- In the event of anastomotic leak, there may be pleural contamination, with severe morbidity and risk of mortality;
- Access to the chest requires single-lung ventilation, with potentially increased pulmonary morbidity.

Minimally invasive McKeown esophagectomy

Advantages:

- Oncologic “en bloc” thoracic esophageal and gastric lymph node resection;
- Higher lymph node yield and potential for more accurate pathological staging;
- Cervical anastomotic leaks may be managed more easily;
- Preservation of Azygos vein, for surgeons who elect to preserve it (56).

Disadvantages:

- Higher incidence of anastomotic leak;
- Higher incidence of recurrent laryngeal dysfunction and oropharyngeal dysphagia.

Minimally invasive THE esophagectomy

Advantages:

- Elimination of a thoracotomy or thoracoscopy, with reduced pulmonary morbidity and pain;
- Cervical anastomotic leaks may be managed more easily.

Disadvantages:

- Higher incidence of recurrent laryngeal dysfunction and oropharyngeal dysphagia;
- Difficult oncologic “en bloc” thoracic esophageal and gastric lymph node resection.

Outcomes of MIE

The principal complication of MIE continues to be anastomotic leak, with a rate that ranges between 0% and 33% in various series (57, 58). Pulmonary complications also remain a cause of concern. Avoiding a thoracotomy in MIE should decrease this rate, but the reported results are still conflicting (59). The surgical and oncological outcomes, in terms of severity of postoperative complications, perioperative mortality and overall survival, (60, 61), have shown improved results and a proven superiority of MIE, as compared to OE in large-scale studies (60, 61).

MINIMALLY INVASIVE MCKEOWN ESOPHAGECTOMY: HOW WE DO IT

The operation is carried out by two surgical teams, one for the thoracoscopic and cervical steps, and one for the laparoscopic step.

Thoracoscopic step

After induction of general anesthesia with double lumen tube intubation and invasive monitoring lines placement, such as central vein, arterial lines and thoracic epidural catheters, the patient is placed in the left lateral position. Lung is deflated and four port video-assisted thoracoscopy is started. A 10 mm. camera port is placed usually in the VII intercostal space posterior to the mid-axillary line; a 10 mm working port is placed in the 8th intercostal space 4 to 5 cm posterior to the first camera port. Another 10 mm port is usually placed in the fourth intercostal space adjacent to nipple and the last 10 mm port is placed in the sixth intercostal space just beneath the tip of the scapula, that helps in retraction and manipulation for the operating surgeon.

After ports placement, the deflated lung is retracted anteriorly, the area of the tumor is identified and general resectability is assessed. Inferior pulmonary ligament is divided, and the esophagus is exposed after incision of the posterior mediastinal pleura. Medial esophageal dissection is performed first, followed by careful lateral dissection from adjacent aorta. Direct branches from aorta to esophagus are individually clipped or coagulated to avoid troublesome hemorrhage. Azygos vein is usually not divided, but only prepared, encircled and retracted to allow esophageal mobilization and lymphadenectomy. Preservation of the azygos vein is aimed at preventing kinking of the gastric tube that will replace the esophagus. It is critical to stay close to the esophagus to avoid lesions of the membranous portion of the trachea which is closely adjacent. The esophagus is mobilized up to the root of the neck, taking care to avoid injury to nearby major vessels. Adequate lymphadenectomy is performed at this stage. Inferiorly the esophagus is mobilized down to the esophageal hiatus after retracting the diaphragm with a sponge on stick. Hemostasis is ascertained, two closed suction chest drains are placed, followed by closure of the chest cavity, following which the patient is moved to supine position.

Laparoscopic step

Five trocars are used. One 12 mm trocar is placed in supraumbilical position, three 12 mm trocars are placed in the right hypochondrium, left hypochondrium and sub-xiphoid region along the midline, the latter with a longitudinal skin incision. One 5 mm trocar is placed along the left midclavicular line and below the transverse umbilical line. The liver is retracted with a grasper introduced from the subxiphoid trocar. The *bursa omentalis* is opened by dividing the gastrocolic ligament up to its origin from the gastroduodenal artery with an advanced energy device (LigaSure™, Medtronic, Minneapolis, Minnesota, USA or Ultracision, Harmonic Scalpel, Ethicon Endo Surgery, Cincinnati, Ohio, USA) following the course of the right gastro-epiploic arch but avoiding to stay too close to these vessels because it is mandatory to preserve them. Division of the gastrocolic ligament continues on the left until the left gastroepiploic artery is divided at its origin from the splenic artery. Next, the short gastric vessels are divided and the left crus of the esophageal hiatus is exposed. After retracting the gastric body upwards, the gastric coronary vein and the left gastric artery are then prepared posteriorly closed with Hem-O-lok clips (Teleflex Medical Europe Ltd, IDA Business and Technology Park, Dublin Road, Athlone, Co Westmeath, Ireland) and divided. The lesser

omentum is opened, and the abdominal esophagus is separated from the right crus of the esophageal hiatus, followed by division of the phreno-esophageal ligament and complete mobilization of the abdominal esophagus. Abdominal D2 lymphadenectomy is carried out in case of tumors located at the esophago-gastric junction. The cervical stage of the procedure follows thereafter.

Cervicototomy incision

The cervical step of the operation is carried out through a left cervical incision. We use an oblique incision on the left side of the neck after dividing the platysma and dissecting along the anterior border of the sternocleidomastoid muscle. If necessary, the middle thyroid vein is ligated and divided to avoid traction injury near its communication with the internal jugular vein. The strap muscles are divided, and the thyroid gland is retracted medially. The cervical esophagus is exposed, and it is then mobilized and divided using an endoGIA 30 stapler (Medtronic, Minneapolis, Minnesota, USA). An umbilical tape is attached to the distal end of the divided esophagus that will subsequently be pulled down into the abdomen through the esophageal hiatus. The umbilical tape will remain in place through the mediastinum during creation of the gastric conduit.

Abdominal part of the procedure and cervical anastomosis technique

After removing the subxyphoid trocar, a 7–8 cm full-thickness service minilaparotomy is created by prolonging the longitudinal skin incision and an Alexis wound protector device (Applied Medical, California, USA) is positioned prior to esophago-gastric extraction. A narrow gastric tube, measuring 3–5 cm in diameter, is created with a GIA 75 mm linear stapler and green cartridges (Medtronic, Minneapolis, Minnesota, USA) after opening the gastric fundus, evacuating any intraluminal content by gentle suction and inserting a 36 fr calibration tube (Teleflex Medical Europe Ltd, IDA Business and Technology Park, Dublin Road, Athlone, Co Westmeath, Ireland) that is laid along the greater gastric curvature. Four to five cartridges are required to suture and to divide the stomach from the fundus to the antrum along the lesser gastric curvature. The surgical specimen, including the esophagus with tumor and the lesser gastric curvature, are removed and hemostasis of the gastric tube is checked. The right gastric artery is usually preserved in case of tumors located in the thoracic esophagus, otherwise it is divided to remove station 5 lymph nodes if the tumor is located at the esophago-gastric junction. After creation of the narrow gastric conduit, Indocyanine Green Near Infrared Fluoroangiography is performed in order to assess its vascular supply.

The gastric tube is then sutured with two or three silk stitches to the distal extremity of the umbilical tape that followed the esophagus upon its withdrawal through the esophageal hiatus, and it is gently pulled up to the neck by pulling on the umbilical tape from the cervicototomy incision. Particular care is taken at this point to avoid torsion or kinking of the vascular pedicle during this maneuver. Our stapled anastomosis technique aimed at preventing fibrous stenosis of the esophago-gastric anastomosis has been previously described (56). The gastric conduit and the proximal esophagus are then prepared for the anastomosis. The proximal end of the gastric graft is transected, and intraluminal content is

evacuated by gentle suction. Two pexing silk sutures between the posterior wall of the esophagus and the posterior wall of the gastric tube are placed. This allows to maintain the cervical esophagus and gastric tube in a side-to-side position, as a double-barreled gun. In order to avoid mucosal retraction, two full thickness silk sutures are placed on the esophagus and on the gastric wall at the level of the gastrotomy to maintain an adequate traction during the anastomosis. The two forks of an endoscopic linear stapler (ETS45 blue cartridge, Ethicon Endo-Surgery) are placed across the two opposite walls with the anvil on the esophageal lumen side and the cartridge on the gastric conduit side. After approximation of the forks and checking the proximal esophagus to avoid any twisting, the stapler is fired, thus accomplishing the posterior part of the anastomosis. At this time, a nasogastric tube is inserted with its tip brought close to the pylorus, and the anterior aspect of the anastomosis is completed by two or more additional firings of the Endo-GIA stapler straight across the raised edges of the stomach and of the esophagus. If necessary, two or three sero-muscular silk stitches are then placed to reinforce the anterior part of the anastomosis. Vascular perfusion of the anastomosis may be checked again at this point by repeating Indocyanine Green Near-Infrared Fluoroangiography. Once completed, the anastomosis drops back into the thoracic inlet. A drainage tube is inserted in the cervical wound. After checking for hemostasis in the peritoneal cavity and at the trocar sites incisions, another drainage tube is positioned below the left hemidiaphragm exiting through the left hypochondrium skin incision. Standard wounds closure follows.

CONCLUSION

EC is increasing in frequency, and it displays a highly aggressive behavior. Early diagnosis in early stages of the disease would be associated with better prognosis but is still difficult to achieve nowadays. The increasing incidence and distribution of EC varies with the tumor type, tumor location and with geographical area. Multiple factors are recognized to play a role, like ethnicity, genetic factors and lifestyle. The incidence rate significantly increases in black men with smoking and alcohol consumption habits. The only known precursor remains Barrett's esophagus. Local endoscopic treatment is sufficient and curative in tumors confined to the mucosa whereas surgical esophagectomy is the current standard treatment in case of tumors invading the submucosa. Preoperative chemotherapy or chemo-radiotherapy is the gold standard treatment for locally advanced disease. The advent of minimally invasive surgical techniques has reduced morbidity and mortality of esophagectomy without compromising the oncological outcomes. Today, minimally invasive esophagectomy is the preferred option when the necessary surgical expertise and technological devices are available.

Conflict of Interest: The authors declare no potential conflict of interest with respect to research, authorship and/or publication of this chapter.

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Gastrointestinal Cancers

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Editor



Professor Jose A. Morgado-Diaz, PhD, is a cell biologist at the Federal University of Rio de Janeiro, Brazil, and a Principal Investigator at the Instituto Nacional de Cancer – INCA, Brazil. From 2012 to 2013, he was a Senior Postdoctoral fellow of the National Institute of Health, Bethesda, MD, USA. In 1997 after serving as Assistant Professor

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