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## REVIEW

- 507 Proteoglycans and their functions in esophageal squamous cell carcinoma  
*Zhu Y, Cheung ALM*
- 522 Therapeutic potential of thymoquinone in combination therapy against cancer and cancer stem cells  
*Fatfat Z, Fatfat M, Gali-Muhtasib H*

## MINIREVIEWS

- 544 Mechanisms of acquired resistance of BRCA1/2-driven tumors to platinum compounds and PARP inhibitors  
*Imyanitov E, Sokolenko A*
- 557 Esophagogastric junction adenocarcinoma: Preoperative chemoradiation or perioperative chemotherapy?  
*Laxague F, Schlottmann F*
- 565 BRCA mutations and gastrointestinal cancers: When to expect the unexpected?  
*Maccaroni E, Giampieri R, Lenci E, Scortichini L, Bianchi F, Belvederesi L, Brugiati C, Pagliaretta S, Ambrosini E, Berardi R*

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## Proteoglycans and their functions in esophageal squamous cell carcinoma

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### Abstract

Esophageal squamous cell carcinoma (ESCC) is a highly malignant disease that has a poor prognosis. Its high lethality is mainly due to the lack of symptoms at early stages, which culminates in diagnosis at a late stage when the tumor has already metastasized. Unfortunately, the common cancer biomarkers have low sensitivity and specificity in esophageal cancer. Therefore, a better understanding of the molecular mechanisms underlying ESCC progression is needed to identify novel diagnostic markers and therapeutic targets for intervention. The invasion of cancer cells into the surrounding tissue is a crucial step for metastasis. During metastasis, tumor cells can interact with extracellular components and secrete proteolytic enzymes to remodel the surrounding tumor microenvironment. Proteoglycans are one of the major components of extracellular matrix. They are involved in multiple processes of cancer cell invasion and metastasis by interacting with soluble bioactive molecules, surrounding matrix, cell surface receptors, and enzymes. Apart from having diverse functions in tumor cells and their surrounding microenvironment, proteoglycans also have diagnostic and prognostic significance in cancer patients. However, the functional significance and underlying mechanisms of proteoglycans in ESCC are not well understood. This review summarizes the proteoglycans that have been studied in ESCC in order to provide a comprehensive view of the role of proteoglycans in the progression of this cancer type. A long term goal would be to exploit these molecules to provide new strategies for therapeutic intervention.

**Key Words:** Esophageal squamous cell carcinoma; Proteoglycan; Glycosaminoglycan; Serglycin; Extracellular matrix; Biomarker

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**Core Tip:** Esophageal squamous cell carcinoma (ESCC) is a highly malignant human cancer because of its early metastasis and late diagnosis. Cancer metastasis involves multiple steps that involve cell-cell and cell-matrix interactions in the tumor microenvironment. Proteoglycans are one of the components of the extracellular matrix that play an important role in cell-matrix interactions. We herein summarize the proteoglycans that have been studied in ESCC to provide a comprehensive view of the role of proteoglycans in ESCC.

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## INTRODUCTION

Esophageal cancer is the 7<sup>th</sup> most common cancer in the world and the sixth highest ranking cancer in terms of mortality rate[1]. The two major subtypes of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). People who develop esophageal cancer usually have no specific symptoms at the early stage. The onset of symptoms is often accompanied by difficulty and pain in swallowing (dysphagia), loss of body weight, and heartburn, by which time the tumor is likely already in the advanced stage. Late diagnosis of esophageal cancer is the primary reason for its high lethality. Tobacco use and alcohol consumption are the two main risk factors of ESCC. In addition, genetic susceptibility also plays a role in ESCC. It has been reported that *TP53* has the highest frequency of mutation in ESCC patients[2]. Other frequently mutated genes in ESCC are *RB1*, *CDKN2A*, *PIK3CA*, *CCND1*, *ZNF750*, *NOTCH1*, *NFE2L2*, *FAT1*, and *FAT2*[3-6]. It is widely accepted that tumor progression not only depends on accumulation of genetic alterations but also on changes within the surrounding microenvironment[7]. The contribution of tumor microenvironment and extracellular matrix (ECM) components to cancer development and progression is increasingly being recognized[7-9]. The ECM in the tumor microenvironment consists of proteoglycans, collagens, fibronectin, and laminins[10]. Interaction of these molecules with growth factors, chemokines, cytokines, and matrix metalloproteinases facilitate tumor cell survival, invasion, and metastasis[11,12]. It is a highly dynamic and complex interaction network. In this review, the characteristics of proteoglycans and their functions in ESCC are summarized.

## STRUCTURE AND CLASSIFICATION OF PROTEOGLYCANS

Proteoglycans typically consist of polysaccharide chains termed glycosaminoglycan (GAG) and a core protein. The GAGs are covalently attached to the serine residues on the core protein. Proteoglycans differ from glycoproteins in several aspects. For example, the carbohydrate content in proteoglycans is 50%-60%, which is much higher than that in glycoproteins. The GAGs in proteoglycans are linear, negatively-charged long chains, whereas the oligosaccharides in glycoproteins are branched short chains that may or may not be negatively-charged. The GAGs of proteoglycans are composed of repeating disaccharide units of hexuronic acid and hexosamine. They may be modified with sulfate groups at various positions to achieve multiple biological functions[13]. The major categories of GAGs are chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS), heparin, and hyaluronan (HA).

CSs consist of repeating disaccharide units of N-acetylgalactosamine (GalNAc) and D-glucuronic acid (GlcA), and can be sulfated at C4 and/or C6 position of the GalNAc unit. The CS chains that contain sulfate group modification at both C4 and C6 positions of the GalNAc unit are named CS-E, whereas those modified exclusively at C4 position of GalNAc are known as CS-A. CS-C contains sulfate group only at C6 position of the GalNAc. CS-D chains have sulfate groups at C6 position of the GalNAc and C2 position of the GlcA. DS chains, also named CS-B, are derived from CS, of which the GlcA residues are epimerized into L-iduronic acid (IdoA). DS chains are

sulfated at C2 position of IdoA and C4/C6 position of the GalNAc. KS chains contain repeating disaccharide units of galactose (Gal) and N-acetylglucosamine (GlcNAc). HS chains consist of GlcA and GlcNAc repeats, while heparins consist of repeating disaccharide units of IdoA and GlcNAc. The sulfate modifications in HS and heparins are in clusters. HA, a linear, protein-free, non-sulfated GAG consists of GlcNAc and GlcA repeating units. The structures of GAG chains are shown in [Figure 1](#).

Proteoglycans can be classified according to their predominant GAG types into CS proteoglycans and HS proteoglycans or according to their cellular and subcellular location, overall gene/protein homology, and the presence of specific protein modules within their respective protein cores into intracellular, cell-surface, pericellular, and extracellular proteoglycans[14]. In this review, the functions of proteoglycans in ESCC will be discussed according to this classification. These proteoglycans are classified and summarized in [Table 1](#).

## INTRACELLULAR PROTEOGLYCANS IN ESCC

Serglycin is the only known intracellular proteoglycan. It was first isolated from rat yolk carcinoma cell line L2 (named as pPG1) in 1985[15]. Human *SRGN* (serglycin) was isolated from promyelocytic leukemia HL-60 cells in 1990[16] and from hematopoietic cells in 1992[17]. Its amino acid sequence was later found to be identical to that of human platelet proteoglycan protein, which was isolated and characterized in 1986[18], and the complete amino acid sequence determined in 1988[19,20].

The human *SRGN* gene is located on chromosome 10 and spans about 16.7 kb with 7% of the gene comprising of exons[16,17,21]. Nicodemus *et al*[16] showed that human *SRGN* gene contains three exons, with exon 1 encoding signal peptide (amino acids 1-27), exon 2 encoding amino acids 28-77, and exon 3 encoding amino acids 77-158, which includes the serine/glycine repeat region (amino acids 94-111). The serine/glycine repeat region is the GAG attachment region that allows the clustering of GAG chains close to the center of the core protein. This structure is unique to serglycin[22].

Serglycin was initially characterized as a hematopoietic proteoglycan[23] and was subsequently found to be present in many other non-hematopoietic cells such as endothelial cells[24], immune cells[25], chondrocytes[26-28], and cancer cells[29,30]. The type and size of the GAG chains of serglycin vary among different cell types and can affect the functions of serglycin. Serglycin synthesized by rat serosal mast cells is mainly modified with heparin or HS, while CS chains are predominant in rat mucosal-like mast cells[31]. HS/CS hybrid GAG chains are found in mouse mastocytoma cells and human erythroleukemia cells[32]. In human umbilical vein endothelial cells, serglycin is modified with CS-GAG chains and has smaller GAG chains than that expressed in platelets[24,33]. In human platelets, the predominant type of GAG chain is CS-4[18].

The enzymes involved in the synthesis of GAG chains have different functions in the particular physiological process of cells. Two enzymes that synthesize CS, namely chondroitin 4-sulfotransferase-1 and GalNAc(4S)-6-O-sulfotransferase, and N-deacetylase/N-sulfotransferase-2, which is essential for heparin synthesis, are positively associated with mast cell maturation, whereas chondroitin 6-sulfotransferase is negatively correlated with mast cell maturation[34]. Serglycin core protein, chondroitin 4-sulfotransferase-1, and GalNAc(4S)-6-O-sulfotransferase are upregulated during mast cell activation and accompanied by downregulation of N-deacetylase/N-sulfotransferase-2[34]. A recent study showed that hyaluronidase-4 can cleave the CS chains of serglycin in human mast cells[35]. These reports suggest that enzymes responsible for GAG synthesis are important in determining the functions of serglycin.

In the past two decades, an ever-increasing number of studies have shown that serglycin plays a significant role in human cancers. It has been reported that serglycin is increased in many human cancers including breast cancer[36-40], multiple myeloma[41-44], acute myeloid leukemia[45], nasopharyngeal carcinoma[46-48], hepatocellular carcinoma (HCC)[49-51], colon cancer[52,53], non-small cell lung cancer[54,55], and glioblastoma[56]. The reported functions of serglycin in promoting cancer progression include regulation of cell adhesion, promoting migration/invasion, inducing angiogenesis, and avoiding immune destruction ([Figure 2](#)).

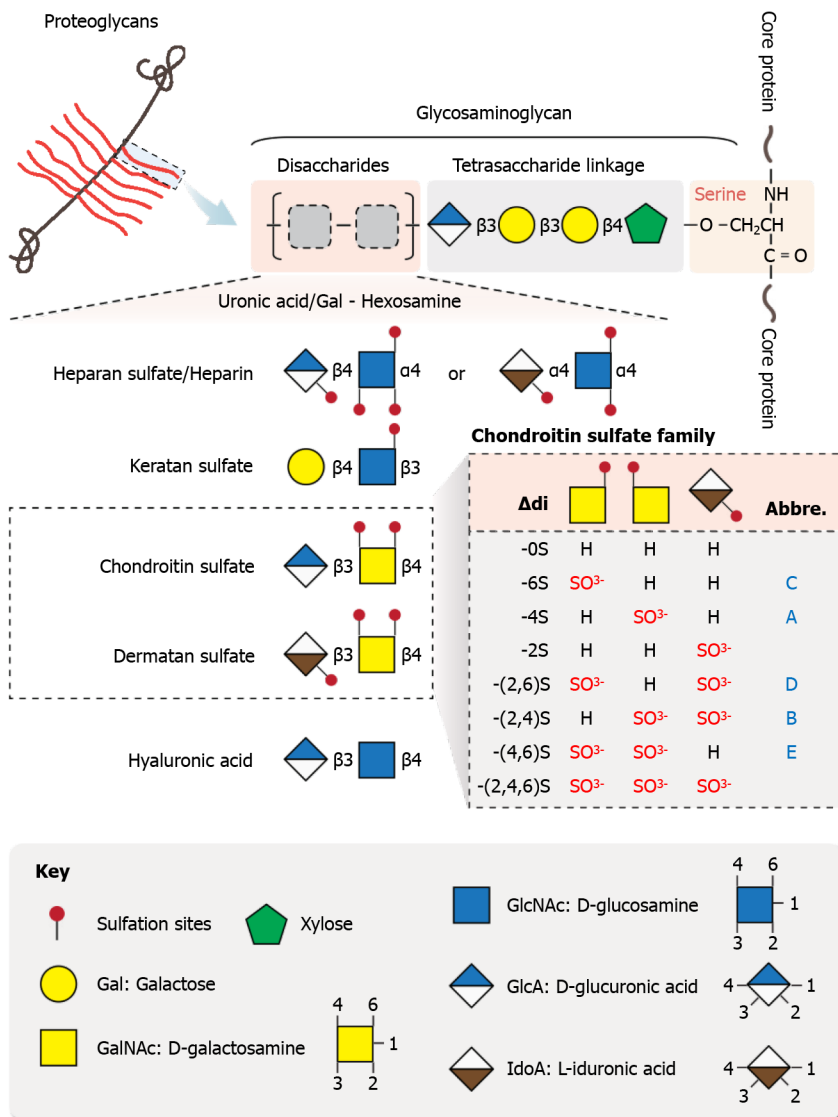
Our recent study showed for the first time that serum serglycin could be a potential non-invasive biomarker with prognostic significance in ESCC[57]. We found that serglycin and its binding partners (including matrix metalloproteinases) could promote ESCC cell invasion, migration, and metastasis. We also identified midkine as a novel GAG-dependent binding partner of serglycin, which contributes to ESCC

**Table 1 Classification of proteoglycans[14] with clinicopathological and/or functional significance in esophageal squamous cell carcinoma**

Location	Classification	Gene symbol	Eponym	Predominant GAG	Studied in ESCC	Ref.
Intracellular	Secretory granules	<i>SRGN</i>	Serglycin	Heparin/CS	Serglycin	Zhu <i>et al</i> [57]
Cell-surface	Transmembrane	<i>SDC</i>	Syndecan 1-4	HS/CS	Syndecan-1, -2	Mikami <i>et al</i> [58], Szumilo <i>et al</i> [60] and Huang <i>et al</i> [61]
		<i>CSPG4</i>	NG2	CS/DS		
		<i>TGFBR3</i>	Betaglycan	HS/CS		
		<i>PTPRZ1</i>	Phosphacan	CS/DS		
	GPI-anchored	<i>GPC</i>	Glypican 1-6	HS	Glypican-1, -3	Hara <i>et al</i> [62], Li <i>et al</i> [63], Harada <i>et al</i> [64] and Song [65]
Pericellular	Basement membrane zone	<i>HSPG2</i>	Perlecan	HS/CS		Zheng <i>et al</i> [71], Zhong <i>et al</i> [72] and Chen <i>et al</i> [73]
		<i>AGRN</i>	Agtrin	HS		
		<i>COL18A1</i>	Collagen XVIII	HS	Endostatin	
		<i>COL15A1</i>	Collagen XV	CS/HS		
Extracellular	Hyalectans	<i>ACAN</i>	Aggrecan	CS/KS		
		<i>VCAN</i>	Versican	CS/DS		
		<i>NCAN</i>	Neurocan	CS/DS		
		<i>BCAN</i>	Brevican	CS/DS		
	SLRPs, Canonical Class I	<i>DCN</i>	Decorin	CS/DS	Decorin	Wu <i>et al</i> [83], Ji <i>et al</i> [84] and Augoff <i>et al</i> [85]
		<i>BGN</i>	Biglycan	CS/DS	Biglycan	Zhu <i>et al</i> [97]
		<i>ASPN</i>	Asporin			
		<i>ECM2</i>	ECM2			
		<i>ECMX</i>	ECMX			
	SLRPs, Canonical Class II	<i>FMOD</i>	Fibromodulin	KS		Kashyap <i>et al</i> [102,103]
		<i>LUM</i>	Lumican	KS	Lumican	
		<i>PRELP</i>	PRELP			
		<i>KERA</i>	Keratocan	KS		
	SLRPs, Canonical Class III	<i>OMD</i>	Osteoadherin	KS		
		<i>EPYC</i>	Epiphycan	CS/DS		
		<i>OPTC</i>	Opticin			
		<i>OGN</i>	Osteoglycin			
	SLRPs, Non-Canonical Class IV	<i>CHAD</i>	Chondroadherin			
		<i>NYX</i>	Nyctalopin			
		<i>TSKU</i>	Tsukushi			
	SLRPs, Non-Canonical Class V	<i>PODN</i>	Podocan			
		<i>PODNL1</i>	Podocan-Like 1			
	SPOCK	<i>SPOCK</i>	Testican 1-3	HS	Testican-1	Song <i>et al</i> [108]

CS: Chondroitin sulfate; DS: Dermatan sulfate; ESCC: Esophageal squamous cell carcinoma; GAG: Glycosaminoglycan; GPI: Glycosylphosphatidylinositol; HS: Heparan sulfate; KS: Keratan sulfate; NG2: Nerve/glia antigen 2; SLRP: Small leucine-rich proteoglycan.

progression by upregulating mitogen-activated protein kinase/extracellular signal-regulated protein kinase signaling and c-Myc expression in an autocrine manner[57].



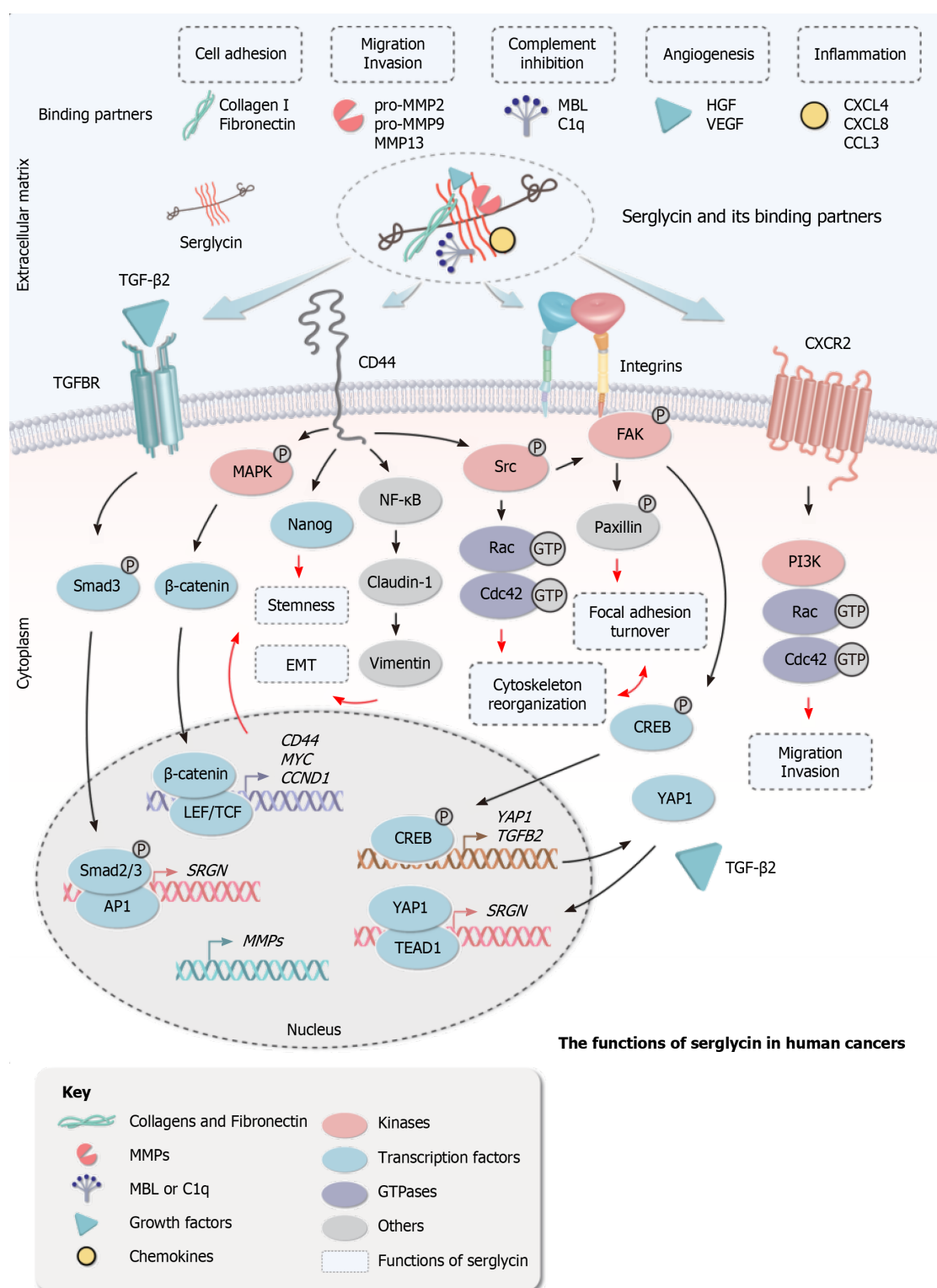
**Figure 1 Structure and components of glycosaminoglycan chains of proteoglycans.** The disaccharide unit of glycosaminoglycan consists of epimers of uronic acid (GlcA, IdoA) or galactose and hexosamine (GlcNAc, GalNAc). The repeating disaccharide units in different types of glycosaminoglycan are shown with the potential sulfation positions. The different disaccharides types ( $\Delta$ di) of chondroitin sulfate family have abbreviated names, which are based on their different sulfation positions.

This study also highlighted the significance of CS-GAGs of serglycin in ESCC cell invasion. Strategies that target serglycin, its binding partners, or its GAG chains where most of the protein interactions take place may be a new direction in ESCC therapy.

## CELL-SURFACE PROTEOGLYCAN IN ESCC

### Syndecan

The syndecan family is a group of transmembrane proteoglycans consisting of syndecan-1, -2, -3, and -4. They generally carry HS-GAG chains. In normal esophageal epithelium, syndecan-1 is strongly expressed in cell membrane, and the expression of syndecan-1 core protein and HS-GAG chains are significantly decreased in T2 and T3 stage specimens compared with lower grade specimens[58]. Although the study by Conejo *et al*[59] found that syndecan-1 messenger RNA level in esophageal malignancies was not significantly different from that in the corresponding normal samples, a histochemical study by Szumilo *et al*[60] showed that well- and moderately-differentiated carcinomas are more frequently syndecan-1-positive compared with poorly differentiated carcinomas. Loss of syndecan-1 was found to be associated with the incidence of lymphatic invasion, and malignant phenotype of ESCC[58]. Notably, the reduction of HS-GAGs on syndecan-1 was more important than that of core protein for



**Figure 2 Overview of serglycin functions in human cancers.** The various binding partners of serglycin contribute to the multiple functions of serglycin in human cancers. Serglycin, with or without binding partner, can bind to receptors on cancer cells and activate the downstream signaling pathways, including  $\beta$ -catenin, Nanog, phosphatidylinositol-3-kinase (PI3K) and transforming growth factor (TGF)  $\beta$  pathways. MMP: Matrix metalloproteinase; NF- $\kappa$ B: Nuclear factor-kappa B.

tumor cell invasion; other pathological parameters such as nodal and distant organ metastasis were negatively correlated with HS-GAG expression but not with syndecan-1 core protein expression[58]. Unlike syndecan-1, which acts as a tumor suppressor in ESCC, syndecan-2 is positively correlated with tumor size in ESCC[61]. Multivariate analysis showed that syndecan-2 is an independent prognostic factor for survival rate of ESCC patients after surgery[61].



### Glypican

Glypican family includes six members, which are glypican-1 to -6. They also carry HS-GAG chains. Glypican-1 was found to be upregulated in ESCC cell lines compared with normal epithelial cells, and its expression in ESCC tissue is negatively correlated with survival rate of patients[62]. Another study confirmed that ESCC tumor samples have higher expression of glypican-1 than that in para-tumor tissues[63]. Functionally, glypican-1 promotes cell motility and induces epithelial-mesenchymal transition (EMT) in ESCC, possibly through activation of AKT/ $\beta$ -catenin pathway[63]. Knock-down of glypican-1 (*GPC1*) significantly inhibited ESCC cell growth by inhibiting epidermal growth factor receptor pathway and inducing cell apoptosis[64]. Systemic administration of anti-GPC1 antibody significantly inhibited growth of tumor xenografts and tumor angiogenesis[64]. Based on these findings, glypican-1 was described as a promising therapeutic target in ESCC[65].

Unlike glypican-1, glypican-3 did not show significant correlation with histological type, tumor stage, tumor grade, or patient survival in ESCC[66,67], although it was reported to be a diagnostic molecule and a therapeutic target in HCC[68,69].

## PERICELLULAR PROTEOGLYCANS

Pericellular proteoglycans include perlecan, agrin, collagen XVIII, and collagen XV (Table 1). The functions of perlecan, agrin, and collagen XV in ESCC have not been elucidated. Endostatin, which is a 20 kDa C-terminal fragment of collagen XVIII, was found to have anti-angiogenic activity[70]. It was later shown to have inhibitory effect on formation of ESCC-related lymphatic vessels[71]. The application of recombinant endostatin protein combined with chemoradiotherapy in ESCC treatment increased the overall survival rate of patients[72]. Recombinant endostatin combined with radiotherapy could significantly inhibit proliferation and migration/invasion of ESCC cells as well as reduce angiogenesis, but there was no effect on cell apoptosis[73].

## EXTRACELLULAR PROTEOGLYCANS IN ESCC

### Decorin

Decorin, also called PG40, belongs to the small leucine-rich proteoglycan (SLRP) family[74]. The core protein of decorin is about 42 kDa. There is a single GAG chain attached to the N-terminus of the core protein[75]. Proteoglycans belonging to SLRP family contain a region with leucine-rich tandem repeats (LRR). The LRR region is modified by N-glycosylation. N-glycosylation and the O-linked GAG side chains are crucial for the interactions of decorin with other molecules. Studies have shown that the DS-GAGs are essential in fibrillar network formation through bridging collagen fibers[76,77]. The GAG chains and LRR region of SLRPs are both involved in ECM assembly.

Decorin was found to be necessary for appropriate fibrillogenesis due to its ability to bind to collagen[74]. The significance of decorin, as well as other SLRPs, in ECM assembly has been intensively investigated and reviewed[78]. Of note, same classes of SLRPs have the same function of binding to collagen and therefore compete with each other. For example, two other class I SLRPs, namely biglycan and asporin, are able to compensate for the loss of decorin[79]. Asporin can compensate for both decorin and biglycan loss[80]. Lumican, a class II SLRP, can compete with fibromodulin, which belongs to the same class[81,82].

The concentration of plasma decorin in 275 ESCC patients was found to be significantly lower than that in normal controls[83]. The expression of decorin in malignant ESCC tissue samples is also lower compared with normal tissue[84,85]. The study by Ji *et al*[84] showed that decorin expression in ESCC is negatively correlated with histological grade, lymph node metastasis, tumor stage, and clinical stage. Low expression of decorin is associated with poor survival rate and is an independent prognostic marker in patients with ESCC[84]. The tumor suppressive property of decorin, as revealed from studies in other types of cancer such as skin squamous cell carcinoma [86] and breast cancer[87,88], is predominantly due to its ability to trap transforming growth factor  $\beta$  (TGF- $\beta$ ) in the ECM. The binding of decorin to TGF- $\beta$  prevents the latter from binding to its receptors. Interestingly, this decorin-TGF- $\beta$  interaction is dependent on decorin-collagen binding[89]. In addition, decorin also acts as a receptor tyrosine kinase inhibitor[90]. It can inhibit the activity of epidermal growth factor

receptor[91], insulin-like growth factor receptor 1, and platelet-derived growth factor receptor  $\alpha/\beta$ [92], thereby suppressing their downstream signaling cascades, and finally inhibiting cancer cell proliferation, migration, and invasion[93]. Systemic administration of recombinant decorin protein can inhibit tumor growth and reduce metastasis of squamous cell carcinoma[86]. Based on these characteristics of decorin, it is regarded as a promising anti-tumor molecule and a potential neoadjuvant therapy for human cancers[94,95].

### Biglycan

Biglycan, a SLRP protein coded by the *BGN* gene, is structurally related to decorin but holds two GAG chains rather than one at the N-terminus of the core protein. The molecular weight of biglycan core protein is about 42 kDa. Although biglycan shares similar structure with decorin, the functions of biglycan in human cancers differ from that of decorin. In ESCC, the gene expression of *BGN* is upregulated in tumor samples compared with non-tumor tissues[96,97], although there is no significant association with patient survival[98]. High expression of biglycan in tumor tissue is positively correlated with tumor invasion, lymph node metastasis, and advanced clinical stage [96,97] and is an independent prognostic marker of ESCC[97]. In addition, higher serum biglycan was found in patients with EAC[99], suggesting that biglycan has diagnostic significance in esophageal cancer. Functionally, biglycan has anti-apoptotic effects on mesangial cells and pro-angiogenesis effects on tumor endothelial cells[100], which contribute to cancer cell survival and metastasis. These studies suggest that targeting biglycan may be a novel approach in anti-angiogenic and anti-tumor therapy for ESCC patients[97,100].

### Lumican

Lumican is a class II SLRP that has up to three KS-GAG chains. There are contradictory reports on the roles of lumican in human cancers. In pancreatic cancer, patients with high expression of stromal lumican have favorable survival after surgery[101]. However, the gene expression of *LUM* was found to be 7-fold higher in ESCC than in normal epithelia[102,103]. Strong positive lumican immunostaining was found in the stromal and epithelial compartments of ESCC specimens but was almost negative in normal epithelium[103]. The concentration of lumican in plasma was identified as a potential biomarker of ESCC *via* a proteomic screen[104]. Nevertheless, a previous study by our group comparing a highly invasive ESCC subline with its parental cells showed that *LUM* decreased 8-fold in the highly invasive subline and was accompanied by activation of AKT pathway[105]. Li *et al*[101] reported that overexpression of *LUM* suppressed AKT activation in pancreatic cancer cells. These findings infer that lumican might be negatively correlated with AKT activation.

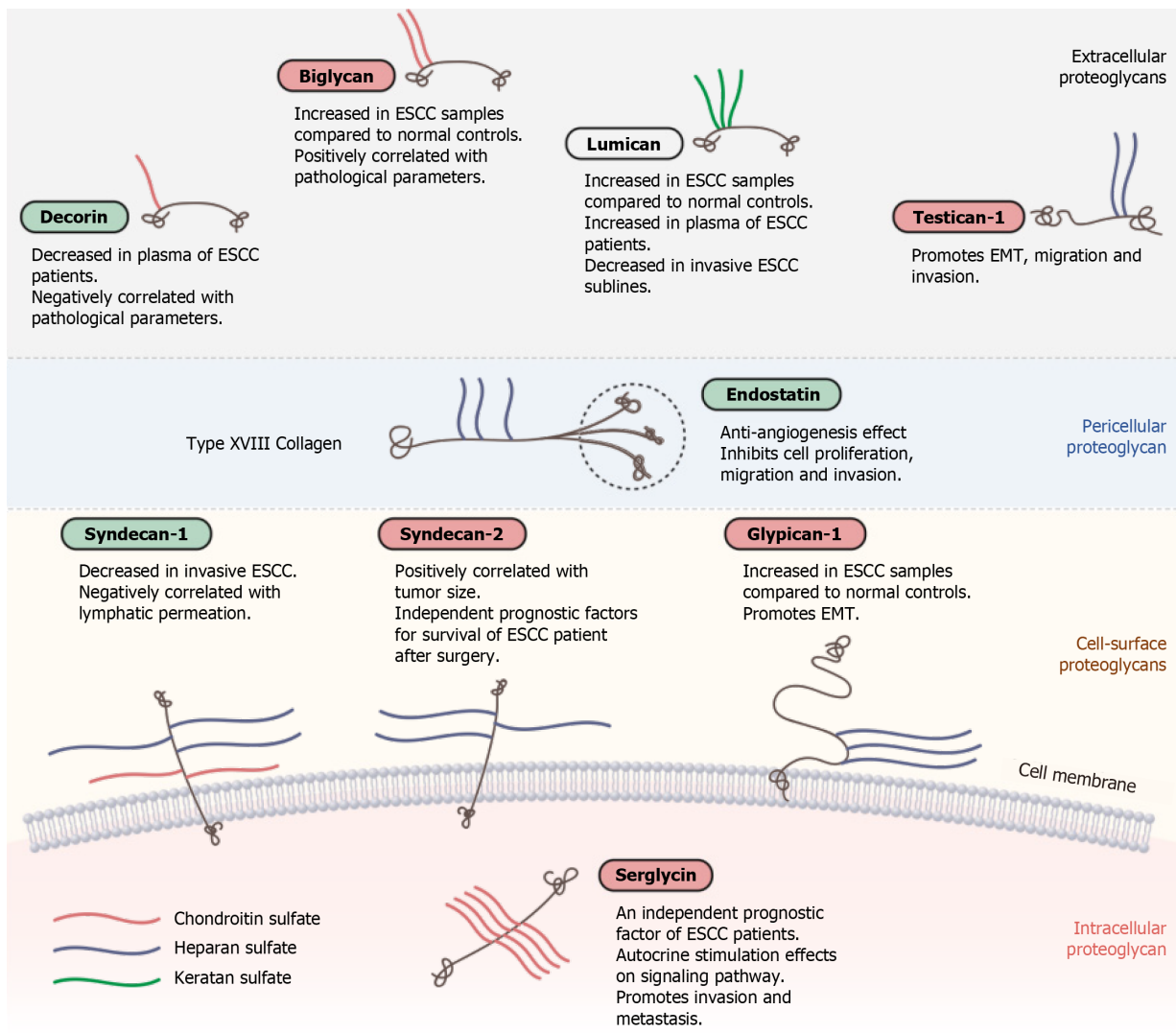
### Testican-1

Testican-1, also known as proteoglycan 1, belongs to the SPOCK family and is encoded by the *SPOCK1* gene. It has been reported to be able to induce EMT in gastric cancer [106]. In colorectal cancer, knockdown of *SPOCK1* could significantly reduce cell proliferation and invasion through the inhibition of phosphatidylinositol-3-kinase/AKT pathway[107]. In ESCC, upregulation of *SPOCK1* also induces EMT and promotes cancer cell migration and invasion[108].

## CONCLUSION

This article reviews the classification and structure of proteoglycans (Table 1 and Figure 1) and the functions of proteoglycans related to ESCC (Figure 3). As one of the components of the ECM, proteoglycans have been studied in several kinds of human cancers due to their roles in matrix organization and regulation of tumor cell-matrix interactions. Proteoglycans have diagnostic and prognostic significance in ESCC and can modulate ESCC cell migration/invasion and EMT. The secreted serglycin can also activate cell signaling in an autocrine manner (Figure 2). In addition, the significance of GAGs attached to the core protein of proteoglycans (*e.g.*, serglycin and syndecan-1) and the expression level of GAGs regulated by multiple enzymes are increasingly gaining attention. Proteoglycans can enhance or inhibit the activity of soluble factors through interacting with them, and such interactions depend largely on the GAG chains. To date, most studies on proteoglycans in ESCC have focused primarily on their diagnostic and/or prognostic significance. In order to utilize or target these dynamic molecules in designing new strategies for treatment of this cancer, more in-





**Figure 3 Classification and schematic representation of proteoglycans studied in esophageal squamous cell carcinoma.** Proteoglycans are classified as extracellular, pericellular, cell-surface, and intracellular according to their cellular and subcellular localization. The ones that have pro-invasive function in esophageal squamous cell carcinoma (ESCC) are highlighted in red bubbles, and the ones that act as the tumor suppressors are highlighted in green bubbles. Lumican is in the transparent bubble because its function in ESCC is still controversial. EMT: Epithelial-mesenchymal transition.

depth research is needed to decipher the complex roles of proteoglycans in ESCC, especially their interactions with other ECM components, receptors, and soluble factors.

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## Therapeutic potential of thymoquinone in combination therapy against cancer and cancer stem cells

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### Abstract

The long-term success of standard anticancer monotherapeutic strategies has been hampered by intolerable side effects, resistance to treatment and cancer relapse. These monotherapeutic strategies shrink the tumor bulk but do not effectively eliminate the population of self-renewing cancer stem cells (CSCs) that are normally present within the tumor. These surviving CSCs develop mechanisms of resistance to treatment and refuel the tumor, thus causing cancer relapse. To ensure durable tumor control, research has moved away from adopting the monotherapy paradigm towards developing and using combination therapy. Combining different therapeutic modalities has demonstrated significant therapeutic outcomes by strengthening the anti-tumor potential of monotherapy against cancer and cancer stem cells, mitigating their toxic adverse effects, and ultimately overcoming resistance. Recently, there has been growing interest in combining natural products from different sources or with clinically used chemotherapeutics to further improve treatment efficacy and tolerability. Thymoquinone (TQ), the main bioactive constituent of *Nigella sativa*, has gained great attention in combination therapy research after demonstrating its low toxicity to normal cells and remarkable anticancer efficacy in extensive preclinical studies in addition to its ability to target chemoresistant CSCs. Here, we provide an overview of the therapeutic responses resulting from combining TQ with conventional therapeutic agents such as alkylating agents, antimetabolites and antimicrotubules as well as with topoisomerase inhibitors and non-coding RNA. We also review data on anticancer effects of TQ when combined with ionizing radiation and several natural products such as vitamin D3, melatonin and other compounds derived from Chinese medicinal plants. The focus of this review is on two outcomes of TQ combination therapy, namely eradicating CSCs and treating various types of cancers. In conclusion, the ability of TQ to potentiate the anticancer activity of many chemotherapeutic agents and sensitize cancer cells to

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radiotherapy makes it a promising molecule that could be used in combination therapy to overcome resistance to standard chemotherapeutic agents and reduce their associated toxicities.

**Key Words:** Thymoquinone; Combination therapy; Cancer cells; Cancer stem cells; Conventional cancer therapy; Natural products

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**Core Tip:** There has been great interest in integrating thymoquinone (TQ) in combination therapy particularly to target cancer stem cells, which are known to be responsible for resistance to treatment and cancer recurrence. The combination of TQ with standard chemotherapeutics and other natural products has exhibited promising anti-cancer responses. TQ was also shown to sensitize cancer cells to radiotherapy and help in overcoming major limitations that restrict the potency of chemotherapy, which are chemoresistance and treatment associated toxic effects.

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## INTRODUCTION

Cancer incidence and mortality are still growing worldwide despite the monumental efforts and the significant progress made in developing therapeutic strategies and improving detection techniques for combatting this disease. Around 19 million new cases and nearly 10 million deaths are estimated globally in 2020[1]. The conventional therapeutic strategies used to treat cancer are surgery, radiotherapy and chemotherapy, in addition to targeted and hormonal therapy. The effectiveness of these approaches has been found to be limited when used in monotherapy strategies due to cancer resistance, tumor relapse and treatment-induced toxicities[2-6]. Ample evidence has demonstrated that the intratumoral heterogeneity is a prominent contributor to cancer resistance to monotherapy and tumor recurrence[7]. The tumor consists of a heterogeneous population of cells that show distinct genetic, epigenetic, and phenotypic features in addition to different sensitivity to the standard therapeutic modalities[8-11]. A growing body of literature has supported the role of cancer stem cells (CSCs) in generating this intratumoral heterogeneity. These CSCs are characterized by their ability to self-renew and to differentiate into various lineages of cancer cells composing the tumor. They are also resistant to the widely used therapeutics measures [12].

Over the last few decades, there has been increased interest in combining cancer treatments rather than using single therapeutic agents. A monotherapeutic strategy having one mode of action eradicates only one subpopulation of tumor cells. Other subpopulations which are less sensitive can escape the treatment and reform a resistant tumor, thus resulting in cancer relapse and treatment failure. In contrast, combined therapeutic agents act simultaneously on multiple targets and eradicate several subpopulations of tumor cells. This results in improving their therapeutic efficacy, limiting their toxicity by lowering the effective therapeutic dose of each agent, preventing the development of resistance and consequently ensuring an effective eradication of the complex heterogeneous nature of the tumor[13]. Bioactive natural products are attracting considerable attention in cancer therapy because they are less toxic and more available and cost effective when compared to synthetic monotargeted drugs[14]. Natural therapeutics have been found to exert effective antineoplastic activity and to potentiate the anticancer effect of conventional therapeutics against CSCs and cancer cells[15,16]. Around 38% of the anticancer drugs approved during the last 40 years are either natural products *per se*, their derivatives or have a pharmacophore derived from a natural product[17].

Thymoquinone (TQ), the major bioactive compound extracted from *Nigella sativa* essential oil, has shown promising antitumor activity *in vitro* and *in vivo* against a wide range of cancer types[18]. What makes TQ an attractive therapeutic agent is its safe profile. It was found to be non-toxic to several normal cells including normal mouse kidney cells[19], normal human lung fibroblasts[20] and normal human intestinal cells [21]. TQ exerts its antineoplastic effects through several modes of action, and its exact molecular target is not known yet. It inhibits cancer cell proliferation and blocks the cell cycle progression. In addition, TQ induces apoptosis by generating reactive oxygen species (ROS), causing DNA damage, upregulating pro-apoptotic factors, activating caspases and causing poly (ADP-ribose) polymerases (PARP) cleavage, disrupting mitochondrial membrane integrity besides modulating several pathways such as p53, wntless/integrated (Wnt), mitogen-activated protein kinase, signal transducer and activator of transcription 3 (STAT3)[22]. It also interrupts metastasis by downregulating the epithelial to mesenchymal transition transcription factors twist-related protein 1 (TWIST1) and E-Cadherin, and inhibits angiogenesis by suppressing the nuclear factor kappa B (NFkB) pathway[22]. Interestingly, TQ was found to inhibit the proliferation of several chemoresistant cancer cells and induce apoptosis in colon CSCs that are resistant to the conventional chemotherapeutic drug 5-fluorouracil (5-FU)[23,24].

These effective anticancer properties of TQ made it an interesting therapeutic candidate for combination therapy with standard therapeutic agents or other natural products to improve cancer treatment efficacy and safety (Figure 1). Here, we shed light on the combinatorial effects of TQ on the activity of these therapeutic agents used in treating CSCs and cancer cells.

## TQ EFFECTS AGAINST CANCER CELLS

### ***TQ in combination with conventional chemotherapeutic agents***

The mode of action of each chemotherapeutic agent as well as the cellular and molecular mechanisms of action of the combination treatment are presented in Table 1.

#### ***Alkylating agents***

**Cyclophosphamide[25]:** Cyclophosphamide has been used in treating a broad spectrum of cancers including leukemia, lymphoma, breast and ovarian cancers[26]. In a study conducted by Khan *et al*[27], TQ was found to amplify the growth inhibitory effects of low doses of cyclophosphamide in breast cancer cells. This combination upregulated the expression of phosphatase and tensin homolog (PTEN) and downregulated the phosphorylation of its downstream signaling molecule Akt in addition to decreasing the expression of cyclin D1. The PTEN/phosphatidylinositol-3-kinase (PI3K)/Akt pathway is known to be an important tumorigenic pathway responsible for cell cycle progression, survival, and migration of malignant cells[28].

**Temozolomide[29]:** Temozolomide (TMZ) has been approved by the Food and Drug Administration for the treatment of glioblastoma multiforme[30]. However, the anti-cancer efficacy of TMZ has been limited by cancer resistance[31]. TQ was found to be a potent enhancer of the anti-proliferative and apoptotic activity of TMZ in glioblastoma cells. The modulation of the apoptotic players including ROS generation, disruption of mitochondrial membrane potential, activation of p53, caspases 9 and 3 was more pronounced in combination treatment compared to separate treatments[32]. Moreover, combining TQ and TMZ caused a stronger inhibitory effect on glioblastoma cells migration, invasion and adhesion than each drug alone. This synergistic inhibitory effect was found to be associated with a decrease in the expression and secretion of matrix metalloproteinases MMP-2 and MMP-9[33] known to promote metastatic spread and to contribute to angiogenesis[34]. Interestingly, TQ was found to block TMZ-induced autophagy, which was suggested to be a prosurvival mechanism of cell resistance to TMZ. TQ suppressed TMZ-induced expression of key players in the autophagy pathway beclin-1 and autophagy-related 7[35].

**Cisplatin[36]:** Cisplatin (CDDP) is one of the most used chemotherapeutic drugs in the treatment of a wide range of cancer types[37]. The primary dose-limiting side effect of CDDP is the dose- dependent nephrotoxicity, which restricts the use of high doses of CDDP to increase its anticancer activity[38]. Numerous studies have demonstrated the anti-neoplastic efficacy of combining TQ with CDDP in different types of cancers as an alternative way to increase CDDP potency. In ovarian cancer, these two agents were

**Table 1 Mode of action of the chemotherapeutics agents and cellular and molecular mechanism of action of the combination treatment in preclinical and clinical studies**

Chemotherapeutic agent	Mode of action	Patients or animal model or cell lines	Cellular and molecular mechanism of action of the combination treatment	Ref.
Cyclophosphamide	Alkylates guanine base and causes the formation of DNA crosslinks leading to cell death	SKBR-3 and MDA-231 breast cancer cells	Increases the percentage of cells in G1 and sub- G1 phases. Downregulates the phosphorylation of Akt and the expression of cyclin D1 and upregulates PTEN	Emadi <i>et al</i> [25], Khan <i>et al</i> [27]
Temozolomide	Methylates DNA at specific sites on guanine and adenine bases causing cell demise	U87MG human glioblastoma multiforme cells	Increases the mitochondrial membrane potential disruption, cytochrome c release, ROS generation, DNA fragmentation and Bax/Bcl-2 ratio. Activates p53, caspases 9 and 3 and reduces NO and GSH levels. Reduces the expression and secretion of MMP-2 and MMP-9. Downregulates beclin-1 and ATG-7	Stupp <i>et al</i> [29], Khazaei <i>et al</i> [32], Pazhouhi <i>et al</i> [33], Pazhouhi <i>et al</i> [35]
Cisplatin	Interacts with purine bases and forms DNA crosslinks resulting in cell death	ID8-NGL mouse ovarian cancer cells. OVCAR3 and NCI/ADR-RES human ovarian cancer cells. BL/6 mice injected with ID8-NGL cells	Increases the level of Bax, pH2AX (ser139), cleaved caspase 3 and PARP. Decreases the level of PCNA and Ki67	Siddik <i>et al</i> [36], Wilson <i>et al</i> [39]
		Eca-109 human esophageal cancer cells. BALB/c nude mice inoculated with Eca-109 cells	Decreases the expression of p-STAT3, p-JAK2, Bcl-2, survivin and cyclin D1. Increases the expression of Bax and activates caspases 3, 7 and 9. Induces chromatin condensation and nuclear fragmentation	Hu <i>et al</i> [40]
		NCI-H460 non-small lung cancer cells. SCID mice injected with NCI-H460 cancer cells	Reduces the ratio of phosphor-Ser529 NFkB/NFkB	Jafri <i>et al</i> [42]
		UMSCC-14C head and neck squamous cancer cells and normal oral epithelial cells	Increases p53 and caspase 9 expression. Decreases Bcl-2 expression	Alaafi <i>et al</i> [43]
		SGC-7901 human gastric cancer cells. BALB/c mice implanted with gastric cancer cells	Increases the level of Bax, AIF, cytochrome c, cleaved caspases 9 and 3. Decreases the level of cyclin D1, Bcl-2, procaspases 9 and 3. Inhibits PI3K/Akt signaling pathway and downregulates P-gp by upregulating PTEN	Ma <i>et al</i> [44]
5-Fluorouracil	A pyrimidine analogue inhibiting the activity of thymidylate synthase enzyme causing the disruption of DNA synthesis and cell death	BGC-823, SGC-7901, MGC-803 and HGC-27 human gastric cancer cells. BALB/c athymic nude mice inoculated with gastric cancer cells	Increases the release of mitochondrial cytochrome c and the level of Bax, caspases 3 and 9. Decreases the level of Bcl-2 and induces nuclear fragmentation and chromatin condensation	Wilson <i>et al</i> [45], Lei <i>et al</i> [48]
		Azoxymethane-induced colorectal tumors in Wistar rats	Increases the expression of DKK-1, CDNK-1A, TGF-β1, TGF-βRII, Smad4 and GPx. Decreases the expression of Wnt, β-catenin, NFkB, COX-2, iNOS, VEGF and TBRAS	Kensara <i>et al</i> [49]
		HCT116, HT29 and SW620 human colon cancer cells SW837 rectal cancer cells. Normal human intestinal epithelial cells. CAM tumors derived from HCT116 cells	Downregulates Wnt/β-catenin and PI3K/Akt pathways	Ndreshkjana <i>et al</i> [50]
		FADU nasopharyngeal cancer cells	Decreases the level of GSH	Williams <i>et al</i> [51]
		MG63 human osteosarcoma cells		Sarman <i>et al</i> [52]
Gemcitabine	A deoxycytidine analog preventing chain elongation during DNA	PANC-1 and MIA PaCa-2 human pancreatic cancer cells	Downregulates PKM2 and decreases the expression of procaspase 3 and PARP	Moysan <i>et al</i> [53], Pandita <i>et al</i> [56]

causing cell death		PANC-1, BxPC-3, and AsPC-1 human pancreatic cancer cell lines. BALB/c nude mice injected with PANC-1 cells	Downregulates Notch1, NICD, Bcl-2, Bcl-xL and XIAP. Inactivates Akt/mTOR/S6 signaling pathway and decreases the phosphorylation and nuclear translocation of p65. Upregulates PTEN, caspases 3 and 9 and Bax and increases cytochrome c release	Mu <i>et al</i> [57]
Paclitaxel	Inhibits microtubules disassembly and induces mitotic arrest	MCF-7 and T47D human breast cancer cells	Increases pre-G1 cell population	Bashmail <i>et al</i> [58]
		4T1 mouse breast cancer cells. Ehrlich tumor cells. Balb/c mice injected with Ehrlich tumor ascites cells	Increases the level of full length and cleaved caspases 3, 7 and 12 and PARP. Reduces phosphorylated p65 and Akt1. Modulates genes involved in apoptosis, cytokine-cytokine receptor interaction, Fas signaling, p53 signaling and JAK/STAT signaling	Ojima <i>et al</i> [59], Şakalar <i>et al</i> [63]
		MCF-7 and T47D human breast cancer cells	Increases pre-G1 cell population. Increases the level of cleaved caspase 3 and PARP and the expression of beclin-1 and LC3-II	Bashmail <i>et al</i> [64]
		MCF-7 human breast cancer cells		Soni <i>et al</i> [65]
Docetaxel	Inhibits microtubules disassembly and induces mitotic arrest	DU-145 human prostate cancer cells	Blocks PI3K/Akt signaling pathway and induces DNA fragmentation	Ojima <i>et al</i> [59], Dirican <i>et al</i> [69]
		DU-145 and C4-2B human prostate cancer cells	Inhibits PI3K/Akt signaling pathway. Increases the expression of Bax, Bid, caspase 3 and PARP and decreases the expression of Bcl-xL	Singh <i>et al</i> [70]
		MCF-7 and MDA-MB-231 human breast cancer cells	Induces DNA damage, cells shrinkage, nuclear fragments, apoptotic bodies and cytoplasmic vacuolation	Alkhatib <i>et al</i> [71]
		MCF-7 and MDA-MB-231 human breast cancer cells		Zafar <i>et al</i> [72]
Cabazitaxel	Inhibits microtubules disassembly and induces mitotic arrest	MCF-7 and MDA-MB-231 human breast cancer cells. Balb/c mice healthy or injected with Ehrlich ascites carcinoma cells	Induces nuclear fragmentation and restores the levels of oxidative stress parameters MDA, SOD and GSH. Prevents the alteration of blood cell count and serum biochemical parameters AST, ALT, creatinine and BUN	Zafar <i>et al</i> [73]
		MCF-7 breast cancer cells		Odeh <i>et al</i> [74]
		MCF-7 and MDA-MB-231 human breast cancer cells	Induces DNA fragmentation and increases the sub-G1 population	Ojima <i>et al</i> [59], Kommineni <i>et al</i> [78]
Doxorubicin	Intercalates DNA, inhibits topoisomerase II, forms free radicals when reduced leading to cell cycle arrest and cell death	Human HTLV-1 positive (HuT-102) and HTLV-1 negative (Jurkat). CD4+ malignant T-cell lines. NOD/SCID mice inoculated with HuT-102 tumor cells	Increases the sub-G1 population and induces ROS production. Disrupts the mitochondrial membrane potential. Downregulates the expression of NFkB and Ki67 and increases the phosphorylation of p53	Meredith <i>et al</i> [79], Fatfat <i>et al</i> [83]
		HL-60 acute myeloid leukemia cells. Dox resistant HT-29 colon carcinoma cells. MCF-7/TOPO multi-drug resistant breast cancer cells	Induces caspases 3 and 8 activity and ROS generation. Disrupts the mitochondrial membrane potential	Effenberger-Neidnicht <i>et al</i> [84]
		BALB/c OlaHsd-foxn1 nude mice injected with MDA-MB-231 breast cancer cells	Induces p38 MAPK phosphorylation and inhibit the expression of XIAP, survivin, Bcl-xL and Bcl-2	Woo <i>et al</i> [85]
		SMMC-7721 and HepG2 hepatocarcinoma cells and human normal liver cells HL-7702	Increases caspase 3 and PARP cleavage	Jehan <i>et al</i> [86]



		MDA-MB-231 human breast cancer cells. MCF-10A and 3T3 non-neoplastic cells	Induces cell shrinkage, membrane blebbing and apoptotic bodies and disrupts the cell membrane. Increases the Sub-G0 population	Ibiyeye <i>et al</i> [87]
		MCF-7 human breast adenocarcinoma and HEPG2 human hepatocellular carcinoma. Albino mice implanted with Heps murine liver cancer cells	Decreases NFkB level and increases that of caspase 3. Increases the level of renal antioxidant enzymes SOD and catalase. Modulates the level of renal oxidative stress biomarkers GSH and MDA. Decreases the level of nephrotoxicity biomarkers BUN and serum creatinine	Zidan <i>et al</i> [88]
		Albino transplanted with Ehrlich carcinoma cells	Upregulates p53 and reduces the level of Bcl-2. Decreases the level of cardiac MDA. Decreases the serum level of cardiac markers lactate and creatine	El-Ashmawy <i>et al</i> [89]
Topotecan	Inhibits DNA topoisomerase I and causes the formation of irreversible DNA double stranded breaks resulting in cell death. Inhibits hypoxia-inducible factor 1 $\alpha$	U937 acute myelogenous leukemia cells	Increases the sub-G1 population. Increases the expression level of Bax/Bcl-2, p53 and p21 and the cleavage of caspases 3 and 9	Robati <i>et al</i> [90], Khalife <i>et al</i> [95]
Bortezomib	Inhibits the proteasome	HT-29 human colon cancer cells	Increases the sub-G1 population. Has no effect on p53, Bax and Bcl-2 expression	Khalife <i>et al</i> [96]
		U266, H929, KMS, RPMI-8226, RPMI-8226-Dox-6 (doxorubicin-resistant clone), RPMI-8226-LR-5 (a melphalan-resistant clone) human multiple myeloma cells. Balb/c mice implanted with U266 cells	Increases the sub-G1 population and the cleavage of caspase 3 and PARP. Reduces the phosphorylation of NFkB (p65) and the expression of Ki67, VEGF, Bcl-2 and the serum levels of IL-6 and TNF- $\alpha$	Siveen <i>et al</i> [99]
Imatinib	Inhibits tyrosine kinase	HCT116 human colorectal cancer cells	Decreases the expression of ABCB1, ABCG2 and hOCT1. Increases the uptake/efflux ratio of imatinib	Thabet <i>et al</i> [103]
Tamoxifen	Competes with estrogen and estradiol for the binding to their receptors and modulates their signaling pathway	MCF-7 and MDA-MB-231 human breast cancer cells		Day <i>et al</i> [104], Ganji-Harsini <i>et al</i> [106]
		MCF-7, MDA-MB-231, MDA-MB-468, T47D, NIH/3T3 and HaCaT human breast cancer cells. Athymic BALB/c mice injected with MDA-MB-231 cells	Decreases the expression of XIAP and the level of p-Akt, p-Bad, p-MAPK and p-GSK-3 $\beta$ and downregulates the expression of Bcl-xL, Bcl-2 and Ki67. Increases the cleavage of caspase 9 and PARP and induces the expression of Bax, AIF, cytochrome c and p27. Increases the percentage of cells in sub-G1 phase and the fragmentation of DNA	Rajput <i>et al</i> [107]
		Breast cancer patients	Increases the tumor tissue catalase, SOD and caspase 3. Decreases the tumor tissue Bcl-2, TGF- $\beta$ 1, MDA, TNF- $\alpha$ and IL-6	Kabel <i>et al</i> [108]
Zoledronic acid	Inhibits osteoclast-mediated bone	PC-3 and DU-145 human prostate cancer cells	Increases DNA fragmentation and activates caspases 3 and 7	Polascik <i>et al</i> [109], Dirican <i>et al</i> [112]
Arsenic trioxide		Human HTLV-I positive (HuT-102 and C91) and HTLV-I negative (CEM and Jurkat) malignant T-cell lines. NOD SCID mice inoculated with HuT-102 cells	Increases the percentage of cells in Pre-G1 phase, the disruption of the mitochondrial membrane potential and the cleavage of PARP and caspase 3. Upregulates p53, Bax and downregulates XIAP and Bcl-2	Houssein <i>et al</i> [117]

PTEN: Phosphatase and tensin homolog; ROS: Reactive oxygen species; Bax: Bcl-2-associated X protein; NO: Nitric oxide; GSH: Glutathione; MMP: Matrix metalloproteinase; ATG-7: Autophagy-related 7; pH2AX: Phospho-histone 2AX; PCNA: Proliferating cell nuclear antigen; JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; NFkB: Nuclear factor kappa B; PI3K: Phosphatidylinositol-3-kinase; AIF: Apoptosis inducing factor; PARP: Poly (ADP-ribose) polymerases; CAM: Chorioallantoic membrane; MAPK: Mitogen-activated protein kinase; COX-2: Cyclooxygenase 2; iNOS:

Inducible nitric oxide synthase; VEGF: Vascular endothelial growth factor; TBRAS: Thiobarbituric acid reactive substances; DKK-1: Dickkopf-related protein-1; CDNK-1A: Cyclin-dependent kinase inhibitor 1A; TGF- $\beta$ 1: Transforming growth factor beta 1; TGF- $\beta$ RII: Transforming growth factor, beta receptor II; GPx: Glutathione peroxidase; GSH: Glutathione; XIAP: X-linked inhibitor of apoptosis protein; mTOR: Mammalian target of rapamycin; PKM2: Pyruvate kinase M2; Bid: BH3 interacting-domain death agonist; AST: Aspartate transaminase; ALT: Alanine transaminase; MDA: Malondialdehyde; SOD: Superoxide dismutase; BUN: Blood urea nitrogen; IL-6: Interleukin 6; TNF- $\alpha$ : Tumor necrosis factor alpha; GSK-3 $\beta$ : Glycogen synthase kinase 3 beta; ABCB1A: ATP-binding cassette subfamily B member 1; ABCG2: ATP-binding cassette subfamily G member 2; hOCT1: Human organic cation transporter 1.

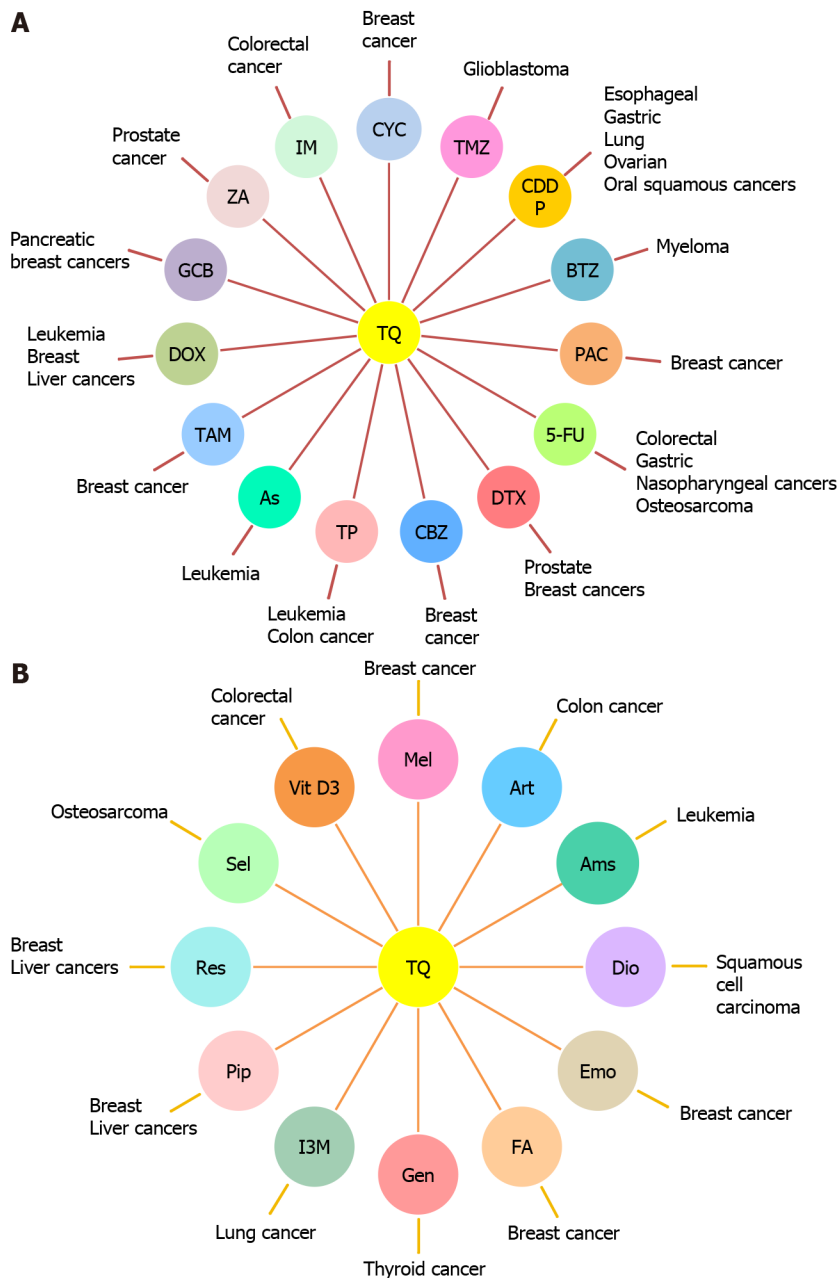
found to synergize to induce apoptosis *in vitro* and in a mouse syngeneic model. The combination was more effective in increasing the levels of Bcl-2-associated X protein (Bax), phospho-histone 2AX on serine 139, cleaved caspase 3 and PARP and in down-regulating proliferating cell nuclear antigen compared to CDDP alone[39]. A study conducted by Hu *et al*[40] found that TQ enhanced the apoptotic effect of CDDP in esophageal carcinoma *in vitro* and *in vivo* through downregulating JAK2/STAT3 pathway known to be involved in cancer cell proliferation, survival, angiogenesis and metastasis[41]. Another study showed the synergistic inhibitory effects of the combination of TQ and CDDP on the proliferation of non-small lung cancer cells and on the growth of lung cancer xenografts through the suppression of NFkB[42]. The improvement of CDDP-induced apoptosis by TQ was also demonstrated in oral squamous carcinoma cells. The combination was more potent in upregulating p53 and caspase 9 and downregulating Bcl-2 than CDDP alone[43]. In addition, combining TQ with CDDP resulted in a superior anti-neoplastic activity in gastric cancer *in vitro* and *in vivo* by further upregulating PTEN expression compared to CDDP alone[44].

### Antimetabolites

**5-FU[45]:** 5-FU is the third most frequently used chemotherapeutic drug in the treatment of a variety of solid cancers, but its clinical efficacy is hampered by drug resistance and treatment-associated toxicities[46,47]. It is the second most frequent chemotherapeutic agent that causes cardiotoxicity symptoms[46]. The potential chemomodulatory effects of TQ on 5-FU anticancer activity have been investigated in various cancer types. TQ was reported to chemosensitize gastric cancer cells to 5-FU-induced apoptosis by upregulating Bax, caspases 3 and 9 and downregulating Bcl-2 [48]. Moreover, the combination of TQ with 5-FU synergistically suppressed azoxymethane-induced colorectal tumors initiation and development in rats without causing nephro- and hepato-toxicities. The dual combination enhanced the decrease in the expression level of pro-oncogenic genes [Wnt,  $\beta$ -catenin, NFkB, cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), and thiobarbituric acid reactive substances] and the increase in the expression level of anti-oncogenic genes [dickkopf-related protein-1 (DKK-1), cyclin-dependent kinase inhibitor 1A (CDNK-1A), transforming growth factor beta 1 (TGF- $\beta$ 1), transforming growth factor, beta receptor II (TGF- $\beta$ RII), Smad4, and glutathione peroxidase] compared to separate treatments[49]. In another study, Ndreshkjana *et al*[50] linked 5-FU with TQ by esterification to form a new hybrid molecule SARB and tested it on colon cancer cells. Both combination and hybrid treatments enhanced the cytotoxic effects of single agents *in vitro*, while SARB was more effective in suppressing the growth of chorioallantoic membrane xenografts *in vivo*. The cytotoxic effects of 5-FU, TQ and the natural product epigallocatechin-3-gallate in triple and double combinations were evaluated in nasopharyngeal cancer cells. The results revealed that the triple combination had the most potent effect in reducing the total number of cancer cells, and the dual combination of TQ and 5-FU was more effective than the combination of TQ and epigallocatechin-3-gallate[51]. In addition, TQ augmented the apoptotic effects of each of 5-FU and the alkylating agent oxaliplatin in osteosarcoma cells. Interestingly, combining TQ with low doses of each of these drugs was found to produce the same anticancer efficacy as higher doses of these agents[52]. Therefore, this treatment strategy may help in alleviating 5-FU and oxaliplatin undesired adverse effects.

**Gemcitabine[53]:** Gemcitabine (GCB) has been approved for treating different types of cancer including pancreatic and breast cancers[54]. The therapeutic application of GCB was compromised by several drawbacks including its short half-life in the blood circulation, poor membrane permeability in addition to the development of chemoresistance[55]. TQ and GCB were found to induce synergistic apoptosis in GCB sensitive and resistant pancreatic cancer cells by downregulating pyruvate kinase M2 expression[56]. In another study, pretreatment of pancreatic cancer cells with TQ followed





**Figure 1 Thymoquinone in combination therapy against different types of cancer.** A: Thymoquinone in combination with conventional chemotherapeutic drugs; B: Thymoquinone in combination with natural products. TQ: Thymoquinone; CYC: Cyclophosphamide; TMZ: Temozolomide; CDDP: Cisplatin; BTZ: Bortezomib; 5-FU: 5-Fluorouracil; GCB: Gemcitabine; PAC: Paclitaxel; DTX: Docetaxel; CBZ: Cabazitaxel; TP: Topotecan; DOX: Doxorubicin; ZA: Zoledronic acid; TAM: Tamoxifen; As: Arsenic trioxide; IM: Imatinib; Vit D3: Vitamin D3; Mel: Melatonin; Res: Resveratrol; Pip: Piperine; Ams: Artemisinin; Art: Artesunic acid; Dio: Diosgenin; Gen: Genistein; I3M: Indirubin3monoxime; FA: Ferulic acid; Emo: Emodin; Sel: Selenium.

by low doses of GCB resulted in a synergistic apoptotic and growth inhibitory responses *in vitro* and *in vivo* by downregulating Notch1/PTEN, PI3K/Akt/mammalian target of rapamycin and NFκB mediated signaling pathways[57]. In the context of breast cancer, TQ boosted the apoptotic activity of GCB against T47D cells. While in the apoptosis defective MCF-7 cells, the combination of TQ with GCB induced significant cell death by autophagy[58].

### Antimicrotubules

**Paclitaxel**[59]: Paclitaxel (PAC) is widely used for the treatment of several cancer types including breast, ovary, colorectal and lung cancers[60]. The major challenges that restrict its curative effect are chemoresistance and adverse effects that are mainly caused by the polyethylated castor oil that is usually added to its formulation to increase its solubility[61,62]. Three studies have evaluated the potential of the combinatorial effect of TQ and PAC in breast cancer. TQ-PAC combination produced a

synergistic anticancer activity through the modulation of genes involved in apoptosis, cytokine-cytokine receptor interaction, Fas signaling, p53 signaling and JAK/STAT signaling[63]. In another study, combining TQ with PAC augmented the necrotic and caspase dependent- apoptotic responses in T47D breast cancer cells compared to PAC alone. While in the apoptosis defective MCF-7 cells, both individual and combined treatments induced significant cell death by autophagy[64]. The co-encapsulation of TQ and PAC in polymeric biodegradable poly (lactide-co-glycolide) nanoparticles lowered PAC effective anticancer dose and reduced cancer cell viability more effectively than PAC loaded nanoparticles or its free counterpart[65]. Therefore, this therapeutic approach may help in hijacking the toxicities associated with the clinical use of PAC.

**Docetaxel:** Docetaxel (DTX) has been approved for the treatment of different type of tumors including prostate cancer and breast cancer[66]. However, low water solubility, treatment related toxicities and drug resistance limit its application in clinical practice[61,67,68]. TQ was found to potentiate the apoptotic activity of DTX in prostate cancer cells by inducing a more prominent suppression of the signaling pathway PI3K/Akt compared to DTX alone[69]. Co-treatment of prostate cancer cells with these two agents resulted in a greater upregulation of Bax, BH3 interacting-domain death agonist (Bid), caspase 3 and PARP and a higher downregulation of Bcl-xL compared to individual treatments[70]. To enhance drug solubility, increase their efficacy and reduce DTX toxicities, multiple nanoparticle drug delivery systems for the co-delivery of TQ and DTX have been developed and evaluated on breast cancer cells. Loading TQ and DTX into a borage nanoemulsion delivery system allowed the lowering of the required effective dose of DTX and enhanced cell death in cancer cells through simultaneous stimulation of apoptosis and autophagy[71]. In another study, co-encapsulating TQ and DTX in low-molecular-weight chitosan coated lipid nanocapsules was found to exhibit stronger cytotoxic and anti-angiogenic responses in cancer cells compared to the free single treatments[72]. The co-delivery of TQ and DTX in Pegylated lipid nanocapsules produced more effective apoptotic and anti-migratory effects in cancer cells in addition to a higher tumor growth inhibition in mice bearing Ehrlich ascites carcinoma compared to free single treatments. Interestingly, these dual drugs loaded lipid nanocapsules prevented the development of DTX-induced hematological, hepato- and nephro- toxicities, an indicator of their protective potential[73]. Moreover, TQ and DTX were co-encapsulated into pegylated liposomes and tested against MCF-7 breast cancer cells. The half maximal inhibitor concentration of each of TQ and DTX co-loaded into liposomes were lower than those of the free individual drugs[74].

**Cabazitaxel:** Cabazitaxel (CBZ) was approved as the second line therapy for metastatic castration-resistant prostate cancer[75]. However, its low aqueous solubility, poor membrane permeability, and severe side effects like neutropenia and anemia are the challenging drawbacks for successful cancer management[76,77]. Combining TQ with CBZ caused synergistic apoptotic effects in breast cancer cells. To address the drug delivery challenge, TQ and CBZ were co-loaded in lipospheres. The combined drugs loaded lipospheres had enhanced apoptotic effects compared to the drug combination in solution[78].

### Cytotoxic antibiotics

**Doxorubicin[79]:** Doxorubicin (DOX) is a primarily adopted chemotherapeutic agent for treating a wide spectrum of solid and liquid tumors[80]. Despite the robust anti-cancer activity of DOX, chemoresistance and severe side effects especially cardiotoxicity weakened its potency[81]. Nearly 11% of the patients treated with this agent develop acute cardiotoxicity[82]. Several studies demonstrated the powerful combinatorial effect of TQ on the anticancer efficacy of DOX. Combining TQ with DOX allowed the lowering of DOX dose by up to 2-fold while maintaining its anticancer potential against adult T cell leukemia (ATL). TQ and DOX synergized to induce caspases and ROS mediated apoptosis in human T-lymphotropic virus-1 positive and human T-lymphotropic virus-1 negative CD4+ malignant T cell lines *in vitro* in addition to suppressing the growth of an ATL xenograft in mice[83]. In addition, co-treatment of HL-60 acute myeloid leukemia cells with TQ and DOX induced two consecutive waves of caspase 3 activity in addition to more than 7-fold increase in ROS generation compared to DOX alone[84]. In breast cancer, TQ potentiated the anti-tumor activity of DOX *in vivo* by inducing apoptosis and inhibiting tumor cell proliferation to a larger extent than separate treatments[85]. Recently, TQ was shown to improve the apoptotic effect of subtoxic doses of DOX in hepatocarcinoma cells by

further increasing the cleavage of caspase 3 and PARP in addition to reducing DOX-induced cytotoxicity to normal liver cells[86]. This synergistic inhibitory effect of TQ and DOX combination was also observed in chemoresistant cancer cells. TQ augmented DOX cell growth inhibitory effect by 2- and 1.2-fold in multi-drug resistant breast cancer cells and in DOX resistant colorectal cancer cells, respectively[84]. To enhance the synergistic effect of these two agents, two nanodrug delivery systems have been developed. Loading TQ and DOX in cockle shell-derived aragonite calcium carbonate nanoparticles (ACNP) showed higher efficacy in inducing apoptosis and reducing migration and invasion in breast cancer cells than the free drugs or the single drug loaded ACNP while being non-toxic to non-neoplastic cells[87]. In addition, incorporating TQ and DOX into F2 gel (poly-N-acetyl glucosamine) nanofibers exhibited superior cellular growth inhibition and apoptosis in breast and liver cancer cells compared to free drugs and single drug loaded nanoparticles. The anticancer potency of this nanodrug co-delivery system was further demonstrated in two *in vivo* cancer models. The dual loading TQ and DOX nanoparticles enhanced tumor suppression *via* apoptosis in mice bearing liver carcinoma by decreasing NFkB level and increasing caspase 3 as well as in mice bearing solid Ehrlich carcinoma by attenuating Bcl-2 level and up-regulating p53. Interestingly, this treatment also reduced the nephro- and cardio-toxicities induced by DOX through the attenuation of the oxidative stress[88,89].

### Topoisomerase inhibitor

**Topotecan[90]:** Topotecan (TP) was approved for the second-line treatment of small cell lung cancer and was recommended to treat platinum resistant ovarian cancer[91, 92]. The instability of the chemical structure of TP in aqueous solutions and in the plasma reduces its anticancer efficacy and causes side effects[93,94]. TQ was found to boost the anti-proliferative and apoptotic effects of non-cytotoxic doses of TP in acute myelogenous leukemia and in colon cancer cells. This effect was exerted by upregulation of p53 and Bax, downregulation of Bcl-2, increase in the cleavage of caspases 9 and 3 in leukemia cells and through p53- and Bax/Bcl-2-independent mechanisms in colon cancer cells. In addition, pretreatment of leukemia cells with TQ followed by TP was found to be more effective than the simultaneous application of both therapeutic agents[95,96].

### Proteasome inhibitor

**Bortezomib:** Bortezomib (BTZ) was approved for the treatment of multiple myeloma [97]. It acts by inhibiting NFkB pathway known to be constitutively activated in multiple myeloma due to genetic aberrations in its components[98]. TQ was found to augment the apoptotic activity of BTZ in multiple myeloma cells *in vitro* by enhancing caspase 3 activation and PARP cleavage. In a xenograft multiple myeloma mouse model, TQ potentiated the anti-neoplastic effects of BTZ by further suppressing NFkB and consequently downregulating the proliferative (Ki67), anti-apoptotic (Bcl-2), angiogenic (VEGF) and inflammatory (interleukin-6 and tumor necrosis factor- $\alpha$ ) effectors. The authors further showed that TQ reduced the proliferation of BTZ resistant multiple myeloma cells[99].

### Tyrosine kinase inhibitor

**Imatinib:** Imatinib (IM) is a potent tyrosine kinase inhibitor that was approved for treating chronic myeloid leukemia and gastrointestinal stromal tumors[100]. Resistance to IM was reported to develop in cancer patients through several mechanisms including the modulation of the expression of drug efflux and influx transporters[101, 102]. In a study conducted by Thabet *et al*[103], TQ was found to improve the anti-proliferative and apoptotic effects of IM in colorectal cancer cells *in vitro*. Interestingly, this was accompanied by a significant decrease in the expression of the drug transporters ATP-binding cassette (ABC) subfamily B member (ABCB) 1, ABCG2 and human organic cation transporter 1 leading to a significant increase in IM uptake /efflux ratio compared to IM alone.

### Hormone receptor modulator

**Tamoxifen[104]:** Tamoxifen (TAM) is one of the first-line therapies for hormone receptor-positive breast cancer patients[105]. A synergistic apoptotic effect was observed by combining TQ and TAM in breast cancer cells *in vitro* regardless of hormone receptor status[106]. Apoptosis was induced through synergistic inhibition of X-linked inhibitor of apoptosis protein (XIAP) resulting in caspase 9 activation and PARP cleavage along with PI3K/Akt pathway inhibition, which caused the downreg-

ulation of Bcl-xL, Bcl-2, and upregulation of Bax, apoptosis inducing factor, cytochrome c and p27. TQ was also found to enhance TAM anti-angiogenic, anti-migratory and anti-invasive effects in breast cancer[107]. In addition, treating breast cancer patients with a combination of TQ and TAM resulted in greater increase in 5-year survival rate and decrease in relapse rate of patients compared to single treatments. At the molecular level, the dual treatment induced a higher increase in tumor tissue antioxidant enzymes (catalase and superoxide dismutase) and increased caspase 3 expression compared to individual treatments. Moreover, the combination of TQ and TAM enhanced the decrease in tumor tissue Bcl-2, TGF- $\beta$ 1, lipid peroxidation product malondialdehyde and pro-inflammatory cytokines tumor necrosis factor- $\alpha$  and interleukin-6 compared to each treatment alone[108].

### **Biphosphonate**

**Zoledronic acid:** Zoledronic acid is a nitrogen-containing bisphosphonate that inhibits osteoclast-mediated bone resorption. It was approved to prevent and reduce the progression of skeletal complications associated with bone metastasis from solid tumors including prostate cancer[109]. Besides its anti-resorption activity, preclinical and clinical data demonstrated its anti-tumor effects in different types of cancer[110, 111]. TQ intensified the apoptotic activity of zoledronic acid in PC-3 (hormone resistant and chemotherapy sensitive) and DU-145 (hormone and chemotherapy resistant) prostate cancer cell lines through a synergistic increase in DNA fragmentation in both cell lines and a synergistic activation of caspases 3 and 7 in PC-3 cells [112].

### **Arsenic trioxide**

Arsenic trioxide was approved for the treatment of acute promyelocytic leukemia [113]. The combination of arsenic trioxide (As) with interferon alpha (IFN- $\alpha$ ) was found to have an effective anti-neoplastic activity in ATL. As and IFN- $\alpha$  synergistically induced apoptosis in ATL leukemia cells *in vitro* and cured murine ATL[114,115]. A phase II trial involving patients with relapsed/refractory adult T-cell leukemia/lymphoma showed that the combination of As and IFN- $\alpha$  exhibited anticancer effects but caused significant toxicity[116]. Combining TQ with As and IFN- $\alpha$  induced synergistic apoptotic activity *in vitro* and *in vivo* and allowed the reduction of the toxic doses of As. TQ alone or TQ/As/IFN- $\alpha$  combination downregulated XIAP and Bcl-2, upregulated Bax and induced cleavage of PARP and caspase 3[117], ultimately leading to enhanced apoptosis.

### **TQ in combination with ionizing radiation**

Radiotherapy is a mainstay therapeutic modality for the treatment of early and advanced solid cancers. Nearly 50% of cancer patients receive radiotherapy during their treatment course[118]. However, its therapeutic potency was found to be compromised by the damage of the surrounding healthy tissue in addition to the development of radioresistance[119]. To overcome these challenges and enhance radiotherapy efficacy, exploring radiosensitizers, molecules that make cancer cells more susceptible to radiations, has attracted great attention[120]. Several studies demonstrated the radiosensitizing role of TQ on cancer cells *in vitro*. TQ augmented the anti-proliferative and apoptotic effects of ionizing radiation and further enriched the sub-G1 population in breast cancer cells[121]. In addition, sensitization with TQ prevented the radiation-induced metastatic progression of breast cancer cells through the restoration of the levels of TGF- $\beta$  and its downstream effectors in addition to epithelial and mesenchymal markers[122]. In melanoma, TQ enhanced the apoptotic responses of low doses of gamma knife irradiation by further inhibiting the phosphorylation of STAT3, which is known to play a key role in cancer cell proliferation, survival, angiogenesis and metastasis[41]. It also improved the gamma knife irradiation-induced immune response by further attenuating the secretion of tumor-related inflammatory cytokines[123]. The cellular and molecular mechanisms of action of TQ in combination with radiation and other therapeutic agents discussed in this review are presented in Table 2.

### **TQ in combination with non-coding RNA**

Gene therapy is a modern therapeutic approach that demonstrated immense and impressive potential against cancer. It consists of delivering therapeutic genetic materials such as small interfering RNA (siRNA), microRNA, and anti-sense oligonucleotides into cancer cells to restore target gene expression, which is modulated and associated with tumorigenesis[124]. miR-34a is a tumor-suppressive microRNA found

**Table 2 Cellular and molecular mechanism of action of the combination treatment in preclinical studies**

Therapeutic agent	Animal model or cell line	Cellular and molecular mechanism of action of the combination treatment	Ref.
Radiation	MCF-7 and T47D human breast cancer cells	Increases the percentage of cells in sub-G1 phase	Velho-Pereira <i>et al</i> [121]
	MCF-7 and MDA-MB-231 human breast cancer cells	Restores the expression levels of TGF- $\beta$ and its downstream molecules NFkB, Smad2, Snail and Twist, adhesion molecules E-cadherin and cytokeratin 19, mesenchymal markers integrin $\alpha$ V, MMP-9, and MMP-2	Rajput <i>et al</i> [122]
	B16-F10 melanoma cells	Inhibits the phosphorylation of JAK2 and STAT3Increases the expression of caspase 3 and Bax. Reduce the expression of Bcl-2 and survivin and the level of VEGF-A, MCP-1, TGF- $\beta$ 1, RANTES and IL-1 $\beta$ . Induces DNA damage	Hatiboglu <i>et al</i> [123]
microRNA-34a	BT-549 metastatic breast cancer cells	Targets and downregulates TWIST1 and ZEB1	Imani <i>et al</i> [126]
Akt-siRNA	Akt-overexpressing MCF-7 and T47D. Tamoxifen resistant MCF-7 and T47D breast cancer cells. BALB/c mice injected with MCF-7/TAM cells	Reduces Akt expression and MDM-2 activation. Activates p53, increases the level of Bax and Bim and decreases the level of Bcl-2 and Ki67	Rajput <i>et al</i> [127]
Vitamin D3	Azoxymethane-induced colorectal tumors in Wistar rats	Reduces the level of Wnt, $\beta$ -catenin, NFkB, COX-2, iNOS, VEGF and HSP-90 and increases that of DKK-1, CDNK-1A, TGF- $\beta$ 1, TGF- $\beta$ /RII and Smad4	Mohamed <i>et al</i> [131]
Melatonin	EMT6/P mouse breast cancer cells. Balb/C mice transplanted with EMT6/P cells	Reduces the expression of VEGF and the serum level of AST and ALT. Increases the serum level of IFN- $\alpha$ and decreases that of IL-4	Odeh <i>et al</i> [134]
Artemisinin	CCRF-CEM and multidrug-resistant CEM/ADR5000 human leukemia cells. Healthy human foreskin fibroblasts		Fröhlich <i>et al</i> [136]
Artesunic acid	HCT116, HT29, Caco-2, DLD-1 colon cancer cells. HCEC nonmalignant colon epithelial cells	Induces ROS generation, DNA damage, PARP and caspase 9 cleavage. Increases the level of $\gamma$ -H2AX	Fröhlich <i>et al</i> [137]
Diosgenin	A431 and Hep2 human squamous cell carcinoma. Swiss albino mice injected with sarcoma 180 cells	Induces DNA fragmentation and cytoskeletal changes. Decreases the expression of CD31 and Ki67	Das <i>et al</i> [138]
Emodin	MCF-7, MDA-MB-231, MDA-MB-468 and T47D human breast cancer cells. CAM inoculated with MCF-7 cells	Increases the percentage of cells in sub-G1 phase. Increases ROS generation, cytochrome c release, expression levels of p53, Bax and cleaved caspase 3. Reduces Bcl-2, pFAK and integrin $\beta$ 1 expression level. Induces nuclear fragmentation, shrinkage, apoptotic body formation, chromatin condensation and membrane blebbing	Bhattacharjee <i>et al</i> [140]
Ferulic acid	MDA-MB-231 human breast cancer cells		Al-Mutairi <i>et al</i> [143]
Genistein	CALC-62 and ACC448 human thyroid cells derived from anaplastic carcinoma CGTH-W1, ACC360 derived from follicular carcinoma	Reduces the expression level of human telomerase reverse transcriptase, VEGF-A and NFkB. Increases the expression level of PTEN and p21 and activates caspase 3	Ozturk <i>et al</i> [145]
Indirubin-3-monoxime	A549 human lung cancer cells. HFL-1 human fetal lung fibroblast. CD1-nude mice injected with A549 cells	Increases the percentage of cells in Sub-G0 phase. Reduces Bcl-2/Bax ratio, TNF- $\alpha$ release and p-Akt (s473), p-mTOR, NFkB/p65, caspase3 and p53 expression level	Dera <i>et al</i> [147]
Piperine	EMT6/P mouse mammary cancer cells. Balb/C female mice injected with EMT6/P cancer cells	Reduces VEGF expression. Increases IFN- $\gamma$ and IL-2 level and caspase 3 activity	Talib <i>et al</i> [149]
	HepG2 human hepatocellular cancer cells	Increase ROS generation and decreases GSH and NADPH level	Das <i>et al</i> [151]
Resveratrol	HepG2 human hepatocellular cancer cells	Increases caspase 3 activity. Decreases GSH and MDA level	Ismail <i>et al</i> [153]
	EMT6/p mouse epithelial breast cancer cells. MCF-7 and T47D human epithelial breast cancer cells kidney epithelial cells. Balb/C mice injected with EMT6/p cancer cells	Induces DNA fragmentation and increases IFN- $\gamma$ and IL-4 level. Reduces VEGF expression	Alobaedi <i>et al</i> [154]



Selenium	MG-63 human osteosarcoma cell line	Increases cellular damage, and decreases the level of alkaline phosphatase and GSH	Barron <i>et al</i> [156]
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PTEN: Phosphatase and tensin homolog; ROS: Reactive oxygen species; Bax: Bcl-2-associated X protein; GSH: Glutathione; MMP: Matrix metalloproteinases;  $\gamma$ -H2AX: Gamma-histone 2AX; JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; NFkB: Nuclear factor kappa B; COX-2: Cyclooxygenase 2; iNOS: Inducible nitric oxide synthase; VEGF: Vascular endothelial growth factor; DKK-1: Dickkopf-related protein-1; CDNK-1A: Cyclin-dependent kinase inhibitor 1A; TGF- $\beta$ 1: Transforming growth factor beta 1; TGF- $\beta$ RII: Transforming growth factor beta receptor II; GSH: Glutathione; mTOR: Mammalian target of rapamycin; AST: Aspartate transaminase; ALT: Alanine transaminase; MDA: Malondialdehyde; IL: Interleukin; INF: Interferon; TNF- $\alpha$ : Tumor necrosis factor alpha; MCP-1: Monocyte chemoattractant protein-1; RANTES: Regulated on activation normal T cell expressed sequence; TWIST1: Twist-related protein 1; ZEB1: Zinc finger E-box binding homeobox 1; MDM-2: Mouse double minute 2; NADPH: Nicotinamide-adenine dinucleotide phosphate; CAM: Chorioallantoic membrane.

to be downregulated in numerous human cancers including breast cancer[125]. Re-introducing miR-34a in metastatic breast cancer cells targeted and inhibited the expression of epithelial to mesenchymal transition-associated proteins TWIST1, zinc finger E-box binding homeobox 1 and NOTCH1 and suppressed breast cancer cell migration and invasion. Moreover, combining TQ with miR-34a synergistically downregulated TWIST1 and zinc finger E-box binding homeobox 1, suggesting the promising therapeutic potential of this combination against breast cancer metastasis [126]. In another study, multilamellar gold niosomes were developed for the co-delivery of therapeutic Akt-siRNA and TQ to overcome chemotherapeutic resistance induced by Akt overexpression in breast cancer. TQ-siRNA dual loaded niosomes produced stronger anti-proliferative and apoptotic effects in breast cancer *in vitro* and *in vivo* compared to free TQ and TQ loaded niosomes. The mechanism of the combination treatment involved an effective decrease of the cellular level of Akt which sensitized breast cancer cells to TQ toxicity leading to inhibition of mouse double minute 2 and therefore induction of p53-dependent apoptosis[127].

### TQ in combination with natural molecules

**Vitamins:** Vitamin D3, the active metabolite of vitamin D, was reported to have potent chemopreventive effects against colorectal cancer *in vitro* and *in vivo*[128,129]. In addition, vitamin D supplementation was demonstrated to have clinically positive effects on survival outcomes in patients with colorectal cancer[130]. TQ was found to enhance the chemopreventive effect of vitamin D3 in suppressing the initiation and progression of colon tumors in an azoxymethane-induced rat model of colon cancer. The combination treatment significantly attenuated the number of grown tumors and large aberrant crypts foci. In addition, it decreased the level of pro-oncogenic (Wnt,  $\beta$ -catenin, NFkB, heat shock protein 90 HSP-90) and angiogenic (VEGF, iNOS and COX2) biomarkers and increased the expression of anti-oncogenic (DKK-1, CDNK-1A, TGF- $\beta$  1, TGF- $\beta$ /RII and Smad4) biomarkers compared with individual treatments[131].

### Hormones

**Melatonin:** Melatonin is a natural hormone involved in different biological activities including regulating the circadian rhythm[132]. Ample evidence revealed that melatonin exerts powerful anti-tumor effects through different modes of action including the activation of anticancer immune responses[133]. The combination of TQ with melatonin in breast cancer bearing mice resulted in 60% of cure in treated mice and produced a stronger apoptotic, necrotic and anti-angiogenetic response in addition to a more potent activation of T helper 1 mediated anticancer immune response compared to separate treatments[134].

### Plant-derived molecules

Numerous studies have tested the anti-neoplastic efficacy of combining TQ with other plant-derived molecules in different types of cancer. Artemisinin is a sesquiterpene lactone extracted from the Chinese medicinal plant *Artemisia annua*[135]. Fröhlich *et al* [136,137] linked each of Artemisinin and its semisynthetic derivative artesunic acid with TQ *via* covalent bonds and tested the anticancer efficacy of the formed hybrid molecules *in vitro*. They found that the ether-linked artemisinin-TQ hybrid exhibited a potent and selective anti-proliferative activity that was superior to that of the conventional drug DOX against sensitive and multidrug-resistant leukemia cells without being toxic to normal human foreskin fibroblasts[136]. They also found that the ester-linked artesunic acid-TQ hybrid promoted apoptosis mediated by ROS-induced DNA damage in colon cancer cells while being non-toxic to normal colon epithelial cells. The hybrid's effect was found superior to each of the conventional drug 5-FU, the dual and

individual treatments[137]. In another study, Das *et al*[138] demonstrated the synergistic anti-proliferative and apoptotic potential of combining TQ with diosgenin, a natural steroidal saponin isolated from several plants such as *Trigonella foenum-graecum*[139], in squamous cell carcinoma *in vitro* and in a sarcoma 180-induced mouse model. Recently, Bhattacharjee *et al*[140] investigated the combined effect of TQ with emodin, which is a natural anthraquinone obtained from various herbs including *Rheum palmatum*[141]. The results revealed that the dual treatment triggered a synergistic apoptotic response in breast cancer cells and enhanced the reduction of cancer cell migration compared to monotreatment by downregulating two important molecular players, namely focal adhesion kinase and integrin  $\beta 1$ . In an *ex ovo* chorioallantoic membrane xenograft model, TQ and emodin were found to suppress the tumor growth and limit the migration of tumor cells to the liver and lung of the chick embryo [140]. Combining low doses of TQ and ferulic acid, obtained from *Ferula asafoetida* plant [142], potently inhibited the proliferation of breast cancer cells, while single treatments did not exhibit any inhibitory effects[143]. Moreover, it has been found that the combination of TQ and genistein, a flavonoid found in soybeans[144], resulted in a higher induction of apoptosis in thyroid cancer cells than treatment with either agent alone[145]. In lung cancer, the combination of TQ and indirubin-3-monoxime, a drug derived from the traditional Chinese herbal remedy Danggui Longhui Wan[146], resulted in synergistic apoptotic and anti-migratory effects *in vitro* and synergistic tumor growth suppression *in vivo*[147]. At the molecular level, the dual treatment decreased the phosphorylation of survival-regulatory proteins Akt, mammalian target of rapamycin and NF $\kappa$ B and activated caspase 3 and p53 in animal tumors[147]. Furthermore, combining TQ and piperine, the major alkaloid found in *Piper nigrum* L [148], resulted in a synergistic inhibition of breast cancer *in vitro* and *in vivo*[149]. It induced a high degree of apoptosis and extensive necrosis, inhibited angiogenesis, and stimulated T helper 1 anticancer immune response with no liver and kidney toxicities. Interestingly, TQ was found to play the major role in inducing the caspase-mediated apoptosis[149].

In another study, the encapsulation of TQ and piperine in micro-vehicles made of a natural polymer guar gum extracted from the seeds of *Cymomopsis tetraganolobus* plant [150] synergistically reduced the viability of hepatocellular carcinoma cells[151]. This was associated with ROS generation as indicated by an enhanced decrease in the level of intracellular antioxidant glutathione and nicotinamide-adenine dinucleotide phosphate[151]. Two studies assessed the anticancer effectiveness of combining TQ with resveratrol, a stilbene polyphenolic compound extracted from over 70 plants including *Polygonum cuspidatum*[152]. TQ and resveratrol combination resulted in a greater cytotoxic effect on hepatocellular carcinoma cells compared to single treatments[153]. In addition, TQ and resveratrol synergized to effectively inhibit breast cancer *in vitro* and *in vivo*. The combined drugs induced apoptosis and necrosis, inhibited angiogenesis and stimulated the anticancer immune response without causing liver and kidney toxicities[154]. Co-treating osteosarcoma cells with TQ and selenium, a micronutrient/trace element found abundantly in *Astragalus bisulcatus* [155], was found to be effective in decreasing cell viability, inducing cellular damage, and attenuating the levels of alkaline phosphatase and glutathione[156].

## TQ EFFECTS AGAINST CANCER STEM CELLS

### TQ in combination with chemotherapeutics agents

TQ was found to potentiate the effects of each of GCB and PAC in depleting the CD44<sup>+</sup>/CD24<sup>-</sup> CSCs population within MCF-7 and T47D breast cancer cells[58,64]. In another study, the co-delivery of DOX and TQ in ACNP effectively eradicated breast CSCs enriched from MDA-MB-231 cells cultured in 3D compared to single drug loaded ACNP and drug combinations in solution. The combined drugs loaded ACNP efficiently attenuated the self-renewal potential of breast CSCs as evidenced by the decrease of their mammospheres forming efficiency. This was accompanied by the reduction of breast CSCs markers CD44 and CD24 expression and aldehyde dehydrogenase 1 activity. In addition, the dual drugs loaded ACNP suppressed breast CSCs migration and invasion[157]. In colorectal cancer, combination of TQ and 5-FU as well as their hybrid SARB downregulated two major stem cell regulatory pathways Wnt/ $\beta$ -catenin and PI3K/Akt. In addition, they were found to effectively reduce the self-renewal potential of colorectal CSCs and eradicate CD133<sup>+</sup> colorectal CSC population [50].



### TQ in combination with natural products

The combined treatment of TQ and emodin improved the elimination of breast CSCs as demonstrated by the enhanced reduction in mammospheres forming efficiency and in CD44<sup>+</sup>/CD24<sup>+</sup> CSCS population compared to single treatments. Moreover, it down-regulated the stemness promoting transcription factors Oct 4 and SOX2[140].

## CONCLUSION

We have emphasized the tremendous potential of TQ in augmenting the anti-neoplastic effects of different therapeutic modalities against a wide range of cancer cells. TQ sensitized cancer cells to radiotherapy and improved outcomes of cancer resistance to conventional chemotherapeutic agents. The use of TQ in combination therapy also lowered the effective doses of standard chemotherapies which helped reduce their associated toxicities while maintaining their therapeutic effectiveness. The combination of TQ with other plant-derived molecules has shown interesting results and merits further investigation to introduce them as potential candidates for treating cancer. Although the studies investigating TQ potency in eliminating CSC in combination therapy are scarce, their results demonstrated great promise. Involving TQ in combination therapy could possibly further eliminate CSCs from tumors and prevent regrowth of neoplasms.

Despite its remarkable anticancer activity, studies reporting TQ anticancer therapeutic potential in clinical settings are still limited due mainly to its hydrophobicity and poor bioavailability. Few studies have supported combined therapies of TQ with nanoparticle formulations to circumvent the drug delivery challenges. These nanoparticles further enhanced the inhibitory effects of the combined agents against cancer or CSC in preclinical studies. Future efforts should be devoted to developing and testing these effective targeted nanoformulations of the combined agents including TQ for potential clinical translation.

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## Mechanisms of acquired resistance of BRCA1/2-driven tumors to platinum compounds and PARP inhibitors

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### Abstract

Molecular pathogenesis of tumors arising in *BRCA1/2* germ-line mutation carriers usually includes somatic inactivation of the remaining allele of the involved gene. Consequently, *BRCA1/2*-driven cancers are sensitive to platinum-based therapy and poly (ADP-ribose) polymerase inhibitors (PARPi). Long-term exposure to these drugs may result in the emergence of secondary *BRCA1/2* mutations, which restore the open-reading frame of the affected allele. This platinum/PARPi cross-resistance mechanism applies both for *BRCA1* and *BRCA2* genes and has been repeatedly validated in various laboratory models and multiple clinical studies. There are some other routes associated with the partial rescue of *BRCA1/2* function or the development of *BRCA1/2*-independent pathways for genomic maintenance; however, their actual clinical relevance remains to be established. In addition, studies on the short-term neoadjuvant therapy for ovarian cancer revealed that even chemonaive *BRCA1*-driven tumors contain a small proportion of *BRCA1*-proficient cells. These pre-existing cells with retained *BRCA1* heterozygosity rapidly repopulate the tumor mass during platinum exposure, but become outcompeted by *BRCA1*-deficient cells during therapy holidays. Understanding of the platinum/PARPi resistance pathways has led to the development of novel therapeutic approaches, which aim to improve the management of *BRCA1/2*-related cancers and are currently undergoing preclinical and clinical evaluation.

**Key Words:** *BRCA1/2* mutations; Platinum-based therapy; Poly (ADP-ribose) polymerase inhibitors; Drug resistance; Secondary mutations; Intratumoral heterogeneity; Neoadjuvant therapy

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**Core Tip:** BRCA1/2-associated tumors are highly sensitive to platinum compounds and poly (ADP-ribose) polymerase inhibitors; however, they eventually acquire resistance to this type of therapy. Restoration of *BRCA1/2* function *via* the second mutation is the most known mechanism of tumor adaptation to the therapeutic pressure. Some studies demonstrate that even chemo-naïve BRCA1-driven tumors contain a small fraction of BRCA1-proficient cells suggesting that the loss of the remaining allele of this gene is not the first event in tumor pathogenesis. These pre-existing platinum-resistant cells rapidly repopulate tumor mass during neoadjuvant therapy for ovarian cancer and explain inevitability of the disease relapses after seemingly successful surgical debulking.

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## INTRODUCTION

Germ-line mutations in *BRCA1* and *BRCA2* genes are the most well-known cause of hereditary cancer predisposition. *BRCA1/2* pathogenic variants contribute to approximately 5%-10% and 15%-30% breast and ovarian cancer morbidity, respectively[1-6]. In addition, both mentioned genes are involved in the pathogenesis of a subset of stomach cancers, and the inheritance of *BRCA2* inactive alleles is associated with an increased risk of prostate and pancreatic malignancies[7,8]. *BRCA1/2*-driven tumors tend to have particular clinical characteristics: Being associated with younger age at onset and highly malignant phenotype[9,10]. Breast carcinomas (BCs) occurring in *BRCA1* mutation carriers usually lack the expression of estrogen and progesterone receptors, and *BRCA1/2*-associated ovarian cancers (OCs) are characterized by serous high-grade histological appearance[10,11].

Breast and ovarian tumors arising in patients with *BRCA1/2*-associated hereditary cancer syndrome usually develop *via* somatic inactivation of the remaining allele of the involved gene. *BRCA1* and *BRCA2* play a key role in the maintenance of genomic integrity. Consequently, cancers lacking functional *BRCA1* or *BRCA2* proteins are deficient in DNA repair by homologous recombination (HR). Platinum compounds and poly (ADP-ribose) polymerase inhibitors (PARPi) induce massive DNA damage, which requires an HR-mediated repair. *BRCA1/2*-null cells are deficient for HR and consequently die upon the action of platinum salts or PARPi. This drug sensitivity is tumor-selective, as the normal cells of the patient retain one functional copy of *BRCA1/2* gene and therefore remain capable of coping with the DNA damage[12-14].

As mentioned above, *BRCA1/2*-related hereditary tumors constitute a significant portion of OCs. In addition, many high-grade serous OCs have other causes of HR deficiency, *e.g.*, somatic biallelic inactivation of *BRCA1/2* genes or the presence of germ-line mutations in other members of DNA repair pathways[1,15]. This explains the high efficacy of platinum-based chemotherapeutic regimens in OC, which were developed empirically before the discovery of *BRCA1/2* genes and constitute a standard-of-care for OC management. As expected, platinum therapy demonstrates increased efficacy in hereditary *vs* sporadic ovarian tumors[2,16]. In contrast to OC, *BRCA1/2* deficiency is characteristic only for a minority of breast tumors; therefore, platinum compounds are not incorporated in the conventional treatment schemes for non-selected BC patients. Several trials demonstrated that platinum salts might outperform other chemotherapeutic agents when applied to *BRCA1/2*-driven BCs[17-19]. PARPi have been developed specifically for targeting tumors characterized by *BRCA1/2* and/or HR deficiency. There are several PARPi approved for clinical use with slightly varying medical indications[20,21].

Although *BRCA1/2*-driven tumors have a clear-cut vulnerability, the use of platinum salts or PARPi does not usually result in a cure from metastatic disease. Platinum- and PARPi-exposed cancers eventually manage to escape from the action of *BRCA1/2*-specific therapy. Multiple preclinical and clinical studies have identified



various BRCA1/2-restoring mechanisms or bypass pathways, which resume resistance to DNA damage in initially HR-deficient tumor cells. Recent investigations also provided evidence for an alternative scenario, where the emergence of the platinum-resistant tumor clone is attributed to a selection of pre-existing BRCA1-proficient cells; these therapy-resistant cells persist in small amounts in chemo-naïve tumors but are enriched in the residual lesion. This paper provides a brief overview of the mechanisms of acquired platinum and PARP-resistance in BRCA1/2-driven tumors.

## RESTORATION *BRCA1/2* BY SECONDARY SOMATIC MUTATIONS

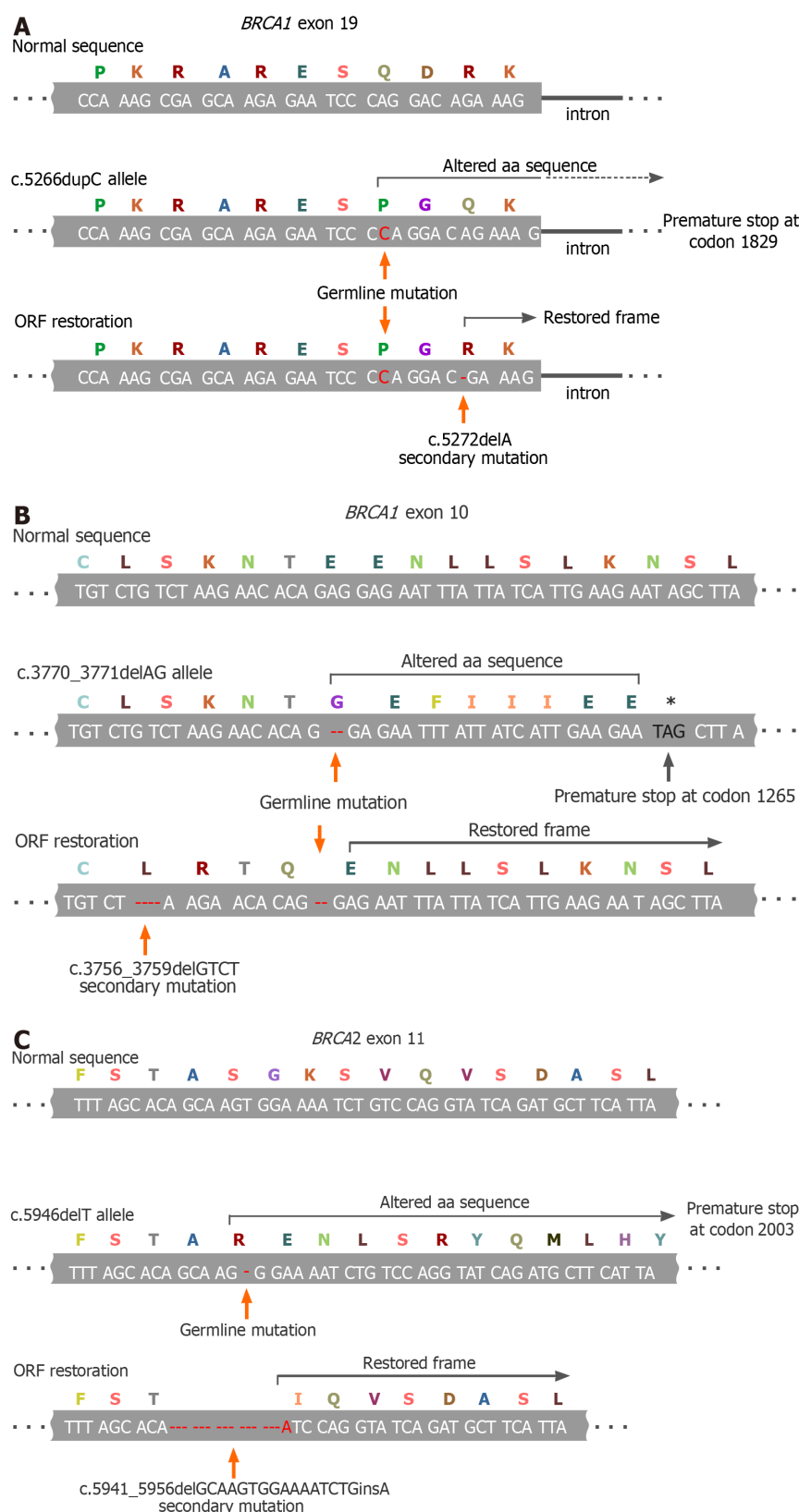
The vast majority of *BRCA1/2* inherited pathogenic alleles are represented by small alterations in the nucleotide sequence, which cause a frameshift and emergence of premature stop-codons. The open reading frame (ORF) can be rescued by a nearby second mutation if it restores an original 3-letter genetic code, or by small deletion, which excises the pathogenic allele and reconstitutes the ORF, or by the true back mutation (Figure 1)[22-24]. A secondary ORF-restoring *BRCA2* mutation was first described in an acute myeloid leukemia cell line obtained from a patient with Fanconi anemia[25]. The discovery of PARPi and the recognition of *BRCA1/2*-specific action of platinum compounds stimulated intense investigations of the mechanisms of tumor resistance to these drugs. A series of studies revealed that the emergence of secondary *BRCA1/2* mutations is the most reproducible hallmark of the acquisition of a drug-resistant phenotype. Indeed, the reversion mutations have been repeatedly observed in the experiments with cell lines, patient-derived xenografts (PDX) and clinical samples[26,27].

For the time being, the development of secondary ORF-restoring mutations in *BRCA1/2* genes is the only clinically proven mechanism of the tumor adaptation to the therapy, which is relevant both to platinum compounds and PARPi, characteristic both for *BRCA1* and *BRCA2* genes, and has been convincingly validated in patient samples. The true incidence of secondary *BRCA1/2* mutations is difficult to presently define due to various selection biases and technical limitations of available molecular genetic assays: They appear to be found in approximately a quarter of PARPi/platinum-resistant tumors, although some studies provide even higher estimates. Importantly, many reports describe the emergence of multiple distinct *BRCA1/2* ORF-restoring mutations in independent drug-resistant clones obtained from the same patient, thus providing evidence for the functional convergence of tumor adaptation pathways[26, 27].

Some data suggest that the genetic reversion is somewhat more characteristic for *BRCA2*- than for *BRCA1*-driven tumors. Distinct pathogenic variants of *BRCA1* and *BRCA2* may differ in their ability to be rescued by the second mutation: It is hypothesized that the genetic reversion is more acceptable for non-conservative regions of the above genes, which are more or less dispensable for their function. Indeed, in all cases, except genuine back mutations, the involved region of *BRCA1* and *BRCA2* genes undergoes subtle alterations (*i.e.* the deletion of a few coding nucleotides or the change of the sequence for a few amino acids); therefore, highly conserved parts of these genes may not tolerate this mechanism of genetic adaptation[14,26,27].

In addition to secondary mutations affecting the coding sequence of *BRCA1/2* genes, there are functionally similar events resulting in the production of hypomorphic but still functional protein. For example, loss of exon 11 is compatible with the participation of *BRCA1* in HR; consequently, alternative splicing resulting in the *BRCA1* exon 11 skipping may contribute to the acquired drug resistance[28]. The mutation located in the N-terminal portion of the *BRCA1* gene can be bypassed by the production of a hypomorphic protein, whose translation starts after the frameshift[29]. Another mechanism of the partial rescue of *BRCA1* function involves gene rearrangements, which terminate *BRCA1* translation before the mutation-containing BCRT domain, consequently preventing the proteasomal degradation of *BRCA1*. These truncated versions of *BRCA1* are capable of maintaining HR and mediate PARPi resistance[30]. Upregulation of HSP90 may stabilize some *BRCA1*-mutant proteins and thus support their function[31]. Amplification of mutated *BRCA2* was shown to compensate for partial loss of *BRCA2* function and rendered PARPi resistance in cell line experiments [32].

Some *BRCA1/2* germ-line pathogenic alleles are represented by so-called large gene rearrangements (LGRs), which may involve deletions of multiple exons. By definition, these tumors cannot be repaired by the second ORF-restoring mutation. One would expect that these tumors are likely to demonstrate a more pronounced and prolonged response to *BRCA1/2*-specific therapy. *BRCA1/2* LGRs are not specifically considered



**Figure 1** Examples of *BRCA1/2* open-reading frame restoration in *BRCA1/2*-mutated tumors during systemic therapy. A: Secondary 1-bp deletion occurring downstream to the germline mutation (*BRCA1* c.5266dupC; described in[22]); B: Secondary 4-bp deletion located upstream to the germline mutation (*BRCA1* c.3770\_3771delAG; an example from[23]); C: Secondary in-frame deletion/insertion excising the mutation-containing gene fragment (*BRCA2* c.5946delT; an example from[24]). ORF: Open-reading frame.

in the studies on tumor drug sensitivity. However, there are case reports supporting exceptional responsiveness of *BRCA1/2* LGR-associated tumors to PARPi[33].

## BYPASS MECHANISMS

There are two key mechanisms of the repair of DNA double-strand breaks (DSB). Accurate correction of DNA sequence can be achieved exclusively by HR. In the absence of functional HR, error-prone non-homologous end-joining (NHEJ) becomes a prevailing mechanism of DSB repair. The choice between HR and NHEJ is mediated by the balance between their regulators, *BRCA1* and *53BP1*. When *BRCA1* is inactivated by mutation, NHEJ-driven DNA repair prevails. This results in the accumulation of multiple DNA lesions and eventual cell death. *BRCA1*-deficient cells may adapt to the platinum or PARPi pressure by down-regulation of *53BP1*. As a result of consequent NHEJ suppression, tumor cells re-activate HR and eventually become resistant to the drug exposure[31,34,35]. Down-regulation of *53BP1* has been observed in some clinical samples that failed platinum-based or PARPi therapy[31,36,37]. *In vitro* studies revealed several other proteins whose loss also contributes to the switch from NHEJ to HR or to other bypass pathways. Noticeably, the involvement of *53BP1* exemplifies the differences between *BRCA1*- and *BRCA2*-mutated tumors, as the loss of *53BP1* or related proteins is relevant only for the treatment escape of *BRCA1*-deficient cancers[34]. Preclinical studies also identified HR-independent platinum/PARPi resistance mechanisms, which involve stabilization of replication forks[38].

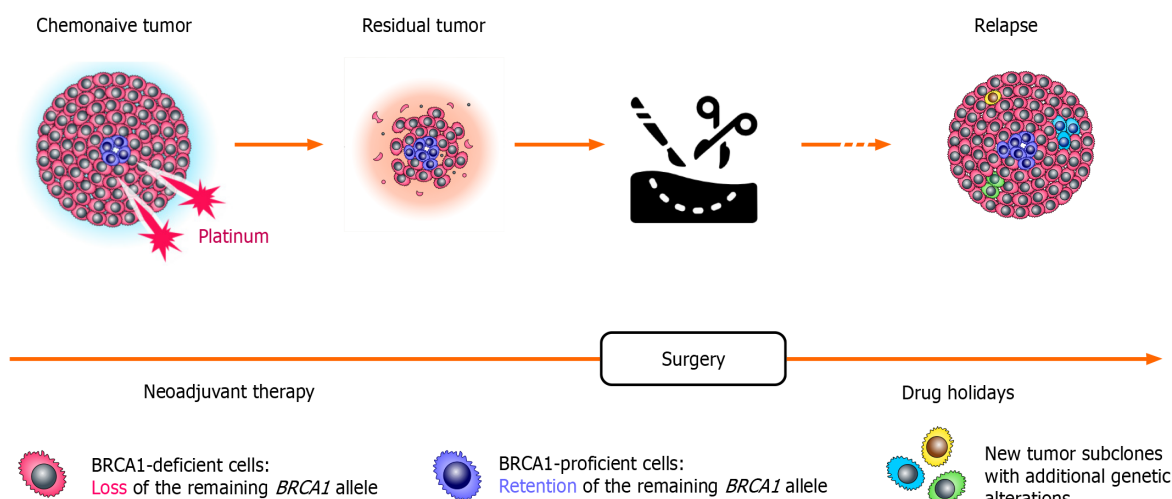
## BRCA1/2-NON-RELATED MECHANISMS OF ACQUIRED RESISTANCE TO PLATINUM COMPOUNDS AND PARPi

The above-described mechanisms of acquired therapy resistance are more or less specific for the *BRCA1/2*-associated action of platinum salts and PARPi. There are also general mechanisms for the adaptation of tumor cells to the therapy, which are indirectly related to the targeted biological pathway and may involve activation of the drug efflux, down-deregulation of apoptosis, preservation of tumor cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT)[39]. Up-regulation of *ABCB1* (MDR1) transporter has been implicated in multidrug resistance. Therapy-resistant ovarian and breast carcinomas are characterized by gene fusions, which result in increased expression of the *ABCB1* gene[40]. The translational implications of these observations are not immediately clear: Drug transporters are involved in multiple physiological processes and are characterized by significant redundancy, so their targeting may be associated with significant adverse events and insufficient clinical efficacy[41]. There are reports demonstrating the selection of CSCs upon PARPi exposure[42]. The role of EMT in the development of PARPi resistance has been shown in preclinical studies involving *BRCA2*-deficient cells[43].

## SELECTION OF PRE-EXISTING BRCA1-PROFICIENT CELLS DURING PLATINUM-BASED THERAPY

A significant portion of OC patients present with the inoperable disease; therefore, they undergo neoadjuvant (first-line) therapy aimed to reduce the tumor burden and permit surgical excision of the remaining cancer lumps. *BRCA1*-driven cancers are particularly sensitive to systemic platinum-based treatment; hence, this category of OC is usually amenable to complete surgical debulking. Despite that presumably efficient platinum-based therapy is administered again after the surgery, it apparently cannot eliminate the residual cancer cells, given that almost all OC treated by this scheme eventually relapse[44].

Comparison of tumor specimens obtained before the start of the treatment and after a few weeks of neoadjuvant therapy revealed surprising findings (Figure 2). While chemonaive *BRCA1*-associated OCs are characterized by somatic loss of heterozygosity (LOH) of the remaining allele, the residual tumors obtained after a few weeks of neoadjuvant therapy often show the retention of the wild-type *BRCA1* copy. This “restoration of heterozygosity” occurs due to the selection of preexisting *BRCA1*-proficient cells, which persist in small amounts in chemonaive tumors; these isolated tumor cells with retained *BRCA1* function can be visualized by various imaging techniques. Importantly, both primary cancers and residual tumor masses were shown to retain the same mutation in the *TP53* gene. Loss of *BRCA1* in normal cells triggers apoptosis, while cells with inactive *TP53* may survive *BRCA1* deficiency. It appears that *TP53* mutation must be acquired in the very initial stages of the tumor evolution,



**Figure 2 Selection of pre-existing BRCA1-proficient cells during platinum-based therapy.** Loss of the remaining *BRCA1* allele is observed in the majority of cells forming the tumor; however, even chemonaive BRCA1-driven ovarian cancers contain a small fraction of transformed cells with retained *BRCA1* heterozygosity. These cells are platinum-resistant and rapidly repopulate tumor mass during neoadjuvant therapy for ovarian cancer. During platinum-free interval, which occurs after the completion of the adjuvant therapy, these BRCA1-proficient cells become outcompeted by cells carrying *BRCA1* LOH. Therefore, ovarian cancer relapses resemble primary tumors with regard to the *BRCA1* status, as they demonstrate again the *BRCA1* deficiency and the sensitivity to platinum compounds.

while *BRCA1* LOH, being a key event in the pathogenesis of BRCA-driven cancers, can emerge and be tolerated only after *TP53* inactivation. The persistence of isolated BRCA1-proficient cells within a gross tumor mass is common for BRCA1-driven cancers, as the “restoration” of *BRCA1* heterozygosity is observed approximately in two-thirds of BRCA1-associated OCs[45].

Intriguingly, the relapse OC tissues obtained from the same patients after therapy holidays show *BRCA1* LOH again, thus providing a mechanistic explanation for the platinum sensitivity of recurrent BRCA1-associated cancers (Figure 2). Exome sequencing revealed that only *TP53* mutation is stably maintained throughout the natural history of BRCA1-driven cancers, while the profiles of somatic point mutations and chromosome number alterations show some variations between chemonaive, post-neoadjuvant and recurrent tumor specimens. Overall, it appears that BRCA1-driven tumors present an ecosystem: While the gross majority of tumor mass is BRCA1-deficient, there are apparently some biological reasons to maintain the persistence of small amounts of BRCA1-proficient cells. In the absence of external hazards, BRCA1-deficient cells clearly outcompete cells with retained *BRCA1* function. However, these cells are sensitive to platinum exposure and perhaps to some other kinds of unfavorable environment, so the maintenance of the reservoir of invulnerable (BRCA1-proficient) cells is important for warranting tumor plasticity. Upon drug pressure, BRCA-proficient cells take advantage and increase their relative fraction in residual tumor mass; however, they again lose the competition after the cessation of the systemic treatment[46]. The above observations fit very well with the concept of tumor “stem cells” as a cause of acquired drug resistance.

The platinum-induced selection of pre-existing BRCA-proficient cells has been demonstrated only for the *BRCA1* gene, while similarly designed studies have not been performed yet for BRCA2-associated tumors. It is not self-explanatory that the same phenomenon is applicable to BRCA2-driven cancers. Indeed, although both *BRCA1* and *BRCA2* proteins are involved in the response to DNA damage, they have essential dissimilarities in their structure and function[14]. Consequently, they demonstrate differences regarding the spectrum of associated tumors, with prostate and pancreatic cancer been strongly linked to *BRCA2* but not to *BRCA1* heterozygosity[7]. Breast carcinomas arising in *BRCA1* germ-line mutation carriers are usually triple-negative with regard to the receptor status (ER, PgR and HER2), while *BRCA2* pathogenic alleles are generally associated with the development of tumors expressing steroid hormone receptors[10,11]. *BRCA1* but not *BRCA2* is essential for taxane-mediated cell death, so the resistance to taxanes is characteristic for BRCA1- but not for BRCA2-deficient cells[12]. The emergence of ORF-restoring secondary mutations in

heavily pretreated tumors appears to be somewhat more common for *BRCA2* than for *BRCA1* gene[26,27]. *BRCA1* deficiency is lethal for normal cells; therefore, the development of cancers in *BRCA1* germ-line mutation carriers always involves mutation-driven inactivation of the *TP53* gene, which results in down-regulation of apoptosis and provides the ground for the survival of *BRCA1*-null cells. In contrast, *BRCA2* inactivation is compatible with cell viability, so *BRCA2*-associated tumors often have wild-type *TP53* status[47]. While the persistence of *BRCA1*-proficient cells in chemo-naïve *BRCA1*-driven tumors is essential for the adaptation of OC to platinum-based therapy, it is unclear how this intratumoral heterogeneity supports the maintenance of tumor mass in “natural” conditions. This intratumoral heterogeneity may not necessarily be characteristic for the cancers arising in *BRCA2* germ-line mutation carriers. Further studies are needed to reveal whether the persistence of isolated HR-proficient “stem” cells is relevant for *BRCA2*-driven tumors or sporadic OCs with BRCAness phenotype.

## CONTROVERSIAL AND UNRESOLVED ISSUES

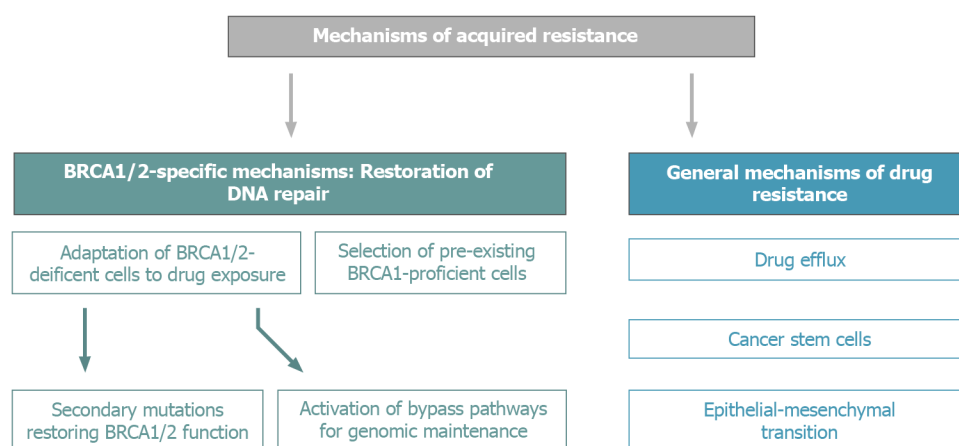
Platinum compounds and PARPi converge in their mechanisms with regard to targeting HR-deficient cells; however, there are also some differences in their action. For example, platinum salts appear to target tumors with deficient nucleotide excision repair[34]. Consequently, while secondary *BRCA1/2* mutations or other HR-restoring events are likely to result in cross-resistance between platinum and PARPi, other modes of tumor adaptation to the therapy may be more drug-specific. Platinum is commonly used for the treatment of ovarian cancer, and the clinical trials demonstrated that the use of PARPi results in significantly better outcomes in platinum-sensitive *vs* platinum-resistant disease[48,49]. Similarly, the advantage of talazoparib was more pronounced in *BRCA1/2*-driven breast cancer patients who did not receive prior cisplatin or carboplatin[50]. However, some presumably platinum-resistant ovarian tumors still demonstrate some sensitivity to PARPi[48,49]. On the other hand, PARPi therapy may result, for example, in the emergence of mutations in the *PARP1* gene, which alter *PARP1* trapping to DNA but are unlikely to affect tumor sensitivity to drugs other than PARPi[51]. It needs to be stressed that in the clinical setting, the platinum sensitivity of ovarian cancer is usually defined not by the actual tumor response to carboplatin or cisplatin but by the time interval exceeding 6 mo since the last platinum exposure. It is not impossible that some tumors may actually restore HR deficiency within a shorter period of time, so their response to PARPi could be explained by conventional PARPi-associated biological mechanisms. While the use of PARPi after chemotherapy has been evaluated in many clinical trials[49], we are unaware of a systematic analysis of chemotherapy response in PARPi-resistant tumors.

*BRCA1* and *BRCA2* germ-line mutations are usually viewed as equivalent in all clinical trials involving DNA damaging treatments. Although this approach is generally well justified, some differences between these two genes need to be acknowledged. Preclinical experiments have demonstrated mechanisms for therapy escape that are relevant for *BRCA1*- but not for *BRCA2*-driven tumors[34]. The spectrum of associated cancers is somewhat different for these two genes; for example, the analysis of PARPi-resistant prostate malignancies is almost entirely limited to *BRCA2* mutation carriers, as *BRCA1* plays a negligible role in the predisposition to this disease[26].

The regimens of administration of platinum salts and PARPi significantly differ. Cisplatin or carboplatin are usually administered in several cycles, so there are peak drug concentrations and significant intervals between chemotherapy infusions. In contrast to this intermittent drug administration of platinum drugs, PARPi are used at a continuous dose for a prolonged period of time. It is very likely that the mode of drug administration may influence the pathways of tumor adaptation to therapeutic intervention. Furthermore, published clinical experiments included very heterogeneous groups of patients with regard to the duration of prior treatment. It appears that the majority of secondary *BRCA1/2* mutations were detected mainly in heavily pretreated patients, while the initial cycles of chemotherapy rarely resulted in the genetic reversion of the *BRCA1/2* sequence[26,27,46,52].

Cell and animal experiments cannot fully recapitulate the complexity of intratumoral heterogeneity, tumor microenvironment, interplay with the immune system, drug dosing, *etc.*, characteristic for a clinical setting. The investigation of biological material obtained from cancer patients is challenging, particularly when it comes to the analysis of acquired therapy resistance. Tumor re-biopsy, by definition, requires





**Figure 3** Mechanisms of acquired resistance of BRCA1/2-driven tumors to platinum compounds and PARP inhibitors.

sound clinical and ethical justification; therefore, some studies relied on circulating tumor DNA (ctDNA). Liquid biopsy is capable, in theory, to uncover the entire spectrum of subclonal secondary mutations, although it may underestimate the frequency of back mutations and does not account for the proportion of BRCA1/2-restored cells within a tumor mass. Current technologies for gene sequencing, which are utilized for the detection of secondary mutations, may miss some large deletions of genetic material[26]. It is highly desirable to continue the collection of platinum- and PARPi-resistant tumor samples from cancer patients, to subject these specimens to comprehensive molecular profiling, and to monitor the response of these tumors to subsequent treatment modalities. This effort may identify gene-response correlation and help to guide the clinical management of BRCA1/2-related cancers after the failure of the standard therapy.

## CONCLUSION

BRCA1/2-driven tumors have a number of in-built mechanisms of adaptation to conventional schemes of platinum-based therapy and PARPi (Figure 3). Nowadays, an increasing number of OC patients are subjected to long-term PARPi maintenance therapy, which certainly affects the biological and clinical properties of recurrent tumors. It is somewhat surprising that the available medical research literature does not put an emphasis on the potential treatment options for tumors arising on the background of continuous PARPi exposure, despite that multiple lines of preclinical and clinical data suggest the involvement of cross-resistance mechanisms[34].

Genome profiling of drug-resistant tumors obtained from BRCA1/2 mutation carriers has not identified recurrent actionable molecular lesions[46]. However, despite the restoration of HR proficiency or the emergence of bypass pathways, these tumors continue to contain the genomic scar of BRCAness, *i.e.* the existence of multiple genomic rearrangements. These genetic lesions may underlie an increased antigenicity of BRCA1/2-driven tumors. Interestingly, second mutations, which are the cause of drug resistance, are often associated with the emergence of additional antigenic epitopes[26]. The feasibility of the use of immune therapy against platinum/PARPi-resistant OCs has not been evaluated systematically, although case series support the promise of this option[53].

The best approach would be to implement treatment that would prevent the appearance of drug-resistant clones. There is a number of ongoing trials evaluating the efficacy of combinations of PARPi with other drugs[34,54]. Several studies demonstrated the potentially curative impact of high-dose chemotherapy for BRCA1/2 mutation carriers; however, the use of this treatment is associated with excessive adverse effects [55]. Neoadjuvant combination of cisplatin and mitomycin C resulted in complete pathological responses, *i.e.* in the elimination of all detectable cancer cells, in some BRCA1/2-driven OCs[44].

There are several recent breakthroughs in the management of BRCA1/2-driven tumors, which resulted in significant improvement of disease outcomes. Continued understanding of the mechanisms of platinum/PARPi resistance inspired the development of a multitude of novel therapeutic approaches, which are likely to

contribute to further advances in cancer treatment. BRCA1/2-associated carcinomas have well-defined vulnerabilities and are characterized by pronounced drug sensitivity. They are similar in this respect to germ-cell tumors and some hematological malignancies, which are generally curable by already available therapeutic tools. There are reasonable chances that cure rates for BRCA1/2-associated malignancies will significantly increase in the near future.

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## Esophagogastric junction adenocarcinoma: Preoperative chemoradiation or perioperative chemotherapy?

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### Abstract

Multimodal treatment is currently the standard of care for locally advanced esophagogastric junction (EGJ) adenocarcinoma due to poor results after surgery alone. Neoadjuvant therapy is intended to shrink the tumor and eliminate potential circulating tumor cells. However, which neoadjuvant treatment is best for patients with EGJ tumors remains controversial. We aimed to compare outcomes of preoperative chemoradiation and perioperative chemotherapy for EGJ adenocarcinomas. For this purpose, we performed a thorough review of the literature describing neoadjuvant treatments for EGJ adenocarcinomas or comparing both therapies. Although some studies have shown better locoregional control and higher rates of complete pathologic response after chemoradiation, data suggest that both types of neoadjuvant therapy have similar survival benefits. As current data are heterogeneous and many studies have included significantly different types of patients in their analysis, future studies with better patient selection are still needed to define which neoadjuvant therapy should be chosen. In addition, targeted therapies and immunotherapy have promising results and should be further explored.

**Key Words:** Esophageal cancer; Esophagogastric junction tumor; Esophageal Adenocarcinoma; Chemotherapy; Chemoradiation; Neoadjuvant therapy

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**Core Tip:** Surgical treatment only has shown poor results in patients with locally advanced esophagogastric junction tumors. Perioperative chemotherapy and

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neoadjuvant chemoradiation are valid treatment modalities for these patients. This evidence-based review explores the results, advantages, and disadvantages of both approaches. In addition, future directions with potentially effective novel drugs are also discussed.

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## INTRODUCTION

Esophagogastric junction (EGJ) adenocarcinoma includes tumors originated from the gastric cardia and the distal esophagus, and is the most common pathological type of esophageal cancer in Western countries[1,2]. The prognosis of this entity is unfavorable in the great majority of patients due to its fast dissemination and advanced disease stages when diagnosed, with an overall 5-year survival of 27%-39%[3,4].

Surgical resection is the gold standard treatment modality for patients without distant disease. The esophagectomy consists of radical resection of the tumor along with the regional lymph nodes[5]. Nevertheless, the poor results after surgical treatment alone have motivated the adoption of neoadjuvant therapies to improve prognosis. Multiple studies have demonstrated that combined preoperative chemoradiotherapy or perioperative chemotherapy plus surgery provide a greater survival benefit than surgery alone[6-9].

Neoadjuvant therapy is intended to shrink the tumor and eliminate potential circulating tumor cells. However, which neoadjuvant treatment is best for patients with EGJ tumors remains controversial. We aimed to compare outcomes of preoperative chemoradiation and perioperative chemotherapy for EGJ adenocarcinomas. For this purpose, we performed a thorough review of the literature describing neoadjuvant treatments for EGJ adenocarcinomas or comparing both therapies.

## NEOADJUVANT AND PERIOPERATIVE THERAPIES OVER TIME

Perioperative chemotherapy for EGJ adenocarcinomas has been explored over time. The first milestone was the British Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial of 2006, which compared patients with gastric and EGJ adenocarcinomas who underwent 3 cycles of epirubicin, cisplatin, and fluorouracil (ECF) before and after the surgery, *vs* surgery alone. Results showed a significant improvement in R0 resections and an overall survival benefit in patients receiving perioperative chemotherapy[9].

In 2011, a multicenter phase III trial was conducted (ACCORD-07) including patients with resectable adenocarcinomas of the stomach, EGJ, and distal esophagus. They compared surgery alone *vs* perioperative chemotherapy with cisplatin and fluorouracil plus surgery. In patients with resectable adenocarcinomas, perioperative chemotherapy significantly improved overall survival, disease-free survival, and curative resection rates[10].

Shortly after, the ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study (CROSS) Group, published the results of a large study that randomized patients with esophageal or EGJ tumors to surgery alone or preoperative chemoradiotherapy followed by surgery. Patients undergoing preoperative chemoradiotherapy with a 5 wk regimen of carboplatin and paclitaxel followed by concurrent radiotherapy, showed a significant improvement in pathological curative resections and overall survival with acceptable adverse events[7,11].

Finally, the German FLOT4 trial in 2019 compared perioperative ECF *vs* perioperative FLOT (fluorouracil, leucovorin, oxaliplatin, and docetaxel) for gastric and EGJ tumors. This trial was able to demonstrate that patients undergoing FLOT had higher rates of pathological remissions and R0 resections than patients undergoing the MAGIC regimen[12].

Several ongoing trials are currently investigating different neoadjuvant and perioperative therapies in patients with EGJ tumors (Figure 1).

## RESULTS OF PREOPERATIVE CHEMORADIOTHERAPY

The CROSS trial included 366 patients [275 (75%) adenocarcinomas, 84 (23%) squamous-cell carcinomas, and 7 (2%) large-cell undifferentiated carcinomas]. The vast majority of patients had distal esophageal cancer, with only 22% of EGJ tumors. Patients were randomly assigned to surgery alone ( $n = 188$ ) or chemoradiotherapy [intravenous carboplatin (AUC 2 mg/mL per min) and intravenous paclitaxel (50 mg/m<sup>2</sup> of body-surface area) for 23 d] with concurrent radiotherapy (41.4 Gy, given in 23 fractions of 1.8 Gy on 5 d/wk) followed by surgery ( $n = 178$ ). The chemoradiotherapy group had significantly higher rates of R0 resections (curative resections) than the surgery alone group (92% *vs* 69%;  $P < 0.001$ ). Furthermore, the overall survival was significantly better in the experimental group (49.4 mo *vs* 24 mo), with a 29% of complete pathological response in the neoadjuvant group. In addition, very few adverse events were reported in the chemoradiotherapy-surgery group (6% leukopenia, 5% anorexia, 3% fatigue, and 2% neutropenia)[7].

Long-term follow-up of the CROSS trial confirmed the benefits of neoadjuvant chemoradiotherapy followed by surgery in patients with EGJ and esophageal cancers. Interestingly, in the subgroup analysis by cancer type, patients with squamous cell carcinomas had a greater overall survival benefit than patients with adenocarcinomas [11].

The addition of radiotherapy to the chemotherapy treatment has shown to improved locoregional control by lymph node downstaging and higher rates of complete pathological response (R0 resections). However, this combination might not be highly effective for reducing the risk of distant metastases[2].

## RESULTS OF PERIOPERATIVE CHEMOTHERAPY

The British MAGIC trial in 2006 introduced the first perioperative chemotherapy regimen for gastric and EGJ tumors, comparing patients who underwent 3 cycles of ECF before and after the surgery, *vs* patients who underwent surgery alone. The study showed a significant improvement in overall and progression-free survival, as well as higher rates of downsizing of the tumor in the chemotherapy group, with similar complications rates between groups (46% *vs* 45%)[9]. It is worth mentioning that this trial included only 11% of patients with EGJ adenocarcinomas. In addition, few patients were able to complete the full perioperative treatment (91% completed the 3 preoperative cycles, 66% started the 3 postoperative cycles, and only 76% of these patients completed the three cycles), with only 42% of the patients completing the full 6-cycle regimen. Furthermore, no complete pathological response was observed[9].

The French Actions Concertées dans les cancers COLORectaux et Digestifs (ACCORD)-07 trial in 2011 compared patients receiving 2 or 3 cycles of cisplatin and fluorouracil before and after surgery with patients undergoing surgery alone. The authors observed better overall survival (38% *vs* 24%), 5 year disease-free survival (34% *vs* 19%), and higher rates of R0 resections in patients with perioperative chemotherapy[10]. In addition, patients receiving chemotherapy had similar morbidity rates than those undergoing surgery alone. In contrast with the MAGIC-trial, 64% of the patients included in the study had EGJ tumors. However, as well as the MAGIC-trial, one of the main disadvantages was that most patients could not finish the complete regimen due to postoperative morbidity[10].

Based on the results of the MAGIC and ACCORD trials, perioperative chemotherapy was widely embraced for EGJ tumors. In 2019, the German FLOT-4 trial (fluorouracil, leucovorin, oxaliplatin, and docetaxel) analyzed the efficacy and safety of perioperative chemotherapy for locally advanced, resectable gastric and EGJ tumors. In this trial, 716 patients (56% with EGJ tumors) were randomly assigned to perioperative FLOT ( $n = 356$ ) or ECF ( $n = 360$ ) plus surgery. An overall survival benefit was observed in the FLOT group (50 mo *vs* 35 mo) and serious adverse events, morbidity, and mortality rates were similar between both groups. These encouraging results have motivated most physicians to adopt FLOT as the standard chemotherapy regimen for patients with EGJ tumors. Remarkably, only 50% and 37% of the patients completed the entire perioperative FLOT or ECF treatment, respectively[12]. Therefore, physicians should be aware that a considerable proportion of patients



**Figure 1 Timeline of neoadjuvant and perioperative therapies in patients with esophagogastric junction cancer.** MAGIC: The British Medical Research Council Adjuvant Gastric Infusional Chemotherapy; CROSS: ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study.

might not be able to receive the entire planned systemic treatment. [Table 1](#) describes relevant characteristics of current available neoadjuvant and perioperative therapies.

## PERIOPERATIVE CHEMOTHERAPY VS PREOPERATIVE CHEMORADIATION

To FLOT or to CROSS: that is the question. Regrettably, which is the most effective neoadjuvant therapy for locally advanced EGJ adenocarcinomas remains unclear. In fact, the most important guidelines recommend either perioperative chemotherapy or preoperative chemoradiotherapy for resectable and locally advanced EGJ tumors[13, 14].

Unfortunately, scarce studies have compared both therapies. A recent meta-analysis of 13 randomized controlled trials with almost 5000 patients found no significant differences in overall survival between both regimens (FLOT reached a non-significant HR of 0.88 (95%CI: 0.46-1.62) compared to CROSS for overall survival in random-effects models)[15]. Petrelli *et al*[2] conducted another large systematic review and meta-analysis including 22 studies comparing perioperative chemotherapy and preoperative chemoradiotherapy for GEJ adenocarcinomas, and showed that both therapies had similar overall survival rates. Interestingly, chemoradiotherapy was associated with better locoregional control and higher R0 resection rates but poorer distant metastases control[2].

A propensity score-matched analysis of patients with locally advanced esophageal and EGJ adenocarcinomas compared 40 patients receiving CROSS and 40 receiving FLOT. The study showed that patients undergoing preoperative chemoradiotherapy had higher rates of complete pathological response (97% vs 85%;  $P = 0.049$ ) and higher rates of negative lymph node metastases (68% vs 40%;  $P = 0.014$ ) than those receiving perioperative chemotherapy. Nevertheless, despite these benefits associated with the CROSS regimen, no difference in overall survival was found between groups[16].

Recently, a study group conducted a propensity score-matched analysis of 3300 patients (1650 for arm) undergoing preoperative chemoradiation vs perioperative chemotherapy for resectable lower esophageal and EGJ adenocarcinomas. The authors hypothesized that chemoradiation was superior to chemotherapy. They found that although patients undergoing chemoradiation achieved higher rates of complete pathological response (2.7 times), overall survival was similar in both groups[17]. Similarly, a 2-center retrospective analysis, failed to demonstrate a greater benefit between different neoadjuvant therapies for resectable EGJ adenocarcinomas. They analyzed 85 patients (33 received neoadjuvant/perioperative chemotherapy and 52 neoadjuvant chemoradiotherapy). There was a significantly higher pathological complete response after chemoradiotherapy (30% vs 12%;  $P = 0.01$ ). However, these differences did not translate into a different disease-free or overall survival[18].

At our institution, neoadjuvant chemoradiation is mostly used for patients with distal squamous cell carcinoma (Siewert type I). This strategy is based on the subgroup analysis by cancer type of the CROSS trial, which showed that patients with squamous cell carcinomas had greater overall survival benefit than patients with adenocarcinomas.

In patients with EGJ adenocarcinoma, we try to avoid the morbidity of radiation and we usually offer perioperative chemotherapy based on the multiple trials showing good outcomes with this approach (MAGIC, ACCORD, and FLOT). Currently, we offer FLOT regimen due to the recent results of the FLOT trial. Radiation is usually added in patients with extensive loco-regional involvement (*i.e.* bulky tumors).



**Table 1 Relevant characteristics of current available neoadjuvant and perioperative therapies for esophagogastric junction tumors**

Study	Year	Number of patients	Included patients	Groups	EGJ tumors	Outcomes
MAGIC	2006	503	Gastric, lower esophagus, and EGJ tumors	ECF + Surgery <i>vs</i> Surgery alone	11%	Perioperative chemotherapy improves overall survival
ACCORD	2011	224	Gastric, lower esophagus and EGJ tumors	CF + Surgery <i>vs</i> Surgery alone	64%	Perioperative chemotherapy improves overall survival, disease-free survival and resectability
CROSS	2012	366	Esophageal and EGJ tumors	Chemoradiation + Surgery <i>vs</i> Surgery alone	22%	Chemoradiotherapy improves overall survival
FLOT	2019	716	Gastric and EGJ tumors	FLOT <i>vs</i> ECF	56%	FLOT improves overall survival

EGJ: Esophagogastric junction; MAGIC: the British Medical Research Council Adjuvant Gastric Infusional Chemotherapy; ECF: Epirubicin, cisplatin, and fluorouracil; CROSS: ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study.

Overall, further studies are needed to clarify which is the best neoadjuvant treatment for EGJ tumors. Each neoadjuvant modality has advantages and disadvantages that should be considered in a case-by-case basis (Table 2).

## FUTURE DIRECTIONS

As no study could demonstrate greater benefit between chemoradiotherapy or perioperative chemotherapy for resectable EGJ adenocarcinomas, efforts to elucidate the best multimodal treatment are still needed.

The ongoing multicenter randomized controlled phase III ESOPEC-trial compares neoadjuvant CROSS *vs* FLOT in patients with resectable and potentially curative esophageal adenocarcinoma. The authors hypothesized that the FLOT regimen might improve overall survival and distant metastases disease control. The results of this trial will hopefully help to decide the most suitable neoadjuvant therapy for patients with EGJ adenocarcinoma[4].

Targeted therapies are designed to inhibit specific molecules overexpressed in patients' tumors and are also currently explored for the treatment of esophageal cancer. The human epidermal growth factor receptor 2 (HER2) is involved in diverse cellular functions such as cell growth, differentiation, and survival. Trastuzumab is a monoclonal antibody targeting the extracellular domain of HER2. The trastuzumab for gastric cancer (TOGA) trial evaluated patients with advanced gastroesophageal adenocarcinoma with overexpression of HER2, and found that the addition of trastuzumab to standard chemotherapy was associated with improved overall survival [19]. A recent trial, however, did not show a survival advantage with the addition of trastuzumab to neoadjuvant chemoradiation in patients with HER2 overexpressing esophageal adenocarcinoma[20]. Pertuzumab is another monoclonal antibody targeting HER2. The PETRARCA trial is currently evaluating the outcomes of perioperative trastuzumab and pertuzumab in combination with FLOT *vs* FLOT alone for patients with HER2-positive resectable esophagogastric adenocarcinoma[21].

The vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) regulate angiogenesis and play a key role in tumor growth. Bevacizumab (monoclonal antibody against VEGF-A), ramucirumab (monoclonal antibody against VEGFR-2), and apatinib (molecule inhibitor selective for VEGF-2) are some of the drugs under investigation[22-25].

The advent of immunotherapy has also brought hope for the treatment of esophageal cancer. Immunotherapy utilizes monoclonal antibodies directed against immune checkpoint proteins such as program death 1 (PD-1) receptor, programmed death ligand 1 (PD-L1) or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). Recent studies have demonstrated survival advantages with monoclonal antibodies targeting PD-1/PD-L1 (*e.g.*, pembrolizumab, nivolumab) in patients with advanced gastric esophageal cancer[26].

Although targeted therapeutics and immunotherapies are indeed promising, further studies are needed to define the safety and efficacy of these drugs.

**Table 2 Potential advantages (+) and disadvantages (-) of preoperative chemoradiation and perioperative chemotherapy**

Preoperative chemoradiotherapy	Perioperative chemotherapy
+ Better loco-regional control	+ Better systemic control
+ High rates of complete pathologic response	+ No adverse events from radiotherapy
- Poorer response in adenocarcinoma (increased radiation sensitivity in squamous cell carcinoma)	- Poorer loco-regional control
- Radiation-induced changes in surgical field	- Many patients are not able to complete the postoperative regimen

## CONCLUSION

Although some studies have shown better locoregional control and higher rates of complete pathologic response after chemoradiation as compared to perioperative chemotherapy, current data suggest that both types of neoadjuvant therapy have similar survival benefits. Future studies comparing both treatment modalities and with better patient selection are still needed to define which neoadjuvant therapy should be chosen. Targeted therapeutics and immunotherapies have promising results and might also be part of the treatment armamentarium in the future.

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## BRCA mutations and gastrointestinal cancers: When to expect the unexpected?

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### Abstract

BRCA1/2 pathogenic variants are widely known as major risk factors mainly for breast and ovarian cancer, while their role in gastrointestinal (GI) malignancies such as colorectal cancer (CRC), gastric cancer and oesophageal cancer (OeC) is still not well established. The main objective of this review is to summarise the available evidence on this matter. The studies included in the review were selected from PubMed/GoogleScholar/ScienceDirect databases to identify published articles where BRCA1/2 pathogenic variants were assessed either as a risk factor or a prognostic/predictive factor in these malignancies. Our review suggests that BRCA1/2 might have a role as a risk factor for colorectal, gastric and OeC, albeit with differences among these diseases: In particular BRCA1 seems to be much more frequently mutated in CRC whereas BRCA2 appears to be much more closely associated with gastric and OeC. Early-onset cancer seems to be also associated with BRCA1/2 mutations and a few studies suggest a positive prognostic role of these mutations. The assessment of a potentially predictive role of these mutations is hampered by the fact that most patients with these diseases have been treated with platinum compounds, where it is expected that a higher probability of response should be seen. A few clinical trials focused on poly (ADP-ribose) polymerase inhibitors use in GI cancers are currently ongoing.

**Key Words:** BRCA; Colorectal cancer; Gastric cancer; Esophageal cancer; Prognosis; Poly (ADP-ribose) polymerase inhibitors



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**Core Tip:** BRCA1/2 pathogenic mutations, other than increasing the risk of breast and ovarian cancer, may increase the risk of developing other types of cancers, such as prostate and pancreatic cancers. Our review highlights that BRCA mutations could also have a predisposing role to gastrointestinal tumours, in particular gastric, esophageal and colorectal cancer, thus playing an important role in terms of surveillance procedures and therapeutic options.

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## INTRODUCTION

BRCA mutations are defined as pathogenic variants in either the *BRCA1* or *BRCA2* gene. These two tumour suppressor genes are involved in different crucial pathways including DNA repair, cell proliferation control and apoptosis. In particular, *BRCA1* and *BRCA2* genes are mainly involved in the homologous recombination (HR) process, responsible for maintenance of genome integrity through an error-free repair pathway for DNA double-strand breaks in response to DNA damage[1,2]. Loss-of-function mutations in *BRCA1/2* may lead to accumulation of DNA double-strand breaks and result in genomic instability and tumour development.

*BRCA1* and *BRCA2*-associated hereditary breast and ovarian cancer syndrome is characterised by an increased risk of breast cancer and ovarian cancer (including fallopian tube and primary peritoneal tumours), up to 84% for breast cancer and 40% for ovarian cancer[3-5]. Moreover, higher risk of male breast cancer has been reported especially in *BRCA2* carriers[6-9].

In *BRCA2* pathogenic variant carriers, familial cancer types different from breast and ovarian, such as prostate, pancreatic cancer, and melanoma, have also been described[10-14].

In addition to that, case-control and family-based studies seem to suggest that *BRCA1/2* pathogenic germline carriers might be at increased risk of other malignancies, although this seems less likely[6,15,16]. It is unclear whether *BRCA1* and *BRCA2* mutations might determine increased risk of tumour types that are already quite common in the general population, such as gastrointestinal (GI) cancers: This might be due to the fact that most data on the subject is derived from cross-sectional studies of family histories of women with *BRCA* pathogenic mutations.

These studies might be susceptible to selection bias; furthermore, misclassification can be present, due to the fact that GI cancer diagnoses were based only on information provided by a family member and this approach may not always be reliable.

Moreover, prospective evidence estimating GI cancer incidence in *BRCA1/2* pathogenic variants carriers are still rare[17]. Predicting GI cancer incidence rates among *BRCA* carriers might convey meaningful implications both for genetic counsellors and tested individuals, as it would aid in the development of appropriate screening policies and risk reduction procedures. Indeed, at the moment, specific guidelines or recommendations regarding the need for gastric and bowel screening procedures for carriers of *BRCA1/2* mutations are lacking.

Furthermore, *BRCA1* and *BRCA2* pathogenic variants have an emerging role for novel target therapies. *BRCA1/2* have essential functions in the HR DNA repair pathway, and genomic alterations in *BRCA* genes lead to impaired DNA repair, called homologous recombination deficiency (HRD). Platinum compounds [such as oxaliplatin, widely used in colorectal and gastric cancer (GC) treatment] and poly (ADP-ribose) polymerase-inhibitors (PARPi) are currently the two main classes of drugs active against cancer cells harbouring HRD alterations. In particular, PARPi exposure promotes synthetic lethality in the setting of defective DNA repair pathway due to irreversible DNA damage, and resulted extremely effective in *BRCA*-mutant ovarian cancer (OvC): In platinum-sensitive OvC patients PARPi maintenance therapy now

represents a mainstay of treatment (Figure 1). Currently PARPi use has also been approved in BRCA-mutant breast, pancreatic and prostate cancer patients[18-21].

In BRCA-mutant GI cancer, the role of HRD alterations is still widely unknown and only few data about their clinical impact are available.

The aim of this review is to highlight a possible association between BRCA pathogenic variants and GI cancer risk and also to investigate the role of BRCA mutations as a prognostic and/or predictive factor in this setting of patients. As the role of BRCA mutations (particularly BRCA2) has already been clarified in pancreatic cancer patients and in such cases PARPi[20] are already used in everyday clinical practice, we decided to focus only on gastric, oesophageal cancer (OeC) and colorectal cancer (CRC).

## LITERATURE SEARCH

The studies included in the present review were selected from PubMed/Google-Scholar/ScienceDirect databases. We used the terms “Gastric Cancer”, “Colon Cancer”, “Rectal Cancer”, “Colorectal Cancer”, “Esophageal Cancer”, “Oesophageal Cancer” for tumour location and also the terms “BRCA1”, “BRCA2”, “BRCA” to select papers focused on BRCA mutations. “Germline” was added as a search term to identify published papers concerning familial BRCA mutations (as opposed to papers where somatic BRCA mutations or all kinds of mutations regardless of their type were included). No limitation as to the year of the published paper was used. Only published papers were included in the analysis (meeting abstracts were excluded). Duplicates were eliminated as well as articles not written in English. The search for articles was conducted independently by two of the co-authors (Maccaroni E and Giampieri R).

## CRC

### **CRC in BRCA carriers: Risk and clinico-pathological features**

CRC is the third most common malignancy in both sexes: It accounts for 10% of new cancer diagnoses and it represents the second estimated leading cause of cancer-related death in 2020 worldwide[22].

One out of 20 CRC patients has a hereditary predisposition. Lynch syndrome (LS, hereditary non-polyposis CRC) is the most common hereditary disorder, justifying 3% of all cases; LS is an autosomal dominant syndrome that causes alterations in the DNA repairing system known as mismatch repair (MMR) proteins. These proteins are able to restore insertions, deletions, as well as A-G and T-C mismatches[23]. Lack of function variants in one of the four genes (hMLH1, hMSH2, hMSH6, PMS2) that encodes the MMR proteins determines, as a final effect, an increase in replication errors in specific areas of the genome where single or double-nucleotide repeats are present, known as microsatellite regions. An increase or decrease in microsatellite length results in what is known as microsatellite instability (MSI) and increases DNA susceptibility to further mutation in oncogenes and tumour suppressor genes. Twelve to fifteen percent of all CRCs harbour a deficient MMR system and the percentage is lower (5%) if we only consider patients with metastatic involvement (mCRCs)[24]. Among these cases, 2/3 are due to sporadic mutations, while only 1/3 of cases are due to germline loss-of-function mutations. Because of this fact, patients with MSI-high status CRC diagnosis should be advised genetic counselling and testing so as not to overlook further diagnosis of LS. Other clinical factors that might lead to suspected LS are Amsterdam I/II criteria and revised Bethesda guidelines[25,26]: Although these criteria might suggest the need for pedigree analysis, it must be considered that genomic profiling remains the gold standard for diagnosis of LS either in patients who fit these criteria or those screened because of a MSI-high CRC tumour.

While the risk of developing CRC in LS is well known, the same does not apply to BRCA1/2 mutation carriers; several efforts have been made to evaluate the lifetime susceptibility of BRCA1/2 mutation carriers to develop CRC and have yielded conflicting results.

Mauri *et al*[27] suggested that BRCA1/2 mutations might determine an increase in CRC diagnoses, particularly in young patients.

Another study of Kim *et al*[28] suggested that even first and second-degree relatives of high-risk BRCA mutated (BRCAm) breast cancer patients are at increased risk of

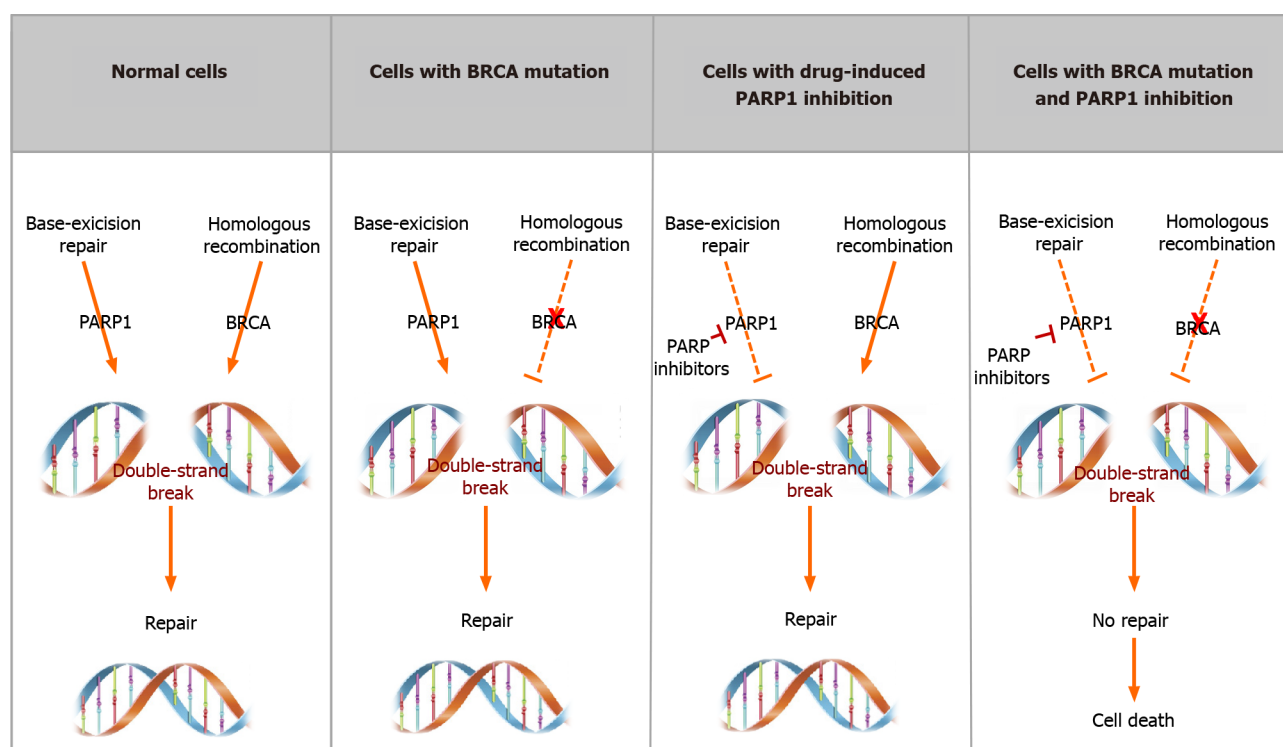


Figure 1 BRCA, PARP-inhibitors and synthetic lethality in gastrointestinal cancers.

non-breast/ovarian cancer.

Phelan *et al*[17] conducted a multicentre prospective study involving a cohort of 7105 female patients with the aim of assessing the CRC incidence in women carrying pathogenic BRCA1/2 mutations. Enrolment was conducted in fifty centres among five countries in Canada, United States and Europe, from 1992 to 2010; the median follow-up was 5.5 years.

When newly diagnosed CRC were matched with population-specific incidence rates, no statistically significant differences were found: Authors reported 21 new CRC diagnoses between mutation carriers, while 23.6 cases were expected. Among them, 16 cases occurred in BRCA1m (*vs* 17.4 expected), and 5 cases in BRCA2m (*vs* 6.1 expected). Although this study failed to show a higher prevalence in all mutated patients, subgroup analysis of BRCA1 carriers showed that early-onset (30-49 years) CRC diagnosis was four times greater than controls. The large population of patients enrolled and the prospective design of this study support these findings; however, the lack of male patients narrows these results to only the female population. Moreover, the relatively small number of incident cases and the ability to retrieve only 2/3 of the pathological reports, may represent another limitation of the study.

Similarly, Oh *et al*[29] presented a meta-analysis of 14 out of 18 studies initially selected, as to estimate the CRC risk in BRCaM carriers. Using a random-effects model, the authors observed a statistically significant increase in the odds of CRC in BRCaM carriers [odd ratio (OR) = 1.24, 95%CI: 1.02 to 1.51,  $P = 0.03$ ]. When the authors focused on specific BRCA1 or BRCA2 carriers, they highlighted that BRCA1 carriers were at a higher risk of developing CRC. On the other hand, they did not observe a higher risk of early onset CRC as previously stated by Phelan *et al*[17].

In contrast, in their systematic review and meta-analysis Cullinane *et al*[30] did not observe any statistically significant difference in terms of CRC risk among BRCaM carriers, regardless of the gene involved. Neither did adjustment for age modify these findings, nor when the analysis was conducted only in the Ashkenazi Jewish heritage population.

It is well established that BRCA1/2 mutations incidence is broader in some ethnic groups, such as the Ashkenazi Jewish population; in this ethnicity there is a high population frequency (approximately 2%) of three founder mutations: BRCA1 185delAG, BRCA1 5382insC, and BRCA2 6174delT[31]. Kirchhoff *et al*[31] collected data from 586 Ashkenazi Jewish patients with pathologically confirmed diagnosis of CRC and from 5012 healthy subjects (controls), to assess whether having one of the three founder mutations could translate into an increased CRC risk. Mutations

incidence in the two groups was matched and after adjusting for age at diagnosis and sex, it was observed that harbouring BRCA1/2 mutations was not associated with higher CRC risk[31].

Similarly, Niel *et al*[32], assessed whether positive breast cancer family history could represent a risk factor for developing other tumour types. The authors performed a case-control study comparing data from 1422 patients with pathologically confirmed CRC, coming from five major hospitals in northern Israel, with 1585 control subjects. They observed that neither BRCA1/2 founder mutations nor breast cancer family history were significantly correlated with increased CRC risk.

Another interesting topic to analyse is whether BRCA-related CRC could be characterised by peculiar clinico-pathological features. Xu *et al*[33] described CRC phenotype differences among suspected LS patients with MMR and BRCA/BRCA-like carrying variants. Among 22833 patients receiving radical surgery for cancer at Fudan University Shanghai Cancer Center, 202 underwent multigene testing covering 139 genes: 42 were carriers of a pathogenic MMR-gene variant, while 20 carried BRCA or a BRCA-like variant. The most relevant differences between the two groups of patients were that the mean CRC age at diagnosis was lower in the MMR group (44.95 years *vs* 56.45 years); furthermore, BRCA/BRCA-like variants were associated with a significantly lower percentage of poorly differentiated CRC (5% *vs* 33%) and lower metachronous CRCs. Moreover, tumour sidedness was different between the two groups, with more left sided colon cancer diagnoses in MMR-gene variants, whereas extra-CRC was significantly higher in BRCA/BRCA-like families. Finally, supposed LS patients carrying BRCA and BRCA-like variants had a longer progression free survival (PFS) compared with the others[33].

Based on histological and molecular features, CRC is actually a heterogeneous group of tumours, characterised by different prognosis and response to medical treatment. Mucinous carcinoma (MC) and adenocarcinoma (AC) are the two most common histological subtypes, accounting for 10%-15% and 85%-90% of cases respectively. In particular, MC is usually associated with worse prognosis: It is more frequently diagnosed in young patients and it is usually diagnosed later than AC, due to the late occurrence of symptoms; most of these tumours occur in the proximal colon. MC is also described as having quicker disease progression. Harpaz *et al*[34] in their paper assessed the correlation between MC and BRCAm CRCs.

One thousand one hundred thirty-four mCRCs were analyzed: A statistically significant higher incidence of BRCA1/2 mutations in MC than in AC patients was observed (14.8% *vs* 4.1%, respectively). Moreover, when considering the “mutation count”, defined as “somatic non-synonymous variants in encoding genes by exome sequencing” for each sample supplied by the database, they also observed that somatic BRCAm CRC has a higher mutation count than wild-type BRCA (BRCAwt); mucinous histology was also the strongest predictor of mutation count, independently of MSI as well as other variables too.

The authors conducted a prospective case-control study to confirm the results of their previous retrospective study: MC and AC CRC patients treated at Hadassah Medical Center in Jerusalem were prospectively enrolled. Thirty out of 53 MC cases were included, together with 40 controls: Authors observed a higher frequency of BRCAm patients in MC (40% *vs* 27%, respectively,  $P = 0.2705$  by chi-squared test). The difference was not statistically significant. Lack of concordance with their previous results might be due to the small cohort and to other limitations: In particular, mutation analysis revealed that variants frequently seen in MC histology such as KRAS, BRAF and PI3K mutations were not observed in this cohort of patients. However, analyzing the BRCA2m group, they noted a trend toward higher frequency of mucinous histology. Moreover, they confirmed the higher tumour mutational burden (TMB) in BRCAm compared to BRCAwt carriers.

While the effects of BRCAm are well known in females, studies focused on males are rarer; indeed, it has been hypothesised that male carriers of BRCA mutations are more likely to develop several types of malignancies, including melanomas, breast, prostatic and pancreatic cancer. However, clinicians used to test far fewer male than female patients for BRCAm, thus limiting our actual knowledge of clinic-pathological and prognostic implications of these kinds of tumours.

Sun *et al*[35] conducted a retrospective pan-tumour survey to identify tumour characteristics in BRCA1/2 male mutation carriers; clinical data of mutated male patients was compared with female patients carrying the same mutation, as well as BRCAwt male patients. Results of the analysis showed that CRC incidence was higher in BRCAm males than in BRCAm female patients and non-BRCAm males. In particular, they found that deleterious mutations in the BRCA2 gene were most frequent in CRC patients. Moreover, women carrying BRCA mutations have better overall



survival (OS) and PFS than BRCAm males, principally due to the differences in tumour types and the therapeutic chances. Finally, males with deleterious BRCAm have increased OS compared to non-BRCAm.

### **Emerging therapeutic options in BRCA-mutated CRC**

Five-years OS rate for patients with mCRC remains poor, around 14%. Standard chemotherapy for these patients includes 5-fluorouracil (5-FU) in association with irinotecan (FOLFIRI), oxaliplatin (FOLFOX) or both (FOLFOXIRI). These regimens are usually associated, particularly in first and second-line settings, with anti-EGFR or anti-VEGF targeted drugs, on the basis of tumour BRAF/RAS mutational status[36].

It has been previously described that BRCA1/2m cancers should have increased sensitivity to platinum compounds; on this basis, a few published case reports suggest that BRCA1/2 mutational testing, particularly in younger CRC patients might guide treatment selection in earlier settings of the disease. Soyano *et al*[37] described the case of a young man with locally advanced rectal cancer [T3N1M0 according to tumour-node-metastasis (TNM) staging]: He was tested for BRCA1/2 mutation and was found to be a BRCA1m carrier. After multidisciplinary evaluation, he started standard chemoradiotherapy with capecitabine as a radiosensitizer; however, when a BRCA1 mutation was found, oxaliplatin was added to standard chemoradiotherapy. It was also decided to opt for a total neoadjuvant strategy as in giving additional two cycles of chemotherapy (mFOLFOX6), after the end of chemoradiotherapy, just before surgery. The pathological report after surgery revealed complete pathological response.

Likewise, Lin *et al*[38] described the case of a 25 years old man affected with locally advanced (cT3N2M0) rectal cancer who had an excellent response to oxaliplatin-based chemotherapy. The patient was due to receive neoadjuvant concurrent standard radio-chemotherapy, but given the increased risk of radiation-induced infertility he refused any radiotherapy; 4 cycles of chemotherapy 5-FU and oxaliplatin-based (FOLFOX6) were then administered. After surgical primary tumour resection performed by laparoscopic total mesorectal excision (TME), the histological report showed complete response. Owing to this excellent but unexpected reaction to oxaliplatin-based treatment, the authors decided to perform next-generation sequencing in both DNA samples taken from the primary tumour sample and the peripheral blood sample; somatic BRCA2 variant was found, as well as high TMB.

This data suggest that the assessment of BRCA-status might be performed when a particularly deep response to platinum-based chemotherapy is reached.

It has already been explained how, in cells that harbour HR system mutations leading to defective DNA double-strand break repair, as in BRCAm patients, genome stability is maintained through the activity of BER and PARP enzymes. However, Paviolo *et al*[39] demonstrated that in PARPi-treated BRCA-defective CRC samples, even though DSBs are generated, they are not directly responsible for cell death. Indeed, more than DSBs, it was proven that rapid and anomalous repair of these defects, through means of DNA repair mechanisms different from HRD, leads to severe chromosomal aberrations which are the cause of cell death.

In clinical practice, several PARPi, such as Olaparib, Niraparib, Rucaparib, are widely used in treating ovarian, breast, pancreatic and prostate cancer harbouring BRCA mutations or HRD signature, and they are under investigation in many other neoplasms.

Veliparib (ABT-888) was the first PARPi to be studied in CRC treatment: Prior works demonstrated its efficacy in BRCA-deficient compared to proficient cells and proved that combining platinum-based compounds with PARPi provided increased response[40]. Harpaz *et al*[34] described the use of Veliparib as maintenance therapy in a patient carrying a germline pathogenic BRCA1m, affected by metastatic KRAS and BRAF wild type rectal adenocarcinoma with pelvic and lung metastases. He underwent first-line chemotherapy with FOLFOX and Panitumumab, and obtained maximal response. Then he started maintenance veliparib, for as long as 2 years; treatment was then stopped after occurrence of new metastases.

Niraparib, a potent and selective PARP1/2i, has shown *in vitro* and *in vivo* efficacy in BRCA mutant cell lines; sensitivity to Niraparib monotherapy is 10-fold in BRCA mutant cell lines compared to BRCAwt cells[41].

A phase 2, multicentre, open-label study evaluating Rucaparib, an orally available PARPi, in patients with unresectable, locally advanced or metastatic solid tumours with deleterious mutations in HR repair genes is currently still recruiting; the trial enrolls patients with either somatic or germline variants and BRCA1 and BRCA2 mutations are allowed. (NCT04171700). Primary outcome is the best overall response rate[42].



In addition to that, a clinical trial evaluating the use of immune checkpoint inhibitors in solid tumours with BRCA mutation is still ongoing[43] (Table 1).

Despite these premises, at the moment there is still no evidence-based data that supports the use of these drugs in CRC patients; because of this it is suggested that PARPi-based clinical trials should be enforced in BRCAm carriers to further validate their use in this setting.

Furthermore, whole-genome sequencing in BRCA1/2m patients allowed identification of different signatures correlated with BRCA status; this BRCA-ness signature was present even in cells without BRCA1/2 mutations, suggesting that the signature might be more likely to predict HRD compared with simple assessment of BRCA mutational status[40]. Should the signature be more widely used, it can be expected that it will be tested to predict PARPi sensitivity.

## GC

Worldwide, GC is one of the most common malignancies and the third leading cause of cancer-related mortality[44]. Approximately 8%-30% of patients have a positive family history[45], but only 1%-3% of GCs are truly hereditary[46]. GC predisposition has been linked to familial cancer syndromes, including hereditary diffuse GC[47], LS [48], Peutz-Jeghers syndrome[49], Li-Fraumeni syndrome[50], familial adenomatous polyposis syndrome[51], adenocarcinoma and proximal polyposis syndrome of the stomach[52].

The association between germline mutation of BRCA genes and risk of GC was investigated in several studies, although some aspects remain unclear.

Historically, carriers of germline BRCA1/2 mutations have shown a four to six-fold increased risk of developing GC compared to the general population[53,54] and the risk appears to be higher in patients with BRCA1 mutations than in patients with BRCA2 mutations[54,55].

Regarding BRCA2 carriers, the Polish study by Jakubowska *et al*[56] assessed the importance of a family history of GC to predict the presence of BRCA2 mutations in ovarian cancer patients. In this study, BRCA2 mutation was found in 8 of 34 women with ovarian cancer and a family history of GC *vs* 3 of 75 women with OR and a family history of ovarian cancer, but not of GC (OR = 7.4; 95%CI: 1.8-30; *P* = 0.004); this finding would confirm that GC is a BRCA2-related tumour.

Remarkably, a study conducted by the same group of authors, suggested that founder BRCA1 mutations reported in Polish breast/ovarian cancer patients do not contribute to increased GC risk[57].

Furthermore, Lorenzo Bermejo *et al*[58] demonstrated that GC before the age of 70 was twice as frequent in families with breast and ovarian cancers as in the general population; similarly, Schlebusch *et al*[59] found a high prevalence of GC in the BRCA2-positive families compared with the general population.

Nevertheless, in a study performed in the Netherlands, 139 BRCA2 families with 66 different pathogenic mutations were analysed and significantly increased risk of developing GC was not observed[16].

These conflicting data could be partially justified by the high prevalence of Ashkenazi Jews in the studies of Schlebusch *et al*[59] and Jakubowska *et al*[56]; in fact, ovarian, pancreatic and gastric cancer as well as non-Hodgkin's lymphoma have a higher incidence rate in the Ashkenazi population[60].

To further assess the impact of founder mutations in the Ashkenazi population, Figer *et al*[61] focused on a founder BRCA2 variant that is present in about 1.5% of the general Ashkenazi population and rarely in non-Ashkenazi Jews. In order to evaluate the contribution of this mutation to non-CRC GI cancer, they tested 70 consecutive unselected Ashkenazi Jews with GI malignancies that carried this specific mutation. They concluded that the rate of the Ashkenazi Jews with BRCA2 founder mutation in patients with GC was 5.7%, approximately five times higher than the general population, supporting the hypothesis of an increased risk of GC in BRCA2-carriers[46].

It has previously been reported that GC in BRCA2 carriers may be sex-related, as it seems to be more frequent in males[6,58]; in an interesting article, Cavanagh *et al*[62] argue that women carrying BRCA1/2 mutations usually develop early-onset breast and ovarian cancer and therefore may not survive long enough to develop GC at an older age; this would explain the high prevalence of GC (and other cancers) in male BRCA-carriers.

Regarding BRCA-associated tumours in males, Sun *et al*[35] recently conducted a retrospective pan-tumour survey on 346 cases of BRCA-associated tumours including

**Table 1 Ongoing trials including patients carrying gastro-intestinal cancer with BRCA1m and/or BRCA2m and solid tumours with BRCA1m and/or BRCA2m**

	Study title	Design	Status	Trial description
NCT03337087	Liposomal irinotecan, fluorouracil, leucovorin calcium, and rucaparib in treating patients with metastatic pancreatic, colorectal, gastroesophageal, or biliary cancer	Phase I	Recruiting	Experimental: nal-IRI, leucovorin, fluorouracil, rucaparib
NCT02286687	Talazoparib in treating patients with recurrent, refractory, advanced, or metastatic cancers and alterations in the BRCA genes	Phase IIa	Recruiting	Experimental: Talazoparib
NCT03428802	Pembrolizumab in treating participants with metastatic, recurrent or locally advanced cancer and genomic instability	Phase IIa	Recruiting	Experimental: Pembrolizumab
NCT04503265	A trial of AMXI-5001 for treatment in patients with advanced malignancies	Phase I-IIa	Recruiting	Experimental: AMXI-5001 (oral PARP and microtubule polymerization inhibitor)
NCT04171700	A study to evaluate rucaparib in patients with solid tumors and with deleterious mutations in HRR genes (LODESTAR)	Phase IIa	Recruiting	Experimental: Rucaparib
NCT03415659	Phase I clinical study of HWH340 tablet in patients with advanced solid tumors	Phase I	Recruiting	Experimental: HWH340 monotherapy
NCT02723864	Veliparib (ABT-888), an Oral PARP inhibitor, and VX-970, an ATR inhibitor, in combination with cisplatin in people with refractory solid tumors	Phase I	Active, not recruiting	Experimental: VX-970 at Days 2 and 9 of each 21-d cycle; veliparib twice a day (BID) days 1-3 and 8-10 of each cycle; cisplatin at day 1 (and day 8 from DL3 onwards) of each cycle
NCT00516373	A study to assess the safety and pharmacokinetics of an inhibitor of Poly ADP-Ribose Polymerase-1 (PARP)	Phase I	Active, not recruiting	Experimental: KU-0059436 (oral PARP inhibitor)
NCT04439227	Testing AZD1775 as a potential targeted treatment in cancers with BRCA genetic changes (MATCH-Subprotocol Z1I)	Phase II	Active, not recruiting	Experimental: Adavosertib
NCT03565991	Javelin BRCA/ATM: Avelumab plus talazoparib in patients with BRCA or ATM mutant solid tumors	Phase II	Active, not recruiting	Experimental: Combination of avelumab and talazoparib
NCT01482715	A study of oral rucaparib in patients with a solid tumor (Phase I) or with gBRCA mutation ovarian cancer (Phase II)	Phase I-IIa	Completed	Experimental: Rucaparib
NCT01989546	Pilot trial of BMN 673, an oral PARP Inhibitor, in patients with advanced solid tumors and deleterious BRCA mutations	Phase I-IIa	Completed	Experimental: Talazoparib (BMN 673)
NCT01286987	Study of Talazoparib, a PARP Inhibitor, in patients with advanced or recurrent solid tumors	Phase I	Completed	Experimental: Talazoparib
NCT03767075	A modular multi-basket trial to improve personalized medicine in cancer patients (BoB)	Phase II	Recruiting	Experimental: Atezolizumab

nal-IRI: Liposomal Irinotecan; PARP: Poly-ADP ribose polymerase; ATR: Ataxia telangiectasia and Rad3-related protein; ATM: Ataxia telangiectasia mutated; BID: Bis in die; DL3: Dose level 3; BoB: Basket of baskets.

GC in males and a comparative analysis among male and female BRCA carriers (349 cases), as well as in male patients who were not BRCA carriers (4577 cases); similar incidences of BRCA mutations (6.0% *vs* 6.6%) and age at diagnosis of BRCA-related tumour (median, 65 *vs* 60 years) were observed both in male and female patients.

Moreover, when evaluating both OS and PFS in patients who developed GC, interesting differences were observed: Compared with females, BRCA mutations in males were associated with decreased OS and PFS; however, subgroup analysis demonstrated that BRCA mutation was associated with increased OS in GC (HR for OS: 0.60  $P = 0.05$ ). Considering the limited data about the outcome of gastric cancer in BRCA carriers, these results are of particular note. However, data regarding the prognostic impact of BRCA pathogenic variants in GC patients are still scarce.

Another study supporting these results was conducted by Halpern *et al* [63], where ten GC patients with BRCA mutations were assessed; 6/10 patients had metastatic disease. Median OS of all ten GC patients was 47.5 mo. Median OS for patients diagnosed with operable disease was 55.5 mo and was 32 mo for patients with metastatic disease. Particularly, patients with metastatic disease have a 1-, 2- and 3-

year survival rate of 100%, 83.3% and 50%, respectively. Albeit the number of patients included in the analysis is rather limited, it is noteworthy, as it seems to support the idea that BRCA mutations might be associated with better survival: Nowadays median OS of patients with metastatic gastric cancer in western countries is usually around 10-12 mo.

In order to clarify the real incidence and outcome in BRCA patients with GC, a trial is currently active; in this prospective trial the investigators aim to evaluate the incidence of BRCA loss in patients with advanced GC and to observe the treatment outcome and the possibility of BRCA loss as a predictive and prognostic factor[64].

As previously discussed, existing clinical studies have suggested that some BRCA-associated tumours are sensitive to PARPi[18]. Therefore, a phase I/II study investigating side effects and best dose of liposomal irinotecan and rucaparib when given together with fluorouracil and leucovorin calcium is currently available for BRCA patients with pancreatic, colorectal, gastroesophageal or respiratory cancer biliary.

Other basket trials available for patients with BRCA mutations are summarised in Table 1.

Finally, several cancers in BRCA carriers have been shown to be particularly sensitive to platinum-based chemotherapy[65,66], but studies evaluating the chemosensitivity of patients with BRCA germline mutations and GC are not available.

However, previous clinical and preclinical studies have reported an association between a low level of BRCA1 expression or a BRCA1 mutation and the chemosensitivity and prognosis of sporadic GC.

According to the American Joint Committee on Cancer, patients with a negative-BRCA1 sporadic GC are more likely to have a high grade of tumour, a high TNM stage and a poorly differentiated tumour[53,67]; at the same time, other studies have revealed that BRCA1negative sporadic GC are more sensitive to platinumbased adjuvant chemotherapy compared with BRCA1positive sporadic GC[68]. These findings indicate that patients with BRCA1negative GC have a longer OS time and improved prognosis, which suggests an important association between BRCA1 expression and platinumbased chemotherapy.

## OEC

OeC is the seventh most common cancer and the sixth leading cause of cancer-related deaths worldwide[69]. The incidence rate of OeC varies considering the location; particularly high frequency is present in East Asia and Eastern/Southern Africa, where oesophageal squamous cell carcinoma (SCC) represents the main histology, while adenocarcinoma is more common in Western countries[70]. Most cases of OeC are sporadic and caused by somatic mutations[71] and oesophageal lesions are rarely described in hereditary CRC syndromes as LS or familial adenomatous polyposis (FAP). However, several studies reported the occurrence of OeC in patients with attenuated FAP or Gardner's syndrome, which are both caused by mutations in the APC gene[72]. The association between BRCA1/2 germline mutations and OeC has been explored in some studies.

Moran *et al*[73], in a family-based study, observed a relative risk increase of OeC (regardless of histology) (relative risk 2.9, 95%CI: 1.1-6) in families with BRCA1 mutations.

Conversely, BRCA2 carriers seem to have a higher risk of developing SCC than Adenocarcinoma.

In fact, contribution of BRCA2 mutations for the development of SCC has been reported in high- and low-risk Chinese populations, in the Turkmen population of Iran and in the very high risk region of northeast India. Specifically, Hu *et al*[74] have reported five different BRCA2 mutations in 6 out of 44 (13%) patients with SCC in high-risk Chinese population, while Akbari *et al*[75] observed a nonsense BRCA2 pathogenic gene variant in eight SCC cases with a family history of OeC in the Turkmen population of Iran. Instead, Kaushal *et al*[76] have investigated 317 cases of SCC in a high-risk region of India and they conducted, performing BRCA2 gene germline mutations, screening in 20 familial and 80 non-familial SCC patients: They found non-synonymous BRCA2 variants in 3 out of 20 patients with familial SCC, while no sequence alterations were found in 80 non-familial SCC cases. Moreover, a study to screen mutations of BRCA2 gene in 47 SCC patients from a low-risk Chinese population was conducted by Zhong *et al*[77]. They found 9 germline missense point mutations in apparently sporadic male patients, with a mutation frequency of 19%. In addition to that, when an additional cohort of 94 healthy controls underwent screening

for the 9 mutations previously identified in SCC cases, only 2 positive individuals (mutation frequency 2%) were found.

Finally, a recent, large-scale study by Ko *et al*[78] included 4517 individuals: 186 familial SCC patients from a high risk region of China, while the rest were healthy East Asian individuals (3289 Henan and 1228 moderate-risk Hong Kong Chinese). They identified BRCA2 Loss-of-function mutations in 3.23% (6/186) familial SCC patients compared to 0.21% in the East Asians (OR = 15.89,  $P = 2.48 \times 10^{-10}$ ).

Regarding the therapeutic implications between BRCA carriers and OeC, few data are still available in the literature. However, encouraging preclinical studies would show that PARPi in combination with a DNA damaging agent might be beneficial in this setting[79]. Furthermore, the PARPi olaparib appears to sensitise OeC cells to fractionated proton irradiation[80].

## DISCUSSION

Since the discovery in the mid-nineties of both *BRCA1/2* genes and their impact on breast and ovarian cancer risk, both surveillance and risk-reducing surgical procedures have been developed as effective means to reduce the risk of developing both malignancies and to overall reduce the risk of death in carriers of these variants. However, recently, BRCA carriers have been recognised to be at risk of developing other kinds of cancer types, such as prostate, pancreatic cancer and melanoma[10-14].

GI cancers are a heterogeneous group of malignancies that, taken together, represent the most common tumour type worldwide. Most tumours of this group are mainly due to environmental and lifestyle risk factors, while the weight of hereditary predisposition is rather low (usually around 5%-10% of all cancers). LS represents the most common form of hereditary predisposition to these kinds of tumours (mainly for CRC), while the role of *BRCA1/2* mutations is less understood.

In our review we tried to summarize the current published evidence on the role of *BRCA1/2* pathogenic variants in colorectal, gastric and OeC risk and prognosis.

As for CRC, there is no definitive consensus regarding increased risk of development of this malignancy: Some studies[17,29] seem to suggest that there might be an increased incidence of CRC in BRCA mutation carriers, particularly for *BRCA1* carriers. In those studies where a positive correlation with increased risk of CRC development was proven, a trend towards younger age of onset was also suggested. On the other hand, other studies[30,31] have failed to confirm these findings, also in selected high-risk populations where the presence of BRCA variants is higher than in the general population (such as Ashkenazi Jews). Both these studies have some limitations that make direct comparison impossible: In the study of Phelan *et al*[17] the population consists entirely of female carriers, thus narrowing their findings to women. Conversely, the negative results of the studies of Kirchhoff and Bethany, conducted in the high-risk Ashkenazi Jewish population, might be influenced by the relatively higher risk of developing CRC in this ethnic group compared to people of other ethnicities[31,32,81].

While there is no consensus on the impact of germline *BRCA1/2* mutations on CRC risk, their prognostic role is more clearly defined: A series of studies have shown how, in patients with CRC, BRCA mutations might increase the likelihood of response to chemotherapy. The evidence is, however, mainly derived from case reports or small retrospective case-control studies where patients are treated with oxaliplatin-based chemotherapy. All these studies suffer from selection biases, as only patients who had particularly significant responses to oxaliplatin-based treatment (and that should have more favourable prognosis), underwent genetic testing; there is a general lack of information concerning whether BRCA mutations may influence CRC prognosis regardless of treatment received. It is hoped that, in the near future, wide screening with multi-panel molecular testing in CRC patients will give us a clearer estimate of the real prevalence and prognosis of BRCA mutations; as multi-panel molecular testing will be used more frequently as a means to identify those patients who will be eligible to receive specific targeted drugs, and we will be able to identify more accurately people that might be carriers of germline BRCA mutations that would not be suspected on the basis of their family history.

The role of BRCA mutations in gastric cancer seems to be more clearly defined compared to CRC: A few studies have indicated an increased risk of gastric cancer in families with either *BRCA1* or *BRCA2* mutations. Even though few papers[54] suggest higher risk in *BRCA1* carriers compared to *BRCA2*, most papers seem to agree that *BRCA2* mutations are more clearly associated with an increased GC risk[56,58,59]. As



well as in CRC, most studies that have shown a positive impact of BRCA mutations on GC risk have also shown an increased likelihood of early onset (< 70 years old) GC.

As stated before, all those studies that have enrolled specific ethnic groups that have a higher risk of harbouring BRCA mutations (such as Ashkenazi Jews) have met with negative results; however, when the analysis was conducted by taking into account this factor, BRCA mutations maintained their causative role. Interestingly, most data that have focused on the prognosis of GC patients harbouring BRCA mutations agree on a positive prognostic role: This might partly be due to the fact that standard treatment of gastric cancer patients includes platinum compounds; it would be interesting to assess whether GC patients who harbour BRCA mutations and that do not receive any kind of platinum-based treatment (as those treated with fluoropyrimidines alone) retain the same positive prognosis.

Surprisingly, although OeC is usually considered as a cancer type where environmental and lifestyle factors predominantly influence the risk of the onset of disease, a lot of published evidence can be found concerning the role of BRCA mutations in this disease. Out of the two main histologies, SCC seems to be much more closely associated with BRCA mutations compared with adenocarcinoma. All papers seem to suggest that BRCA2 mutations are more frequently observed in this tumour type compared with BRCA1 mutations. Although studies that focused on the prognostic or predictive role of BRCA1/2 are lacking, it is expected that, based on the standard treatment options for this kind of disease that include platinum compounds and radiotherapy, more favourable outcome for patients who are carriers of these mutations can be expected.

The retrospective nature of these studies represents a major limitation; it is however interesting to notice that most of them indicate a positive correlation of germline BRCA1/2 mutations and an increased GI cancer risk. BRCA1/2 variant assessment will soon become one of the most promising fields of research in these kinds of diseases: Most of these tumours are treated with platinum compounds (either Oxaliplatin or Cisplatin) and it is expected that BRCA mutation carriers should have greater likelihood of response to treatment compared to wild type BRCA individuals. Furthermore, as in pancreatic cancer where PARPi have now become the standard care for patients with BRCA germline mutations[20], we can expect that the same might apply to other cancers arising from other areas of the GI tract: Indeed, PARPi are currently being evaluated in a wide number of clinical trials (Table 1).

In addition to that, it is actually recognised that, in CRC, GC and OeC, completely different clinical presentations are seen: While older patients have experienced in the last decade an improvement in survival (mainly due to introduction of screening procedures and advances in adjuvant therapy[36], younger patients are still those whose survival has not changed; most papers that have focused on BRCA1/2 mutations came to the conclusion that BRCA germline carriers are those who have the highest likelihood of early onset GI presentation. We believe that further research on early onset GI cancer might identify a greater number of patients where germline defects rather than environmental risk factors are the main causes of disease; this might also be associated with differences in treatment response. Moreover, it would advocate different surveillance procedures, as younger patients are not usually taken into account in most screening programmes.

## CONCLUSION

In conclusion, we believe that this review has highlighted the importance of BRCA1/2 germline mutations as risk factors in GI malignancies and has drawn attention to future applications of this knowledge in clinical practice.

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