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Review

Apoptosis signaling proteins as prognostic biomarkers in colorectal cancer: A review

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ABSTRACT

Colorectal cancer is a leading cause of cancer related mortality in the Western world. In recent years, combination 5-fluorouracil based adjuvant chemotherapy as first line treatment of this disease has led to improved disease free and overall survival. However drug resistance, both innate and acquired, remains an obstacle in the effective treatment of this disease. Apoptotic pathways are frequently altered in both tumor progression and drug resistance; therefore proteins associated with this pathway may have potential as prognostic biomarkers for this disease. Identification of clinical biomarkers that are able to identify patients who are more likely to respond to specific chemotherapy will lead to more personalized, effective, and less toxic therapy. This review focuses on the current status of apoptosis related proteins as biomarkers for colorectal cancer and discusses the possible application of systems approaches in this context.

Contents

1. Introduction

1.1. Current prognosis of colorectal cancer

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer related mortality in the Western

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world. The current standard of care for colorectal cancer patients is primarily dictated by disease stage. When diagnosed at an early localized disease stage (Dukes A), patients undergo curative surgical resection. Dukes B patients, in which there is localized spread with no lymph node involvement, are treated surgically with or without 5-fluorouracil (5FU) based adjuvant chemotherapy. The 5-year survival of Dukes B patients undergoing surgical resection alone is approximately 75%, indicating that only/maximal 25% of Dukes B patients may potentially benefit from adjuvant chemotherapy. In the advanced disease setting, in which there is lymph node involvement (Dukes C) or metastasis to other organs (Dukes D), the current treatment paradigm is surgical resection followed by adjuvant 5FU-based chemotherapy. As monotherapy, the anti-metabolite 5FU has response rates of approximately 10–15%, recently however response rates and disease-free survival have improved due to shifts in treatment paradigms to 5FU-based combination therapy, namely with the platinum agent oxaliplatin [1,2] or the topoisomerase I inhibitor, irinotecan (CPT-11) [3].

Currently there are no clinically-used/routine biomarkers which accurately predict whether colorectal cancer patients will or will not respond to adjuvant chemotherapy. Therefore there is an urgent need for the determination of useful prognostic markers at both the pathological and biochemical/molecular level. Identification of such biomarkers is particularly important for the stratification of Dukes B patients as the benefit of adjuvant chemotherapy in this group of patients is not clearly defined [4].

1.2. Biomarkers: where we stand now

The key prognostic indicator for colorectal cancer is tumor staging, with other possible indicators for disease prognosis including microsatellite instability (MSI) [5–8], anatomic location of tumor [9], and DNA content [7]. In 2002, Peterson et al. identified four pathologically defined markers to potentially be used in stratifying Dukes B patients for relapse following surgical resection, and therefore would benefit from adjuvant chemotherapy [10]. These pathological markers were defined as peritoneal spread, venous spread, surgical margin spread, and tumor perforation. Each were scored to generate a prognostic index (PI) with patients classified as low risk (PI=0 or 1) or high risk (PI≥2) for recurrence. The effectiveness of this method of stratification of Dukes B patients was recently shown to be limited due to often inadequate pathological reporting [11], indicating an ever pressing need for the identification of useful biochemical markers.

Current chemotherapy regimens for colorectal cancer consist of classical cytotoxic chemotherapeutic agents, however this is poised to change as discovery of disease biomarkers may represent novel therapeutic targets. Categorizing patients for therapy based on the presence or absence of certain markers may ultimately lead to more individualized therapies which are more effective and less toxic. Genes and/or proteins which may be potential response biomarkers that are frequently determined in the *in vitro* preclinical setting and are often identified as knowledge of drug mechanisms of action and/or resistance are determined. Accordingly insight into drug action and resistance, along with knowledge of disease progression often has served as a spring board for translational studies aiming to determine their effectiveness as response biomarkers. In colorectal cancer, several such biomarkers have been identified for 5FU and oxaliplatin response, namely thymidylate synthase [12,13], thymidine phosphorylase [14,15], dihydropyrimidine dehydrogenase [16] and ERCC-1 (excision repair cross-complementing 1) [13].

More recently, advances in technology (i.e. DNA microarrays) have provided additional insight into disease progression as well as into mechanisms of drug action and resistance. Preclinical microarray studies aimed at further understanding the mechanisms of action and resistance to traditional chemotherapies have identified markers which may represent novel drug targets or play a role in determining colorectal cancer prognosis [17–19]. Clinical studies of other malignancies have also identified 'gene signatures' which may be important in diagnosis and treatment selection [20,21].

This review will focus on the current status of prognostic markers for colorectal cancer, with a focus on apoptosis associated biomarkers.

2. Apoptosis resistance and colorectal cancer

5FU, oxaliplatin and irinotecan exert their cytotoxic effects via the induction of the DNA damage response, which leads to cell cycle arrest and/or the induction of apoptosis. Apoptosis occurs through the intrinsic and extrinsic pathways (Fig. 1), both of which can be induced by these chemotherapeutics.

The intrinsic or mitochondrial pathway is characterized by the release of cytochrome *c* from the mitochondria leading to the activation of caspases, a family of cysteine proteases. This process is controlled by the BCL-2 family of proteins. While having different molecular functions, all the Bcl-2 family proteins share sequence homology in varying numbers of the alpha-helical **B**cl-2 **h**omology, or **BH**, domains. The anti-apoptotic Bcl-2 like proteins are the only family to contain 4 BH domains (BH1–4) with the pro-apoptotic Bax family of proteins and the BH3-only proteins sharing sequence homology in the BH1–3 and BH3 domains respectively. Apoptotic signaling triggers a conformational change in the pro-apoptotic Bax and Bak proteins which allows them to insert into the outer mitochondrial membrane, causing the release of cytochrome *c* and other pro-apoptotic molecules. Activation of Bax and Bak and subsequent mitochondrial membrane permeabilization is modulated by the Bcl-2 family of proteins and the pro-apoptotic BH3 only families of proteins. BH3 only proteins bind to and inhibit the pro-survival Bcl-2 like family members and may potentially also directly activate Bax and Bak. The release of cytochrome *c* from the mitochondria triggers the activation of the initiator caspase 9, which goes on to activate the effector caspases (caspases 3, 7).

The extrinsic apoptotic pathway is triggered through the activation of death receptors on the cell membrane and can activate caspases partially independent of the mitochondria. In this pathway, activation of death receptors [Fas, Death Receptors 4 and 5 (DR4, DR5)] by their respective ligands [FasL and TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand)] leads to recruitment of the adaptor molecule FADD (Fas associated death domain) which in turn results in the activation of caspase 8 which in turn activates the effector caspases [22]. Caspase 8 activation also leads to the cleavage and activation of the BH3 only protein Bid, linking the extrinsic to the intrinsic apoptotic pathway (Fig. 1). Both the extrinsic and intrinsic apoptotic pathways are tightly regulated and can also be controlled by the inhibitor of apoptosis (IAP) proteins (Survivin, XIAP, cIAP1 and cIAP2). IAP proteins inhibit the enzymatic activity of caspases and also trigger their proteasomal degradation. The mitochondrial release of the pro-apoptotic SMAC/DIABLO protein during apoptosis can overcome this block.

Defects in the apoptotic pathway have the potential to confer a survival advantage to cells, contributing to the malignant phenotype. Importantly, alterations in the expression of and mutations in apoptosis associated proteins represent important mechanisms for tumors to become chemoresistant. This chemoresistance may be inherent leading to lack of response, or maybe acquired during the course of treatment leading to disease recurrence. The key role of the apoptotic pathway in cancer progression and response to therapy therefore indicates that associated proteins have potential as cancer prognostic biomarkers. New targeted drug therapies aimed at interfering with apoptotic signaling have been developed and have been examined in preclinical studies and tested in clinical trials. TRAIL agonist and activating antibodies, Bcl-2 antagonists/

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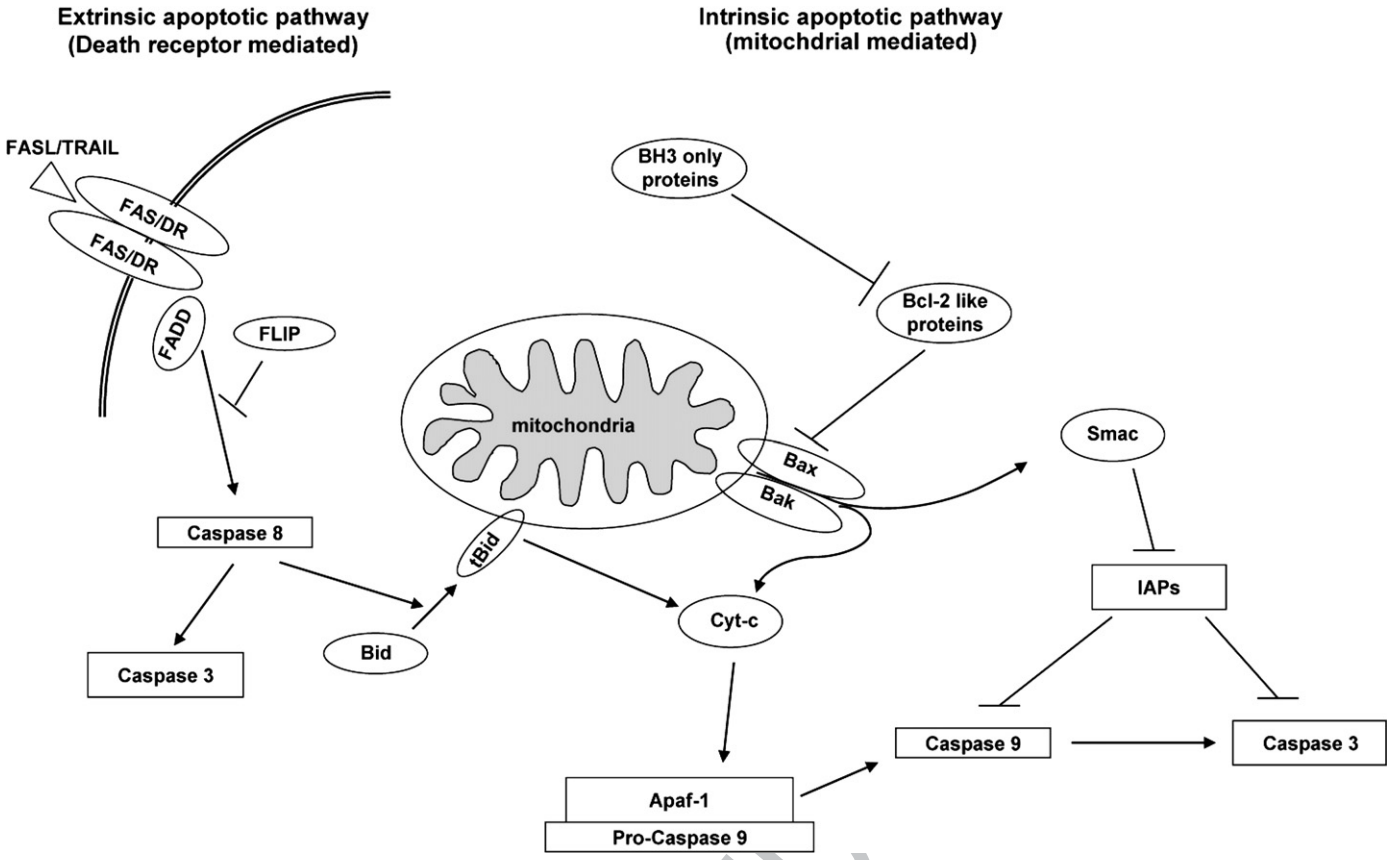

Fig. 1. The extrinsic and intrinsic apoptotic pathways.

BH3 mimetics, SMAC mimetics, and proteasome inhibitors are examples of these targeted therapies which may be effective in the treatment of colorectal cancer. The identification of patients who will benefit from these therapies will be facilitated by identification of these biomarkers.

3. Upstream of the mitochondria: Bcl-2 family members as biomarkers for CRC

Mitochondrial mediated apoptosis associated proteins are frequently studied for use as biomarkers in cancer. In particular, as key

Table 1
Bcl-2 family apoptotic proteins in colorectal cancer: clinical findings

Protein		Function	Clinical findings	References
Bcl-2 like family	Bcl-2	Pro-survival	Protein overexpression associated with better disease free survival	[41–43]
			Protein overexpression associated with better overall survival	[42–48]
			Protein overexpression does not correlate with survival	[49–51]
			Protein overexpression associated with unfavorable disease outcome	[52]
	Bcl-X_L	Pro-survival	Protein overexpression in patients developing from ulcerative colitis	[55]
			Protein overexpression not associated with survival	[45,54]
	Mcl-1	Pro-survival	Decreased expression in tumors	[60]
			Diffuse IHC staining in poorly differentiated tumors	[61]
			Perinuclear staining associated with response to 5FU based chemotherapy	[62]
	Bcl-W	Pro-survival	Overexpressed in tumors, no associations with outcome made	[63]
Bax family	Bax	Pro-apoptotic	Bax (+) tumors associated with better survival	[70–73]
			No differential protein expression between normal and tumor tissue	[45]
			In MSI (+) patients, genetic mutations correlate with poor prognosis	[74–79]
			Bax (+) and p53 (−) tumors exhibit greater response to 5FU based therapies	[71,72]
			Bax (−) and p53 (−) tumors exhibit greater response to 5FU based therapies	[170]
	Bak	Pro-apoptotic	Genetic mutations infrequent in colorectal cancer	[80]
BH3 only family	Bad	Pro-apoptotic	Inactivating genetic mutations in tumors	[84]
			Inactivated phosphorylated Bad increased in tumors	[85,86]
			Elevated expression in tumors correlates with longer disease free and overall survival in patients receiving 5FU based therapy	[87]
	PUMA	Pro-apoptotic	Higher protein expression in tumors, with no genetic mutations	[86]
			No changes in gene expression in tumors	[92]
	NOXA	Pro-apoptotic	No differential mRNA or protein expression between normal and tumor tissue	[93]
	Bik/nbk	Pro-apoptotic	Genetic mutations infrequent in colorectal cancer	[94]
	Bid	Pro-apoptotic	Elevated protein expression in tumors did not correlate with disease survival.	[151]
			No difference in expression between tumor and matched normal tissue	[45]
			Elevated expression in tumors correlates with longer disease free and overall survival in patients receiving 5FU based therapy	[87]

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regulators of this pathway, the Bcl-2 family members are among the most frequently studied as apoptotic biomarkers in a variety of tumor types, including colorectal cancer. The three subfamilies and their current status as biomarkers for colorectal cancer are discussed below and summarized in Table 1.

3.1. Bcl-2 like proteins

The pro-survival Bcl-2 like subfamily members include Bcl-2 [23,24], Bcl-X_L [25], Mcl-1 [26,27] and Bcl-w [28]; all of which have been examined for their use as biomarkers in a variety of human cancers, including colorectal cancer. Bcl-2 was first identified as an oncogene in B-cell lymphoma with constitutive over-expression resulting from the *t*(14:18) chromosomal translocation [29] and was subsequently identified as a key inhibitor of apoptosis [23,24]. Bcl-2 is one of the most frequently examined apoptotic protein for potential clinical use as a prognostic biomarker in cancer. In addition to its role in apoptosis regulation, Bcl-2 has also been shown to regulate autophagy, playing a protective role [30]. Aberrant expression of this protein has been shown in a number of solid tumors [31–33]. In normal colonic mucosa, Bcl-2 has a distinct expression protein pattern, expressed solely in the base portion of colonic crypts where there is very low levels of physiological apoptosis [34], an expression pattern which is lost in the progression to colorectal cancer [35,36]. In addition, overexpression of Bcl-2 has also been associated with resistance to cytotoxic drugs such as 5FU, CPT-11, and cisplatin in various cancer model systems [37–40]. Since the mid-1990s, there have been many immunohistochemical studies carried out with the aim of determining the clinical utility of Bcl-2 as a prognostic biomarker. Surprisingly, in studies which have shown an association between Bcl-2 protein expression and survival, overexpression is frequently associated with either better disease free survival [41–43] and/or better overall survival [42–48]. However some studies have shown either no correlation between Bcl-2 expression and survival [49–51] or that overexpression of Bcl-2 correlates with unfavorable outcome in colorectal cancer patients [52].

Based on preclinical findings linking Bcl-2 expression to chemoresistance, the correlation between overexpression of Bcl-2 and improved survival seems counterintuitive. A possible explanation for the paradoxical relationship between Bcl-2 expression and clinical outcome is its probable role in colorectal cancer progression. Studies have shown that aberrant Bcl-2 expression facilitates tumor progression in the early stages of colon cancer when a patient's prognosis is more favorable [35,49,53,54]. Sinicrope et al. which showed that while Bcl-2 expression did not correlate with overall survival in Dukes B patients its expression did correlate with probable favorable prognostic features such as DNA content and low proliferative index [42]. Taken together all of these studies indicate that while Bcl-2 may be a useful biomarker for colorectal cancer prognosis, its true clinical utility is yet to be fully realized.

As a member of the anti-apoptotic Bcl-2 like family, over-expression of Bcl-X_L should give cells a survival advantage and confer chemoresistance [38]. In the clinical setting, there have been few studies examining the prognostic value of the Bcl-X_L in colorectal cancer. In a small study, van der Woude et al. examined the expression of Bcl-X_L in the progression from chronic ulcerative colitis to malignant colorectal cancer [55]. This study found that Bcl-X_L is overexpressed in patients with cancers developing with ulcerative colitis, but not in the development of malignancy from normal colonic mucosa [55]. Furthermore, this study finds that the expression patterns seen in this study closely resemble those seen in the development of esophageal cancer from Barrett's esophagus [56], indicating a possible role of inflammation in the development of these cancers. In another study, Han et al. suggest that aberrant expression of Bcl-X_L, like Bcl-2, may play a role in the development of colorectal cancer [54]. However unlike Bcl-2, the expression of Bcl-X_L has not been found to be associated with survival in these patients [45,54].

A possible reason for the lack of association between expression of Bcl-2 and Bcl-X_L and survival may be due to the fact that while these proteins are pro-survival, *in vitro* studies have shown that over-expression of Bcl-2 and Bcl-X_L results in not only an inhibition of apoptosis but also G_0/G_1 cell cycle arrest and subsequent decreased cellular proliferation [57–59]. While this may aid in explaining why Bcl-2 expression associated with favorable prognostic markers such as low proliferative index found by Sinicrope et al. [42] it also serves to further complicate the use of these proteins as prognostic markers.

Similarly to Bcl-X_L, there have been only a few studies which have examined whether the pro-survival Mcl-1 and Bcl-w proteins have any clinical importance as a biomarker in colorectal cancer. One early study which examined the immunohistochemical expression of Mcl-1 in primary tumors and adenomatous polyps found that Mcl-1 staining was significantly decreased in tumors but not in benign polyps indicating that decreased expression of Mcl-1 may be a later event in malignant progression and/or tumor dedifferentiation [60]. Two studies by Backus et al. both indicate that the staining patterns, rather than overall expression of Mcl-1 protein in tumors may be important [61,62]. The authors hypothesized that perinuclear staining may indicate the presence of Mcl-1 at the mitochondria and subsequent inhibition of apoptosis, whereas as in tumors with diffuse staining inhibition of apoptosis by Mcl-1 is altered allowing for increased tumor growth. An immunohistochemical analysis of Bcl-w expression in colon cancer showed that this protein was expressed in 92% of 75 colonic adenomocarcinomas with little expression in patients with adenomas and no staining in normal tissue [63]. These studies showed stronger staining in Dukes C patients than Dukes B indicating a possible role of this protein in tumor progression, however there was lack of sufficient follow-up time with which to make any correlations between Bcl-w expression and treatment response or patient survival [63].

3.2. Bax family

Bax and Bak are essential in mitochondrial mediated apoptosis, as their insertion into the mitochondrial membrane triggers the release of cytochrome *c* release into the cytosol, leading to the caspase activation and committing the cell to apoptosis [64–67]. Bax and Bak are highly redundant in function, such that knocking out one does not alter the cells ability to undergo apoptosis, while knocking out both Bax and Bak leads to prevention of apoptosis [67–69].

Of the Bax family of proteins, Bax is the most extensively studied in colorectal cancer. Two studies have shown that Bax positivity correlated with better survival outcomes than Bax negative tumors in advanced metastatic colorectal cancer [70–73]. Sturm et al. showed that with improved outcome in patients with both wild type p53 and Bax-positive tumors [71]. In another study examining the expression of fifteen apoptosis related proteins, there was no significant difference found between Bax expression in tumor tissue and normal colonic mucosa and no correlation between Bax expression and survival was found [45]. Of note, genetic studies indicate that in approximately half of MSI positive colorectal tumors have frameshift mutations in the *bax* gene [74–79]. Inactivating mutations such as these may contribute to tumor progression by disrupting the apoptotic pathway in MSI positive patients and have been correlated with poor prognosis [74].

Bak has been the focus of a genomic study looking for common mutations and single nucleotide polymorphisms (SNPs) in colorectal and gastric cancers. In a cohort of 192 patients, no somatic mutations were found in the *bak* gene and any SNPs found in the coding sequence were also found in normal samples, indicating that genetic mutations in this gene are rare [80]. Due to the importance of these proteins in the apoptotic pathway, it is surprising that there have been so few studies examining their prognostic potential and future studies examining this are warranted.

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3.3. BH3 only proteins

BH3 only proteins are an increasingly important pro-apoptotic subfamily of the Bcl-2 family of proteins. BH3 only protein genes are transcriptional targets of stress activated transcription factors and BH3 only proteins are targets of stress-induced signaling cascades. The BH3 only family members include Bad, Bim, Bid, PUMA, NOXA, Bmk, and NBk. Most of these BH3 only family members are predominantly under transcriptional control, with the exception of Bad and Bid which are constitutively expressed and require post-translational phosphorylation and cleavage for activation respectively. Due to its role in the extrinsic apoptosis pathway, Bid will be discussed in Section 5.

There have been several recent studies examining the role of BH3 only proteins as biomarkers in colorectal cancer. Bad exists in both an unphosphorylated active and a phosphorylated inactive form. In the active form, Bad dimerizes with either Bcl-2 or Bcl-X_L preventing their sequestration of Bax and promoting apoptosis [81]. Phosphorylation of Bad results in its inactivation and sequestration on the cytosol, allowing for cell survival [82], this survival switch is regulated by the survival kinase Akt [83]. Bad has been examined at both the genetic and protein level in colorectal cancer tissue. At the genetic level, Lee et al. has identified two missense inactivating mutations in the BH3 homology domain of *bad*, a region essential of for its pro-apoptotic function in sequestration of Bcl-2 and Bcl-X_L [84]. Studies have also shown that colon cancer tumor tissue has stronger immunohistochemical staining for phosphorylated Bad, specifically at serine 136 [85] and serine 112 [86] compared to normal tissue indicating that increased inactivation of Bad may contribute to the dysregulation of apoptosis in colorectal cancer progression. A recent study examining Bad protein expression in 5FU treated colorectal cancer patients showed that pretreatment Bad protein levels were higher in tumors than in adjacent normal mucosa and that higher protein expression of Bad correlated with longer overall survival [87].

PUMA and NOXA are BH3-only proteins which are unique in that they are transcriptionally regulated by p53 [88–90]. p53 is frequently mutated in colorectal and other cancers and therefore alterations in PUMA and NOXA mutational status and/or expression have biomarker potential for progression of treatment response. In fact, a recent study has identified both of these proteins as potential recurrence biomarkers for prostate cancer [91]; however in colorectal cancer these molecules have not been very well studied. One recent study has shown that while PUMA protein is expressed in both normal and colorectal tumor tissue, expression in tumors is higher than in normal mucosa, with no concurrent genetic mutations in the BH3 domain [86]. Another study examining PUMA gene expression and immunohistochemical staining of colorectal tumors compared to normal tissue showed higher protein expression in 29% of tumors, with no significant changes at the level of gene expression [92]. Genetic and protein studies of NOXA in colorectal tumors and normal tissue revealed that there were no significant differences in expression of NOXA at the mRNA or protein level, and that this gene does not appear to be mutated in colorectal cancer [93], indicating that NOXA may not be important in colorectal cancer.

Mutational analysis of the *bik/nbk* gene in MSI+ colon cancers were not able to show any mutations [94], with the authors indicating that this protein likewise may have little if any role in colorectal cancer progression.

4. Downstream of the mitochondria

4.1. Caspase activation in the intrinsic apoptosis pathway

Following release from the mitochondria, cytochrome *c* complexes with APAF-1 (apoptotic peptidase activating factor 1) to form the apoptosome, which then recruits and activates pro-caspase 9 [95,96]. While cytochrome *c* is the key component of the apoptosome, it has seldom been studied as a biomarker in cancer and to the best of our knowledge has not been examined in colorectal cancer. One study examined serum cytochrome *c* levels in a variety of tumor types treated with different chemotherapeutics, showing that high serum cytochrome *c* was a negative prognostic marker in chemotherapy treatment. Importantly, the authors suggest that serum cytochrome *c* levels may in the future be used in combination with other tumor markers for stratifying high risk patients [97].

With its role in promoting apoptosis, it would be expected that APAF-1 be associated with better overall survival. One study indicated that APAF-1 expression correlated with longer overall survival in early stage colorectal cancer [45]. Similarly another study has shown that in rectal cancer, less than 50% of pretreatment biopsies stained positively for APAF-1 and that positivity significantly correlated with complete response to radiotherapy [98]. A recent study has shown that loss of APAF-1 expression at the protein level was correlated with shorter overall survival without making any correlations to response to chemotherapy [99]. These studies indicate that APAF-1 is promising as a potential prognostic biomarker. However, it should also be taken into consideration that the APAF-1 gene has been shown to be inactivated through methylation of its promoter in melanoma patients [100]. Epigenetic silencing of APAF-1 may therefore contribute to any loss of protein expression seen in colorectal cancer patients and should be examined.

4.2. The caspase family

The key effector molecules in the apoptotic pathway are caspases, a family of cysteine proteases whose proforms are activated thorough proteolytic cleavage in both the mitochondrial and death receptor mediated apoptotic pathways. The caspase family consists of two groups, the initiator and effector caspases. Following their activation, the initiator caspases go on to activate the effector caspases. As previously stated, in mitochondrial mediated apoptosis formation of the apoptosome leads to the activation of the initiator caspase 9 which in turn activates the effector caspases 3 and 7. The effector caspases 3 and 7 are highly similar in function, such that in knock out model systems, a cell susceptibility to apoptosis is not completely abrogated unless both are concurrently knocked out [101]. Due to their key role in cell death, caspases and modulators of their activity have potential as serve as biomarkers of response to chemotherapy.

There is some evidence of alterations in the expression of caspase family members in colorectal cancer, at both the genetic and protein levels. Palmerini et al. examined the protein expression of caspases 7 and 9 in matched tumor and normal tissue by immunohistochemistry [102], and showed a decrease in their protein expression. Caspase 7 showed the largest differential expression, with 85% of tumors showing marked downregulation of protein. Two genetic studies by Soung et al. identified mutations in both the *caspase 3* [103] and *caspase 7* [104] genes in a number of tumor types, including a small percentage of colon cancers. One recent study has shown elevated procaspase 3 protein in colorectal cancer tumor tissue compared to adjacent normal [105]. However, the clinical implications of these findings have not been fully studied. Leonardos et al. showed that the enzyme activity of caspase 3-like proteases was significantly elevated in colorectal carcinomas compared to matched normal tissue. However, this upregulation did not correlate with prognostic factors such as tumor stage, grade, location or patient age [106].

The number of studies examining the expression of caspases in colorectal cancer is very limited and they have not examined any correlation between expression and patient survival (summarized in Table 2). Therefore the clinical utility of caspases as colorectal cancer prognostic markers needs further investigation.

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Table 2
Caspases and modulators of their activity in colorectal cancer: clinical findings

	Protein	Clinical findings	References
Caspase family	Caspase 9	Decreased protein expression in tumors	[102]
	Caspase 7	Decreased protein expression in tumors	[102]
		Genetic mutations in gene identified	[104]
	Caspase 3	Genetic mutations in gene identified	[103]
		Elevated caspase 3 protein in tumor tissue	[105]
		Caspase 3-like activity elevated in tumor tissue	[106]
	Caspase 8	Inactivating genetic mutation in a small subset of patients with invasive colorectal carcinoma	[143]
		Moderate increase in caspase 8 protein expression in tumors	[102]
Modulators of caspase activity	Survivin	mRNA overexpression associated with poor survival rates	[116]
		Increased protein expression in tumors compared to matched normal	[45]
		Protein overexpression associated with poor survival rates	[113, 116]
		Cytoplasmic staining associated with better overall survival	[112]
	cIAP1	Increased protein expression in tumors compared to matched normal	[45]
	cIAP2	Increased protein expression in tumors compared to matched normal	[45]
		Increased protein expression correlated with shorter overall survival	[45]
	XIAP	Increased protein expression in tumors compared to matched normal	[45]
	APAF-1	Protein expression correlates with better overall survival	[45]
		IHC positive rectal cancer patients correlates with complete response to radiotherapy	[98]
		Loss of protein expression correlates with shorter overall survival	[99]
	SMAC	Overexpressed in tumor tissue, no correlation to survival	[45]

4.3. Inhibitors of apoptosis proteins

The IAP family of proteins are important regulators of caspase activity and apoptosis with family including cIAP1, cIAP2 [107], XIAP [108] and Survivin [109]. All of these IAP family members have been shown to be more highly expressed in tumor tissue than normal tissue in a cohort of Dukes B patients [45]. Coupled with their biological role in cell survival, IAP family members have potential as prognostic markers and have been studied in a variety of tumor types, [reviewed in [110]], including those of the gastrointestinal tract [111–113].

Survivin has received the greatest amount of attention in studies evaluating the prognostic potential of IAP family members in colorectal cancer. Survivin has also been shown to play a role in cell division as it functions as a regulator for mitosis via its role in regulating microtubule dynamics, indicating that the overexpression of this protein may not only inhibit apoptosis but also lead to aberrant mitosis and malignancy [114,115]. Due to its roles in both cell survival and in cell division, Survivin is an attractive candidate as a prognostic biomarker for colorectal cancer. Several studies have shown poorer survival rates in colorectal cancer patients overexpressing Survivin mRNA [116] and protein [113,117]. Focusing on Dukes B patients, an immunohistochemical study conducted by Sarela, et al. demonstrated that Survivin negative patients had a 94% 5-year survival rate following curative resection compared to 52% for Survivin positive patients, indicating that expression may aid in stratifying Dukes B patients for adjuvant chemotherapy [113]. Aside from the expression of Survivin at the mRNA and protein levels, the subcellular localization of Survivin may also have prognostic potential. One study has shown that elevated Survivin cytoplasmic staining correlates with better overall survival in colorectal cancer patients [112]. Survivin has also been studied as a marker for cancer diagnosis, Rohayem et al. detected survivin auto-antibodies in the sera of both lung cancer and colorectal cancer patients [118]. Monitoring survivin mRNA levels in the blood of gastrointestinal cancer patients following surgical resection may play a role similar to carcinoembryonic antigen (CEA) in monitoring a patient for recurrence [111].

Far less is known about the prognostic role of other IAP family members in colorectal cancer, in particular XIAP which is the most potent and long-lived caspase inhibitor of this pathway. The study by Krajewska et al. involving a large panel of apoptosis biomarkers demonstrated a correlation between elevated cIAP2 and shorter survival, with no correlation between survival and the expression of cIAP1, XIAP, and survivin [45]. These findings are similar to a study examining the levels of these proteins in prostate cancer [119].

While the prognostic importance of IAP family members remains unclear, regulators of their activities such as SMAC and XIAP-associated factor 1 (XAF1) may also have biomarker potential. SMAC is released in conjunction with cytochrome *c*, where it then goes on to bind to IAPs and allow for caspase activation. SMAC protein has been shown to be highly expressed in solid tumors of the stomach, colon, lung, ovaries, and prostate [120]. An immunohistochemical study demonstrated that SMAC is overexpressed in Dukes B colorectal tumors, compared to normal mucosa, but expression was not correlated with survival [45]. A recent study examining the gene expression of XAF1, a negative regulator of XIAP, in colon carcinoma, benign adenomas, and polyps showed that XAF-1 mRNA levels are higher in colon carcinoma than in benign adenomas and polyps, with the authors suggesting that this molecule may play a role in colon cancer progression [121]. Clinical studies examining the expression of IAP family members and their modulators are summarized in Table 2.

5. Bypassing the mitochondria (or not): outside signals, the extrinsic pathway of apoptosis

Due to its role in cell death, members of the extrinsic apoptotic pathway hold potential as biomarkers and therapeutic targets in colorectal cancer. Activation of this pathway occurs through the binding of death receptors (Fas, DR4 and DR5) with their respective ligands (FasL and TRAIL) (Fig. 1). In colorectal cancer the death receptor Fas (CD95), a TNFR (Tumor necrosis family receptor) superfamily member and its ligand FasL are thought to be involved in disease progression. This may be in part due to the FasL overexpression of colon cancer cells which allows cells to avoid cell death by the immune response [122], although there is no consensus on role of FasL in this role [123–126]. Immunohistochemical staining of FasL is frequently elevated in tumors and that FasL positivity is associated with later stage of disease and poorer survival [127,128]. However, another study has shown conflicting results, with greater FasL expression associated with better disease outcome and early stage [129]. The Fas pathway has also been implicated in the mechanisms of action of 5FU [130] and therefore its potential as a chemotherapy response marker has also warranted examination. In a study of patients of metastatic Dukes D patients, the expression of neither Fas nor FasL correlated with response to 5FU [131]. Serum

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levels of Fas have been shown to be elevated in patients with colon cancer [132] indicating that serum levels may have ability to serve as a prognostic marker. One study by Nadal et al. has shown that serum Fas levels are further elevated following treatment with oxaliplatin. When measured in conjunction with serum FasL, a greater than 1.2 ratio of Fas/FasL is associated with response to oxaliplatin chemotherapy, with ratios less than this associated with oxaliplatin resistance [133].

The binding of TRAIL to its receptors DR4 and DR5 also activates the extrinsic apoptotic pathway and it has been shown that cancer cells are more susceptible to apoptosis induced by TRAIL than normal cells [134,135]. Therefore potential cancer therapeutics targeting this pathway have been developed in order to increase the amount of apoptotic cell death by direct activation of the TRAIL receptor mediated cell death. These targeted therapies have potential in the cancer therapy and include recombinant TRAIL and monoclonal agonist antibodies directed against the TRAIL receptors [136]. There have been few studies examining the role of TRAIL and its receptors DR4 and DR5 as colon cancer biomarkers. In comparisons of colorectal tumors and matched normal mucosa, studies have shown that tumoral expression of TRAIL is frequently lower than in normal mucosa [137,138]; in contrast one has shown higher tumoral TRAIL levels [139]. Studies examining the levels of the TRAIL receptors DR4 and DR5 all show higher expression of both receptors in colonic tumors compared to normal tissue [137–139]. In the study by van Geelan et al. overexpression of DR4, but not TRAIL or DR5 correlated with worse disease free survival and shorter time to recurrence in Dukes C patients [139], indicating that levels of this protein may have some clinical use in deciding a patients chemotherapy regimen. In addition to the DR4 and DR5, the decoy receptors DcR1, DcR2 and DcR3 may have clinical importance. These receptors bind to TRAIL, but lack a death domain and therefore have pro-survival rather than pro-death effects [22,140,141]. In a study examining the levels of DcR3 in colorectal patients and response to chemotherapy, Mild et al. found higher genetic copy numbers and protein overexpression of DcR3 in patients with colorectal cancer, and that patients with higher gene copy number had worse disease free and overall survival compared to patients with normal copy number [142].

Following death receptor activation, caspase 8 is activated via its interactions with FADD. Mutational analysis of the *caspase 8* gene revealed the presence of an inactivating mutation in a small subset of patients with invasive colorectal carcinoma [143]. These mutations were not present in colonic adenomas, indicating that these mutations may contribute to the pathogenesis of disease. In examining the immunohistochemical expression of a number of caspases in colorectal cancer, Palmerini et al. showed a moderate increase in caspase 8 expression in tumors [102]. Activation of caspase 8 by FADD can be inhibited by cFLIP, which has been shown to inhibit TRAIL induced apoptosis in a number of cancer model systems [144]. cFLIP exists in two forms, a long form cFLIP-L and a short form cFLIP-s, both of which are capable of inhibiting caspase 8 activation and TRAIL induced cell death [145]. cFLIP-L has been shown to be more highly expressed in adenomocarcinomas of the colon compared to premalignant polyps and normal colonic tissue at both the mRNA and protein levels, indicating that alterations in cFLIP-L levels may contribute to the malignant phenotype [146]. A recent study correlating cFLIP expression to colorectal cancer patient survival indicates that strong immunohistochemical staining of cFLIP-L, but not cFLIP-s correlates with poor prognosis [147].

In addition to the activation of caspases independent of the mitochondria, the death receptor signaling pathway can also contribute to release of cytochrome *c* from the mitochondria. Following activation by FADD, caspase 8 goes on to cleave and activate the BH3 only protein Bid [148,149]. Truncated Bid (tBid) can then insert itself into the mitochondria, leading to the release of cytochrome *c* and amplification of the cell death signal [149,150]. There has been very little work done in examining the role of Bid as a biomarker in colorectal cancer. One immunohistochemical study of Dukes B patients indicated that in a comparison of tumor and matched adjacent normal tissue; Bid was elevated in 57% of 60 patients but that this did not correlate with disease survival [151]. Further analysis by this same group and extension of the patient cohort ($n=100$) found no difference in Bid expression between tumor and matched normal tissue [45]. Another recent immunohistochemical study by Sinicrope et al. showed that in addition to elevated expression of Bid protein in tumors compared to normal tissue, there was also a correlation between high Bid expression and longer overall survival in Dukes B and C patients receiving 5FU based chemotherapy [87].

6. Caspase independent death

Cell death is not limited to the apoptotic pathway, but also occurs in ways which are caspase independent. Caspase independent cell death pathways include autophagy, AIF (apoptosis initiating factor)-induced cell death and potentially autophagic cell death. While there is little known regarding the role of caspase independent cell death in colorectal cancer prognosis or response to treatment, these pathways may play a role in cell death associated with chemotherapy regimens and therefore may have some potential as biomarkers.

6.1. AIF

AIF induces cell death following release from the mitochondria, triggered through both caspase dependent and caspase independent pathways. In caspase dependent cell death, AIF is released along with cytochrome *c* following permeabilization of the mitochondrial membrane [152]. AIF is capable of causing caspase independent cell death through its role in chromatin condensation and DNA fragmentation [152,153]. In addition to its role in apoptosis, AIF which works to promote survival through its role as a NADH oxidase in the mitochondria and in colon cancer cell lines has been shown to suppress cell death [154] indicating that this protein may play a role in malignancy. Examination of AIF in colorectal cancer found that while somatic mutations in AIF are rare, the majority of colorectal cancer tumors expressed higher levels of AIF protein than normal mucosa [155]. Immunohistochemical analysis of AIF expression in matched tumor and normal tissue showed no difference in protein expression [45].

6.2. Autophagy

Autophagy refers to the degradation and recycling of intracellular proteins and cellular organelles [156,157]. This can have a protective effect as it is plays a role in protein turnover and response to lower cellular energy levels [158], and it can also result in autophagic cell death when the levels of damage are high enough. Importantly this process may also be controlled by the Bcl-2 family of proteins. Beclin-1 is a regulator of autophagy and has been identified as a possible tumor suppressor [159]. Beclin-1 is kept inactive via binding to Bcl-2; inhibition of this interaction can release Beclin-1 and trigger autophagy. Its mutational status and expression have been examined in malignancies, including colorectal cancer [160,161]. These studies found that mutations in the *beclin-1* gene are rare [161], but that protein was expressed in 95% of tumors, with little or no expression in normal tissue [160]. Further studies examining the role of the new field of autophagy are clearly warranted.

7. Multiple marker studies

Many of the studies reviewed here examine the biomarker potential of multiple proteins, but they are limited in that these proteins are examined individually and not in combination. Indeed, the use of multiple markers in combination with each other is an

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excellent way in which the specificity of a prognostic or diagnostic test can be increased. There are a handful of studies which have examined the prognostic significance of marker combinations in colorectal cancer progression. Generally these studies have focused on a very small number of empirically chosen proteins believed to be of particular importance, with the focus being on Bcl-2 and/or p53 protein. These proteins are often examined either as a pair [35,42,43,46,48,162] or in combination with the proliferation marker Ki-67 [36,41,51,163] and/or with other molecules involved in the regulation of cellular proliferation such as c-Myc [36,52], cyclin D1 [163], p21 and p27 [164].

Several studies have explored the role of p53 status in conjunction with apoptosis regulating proteins. The tumor suppressor protein p53 has a variety of molecular functions, including the induction of apoptosis in response to cellular stress [165] via transcriptional activation of a number of pro-apoptotic target genes including the BH3 only proteins PUMA [166] and NOXA, but also Bax, Bid, APAF-1, and caspases 9, 3, and 8 [167]. p53 mutations are a hallmark in colorectal cancer progression, leading to stabilization of protein and elevated immunohistochemical staining. The ability of p53 to serve as a prognostic biomarker has been extensively studied in CRC, with most studies focusing on increased immunohistochemical staining [reviewed in [168]]. Studies have suggested that p53 protein stabilization and mutations are associated with poor clinical prognosis [36,168,169]. In a study examining whether the expression of p53 AND Bcl-2 in rectal cancer predicts for survival following surgical resection, Schwander et al. found that certain combinations of the two proteins were effective in separating patients with poor and favorable outcomes. Namely, patients which were p53 positive and Bcl-2 negative had a significantly poorer prognosis than patients which were p53 negative and Bcl-2 positive [162]. Buglioni et al. found similar results in a cohort of colorectal cancer patients which focused on patients which were Dukes stage A or B [43], indicating that this combination may be of importance in the stratification of early stages colorectal cancer for adjuvant chemotherapy. Aside from p53 and Bcl-2 combinations, other combinations of markers have been identified as colorectal cancer prognostic markers. Tornillo et al. found that alone, the expression of p53, Bcl-2, p21 and p27 on a tissue microarray had prognostic significance. However combinations of these markers, namely p21/p27/p53 and p21/p27/Bcl-2 were predictive of survival [164].

One recent study has shown that while there was no correlation between Bax expression and Bcl-2 expression and survival, there was a correlation between p53 negative and high Bax expression had better survival rates than tumors which were p53 positive and had high Bax expression [72] in patients receiving 5FU based chemotherapy. Likewise, Sturm et al. have shown that Bax expression was indicative of longer survival in patients with advanced metastatic disease, and this effect was enhanced in patients whose tumors were Bax positive and p53 wild type [71]. However another immunohistochemical study aimed at correlating the expression of these proteins to response to therapy showed that tumors which were p53 and Bax negative had greater response rates to 5FU based therapy [170]. These contradictory studies indicate that the relationship between p53 and Bax needs to be more closely examined. Another study has shown that tumors expressing high levels of both the BH3 only proteins Bid and Bad had longer disease free and overall survival in a cohort of Dukes B and C colon cancer patients undergoing 5FU based chemotherapy, indicating that these markers may aid in deciding treatment regimens for patients [87].

Recently, Krajewska et al. carried out a large tissue microarray study examining the expression of a large 15 apoptosis related proteins in Dukes B colorectal cancer patients [45]. Protein expression was determined in matched tumor and normal tissue and any correlations with clinical outcome were determined. As individual markers, better survival outcome was associated with low tumoral levels of expression of cIAP2 and the caspase 9 antagonist TUCAN and high tumoral levels of APAF1, and Bcl-2. Analysis to determine the correlations between the expression of multiple proteins and survival indicated that certain pairwise combinations of proteins whose expression correlated with outcome were very highly predictive of patient outcome in Dukes B patients. Specifically, tumors expressing the combinations of low cIAP2/low TUCAN, low cIAP2/high APAF1, and low TUCAN/high Bcl-2 were predictive of better outcome, with 97–100% of patients with these phenotypes being alive at 5 years. Patients expressing combinations of proteins which correlated negatively with outcome; low tumoral APAF1/high TUCAN or high cIAP2/low Bcl-2, were more likely to die from disease. This study in particular shows the importance of examining the expression of multiple markers for increasing specificity for better or worse prognosis.

8. Future directions: towards a systems analysis?

The majority of clinical studies discussed in this review examine the qualitative expression of a small number of apoptosis related proteins and any correlation to clinical outcome. These studies have often shown differential protein expression between tumor and normal tissue, but as discussed above correlating these differences with clinical outcome has frequently produced inconsistent results. While informative, these multiple marker studies fail to take into account the complex nature of biological processes, and therefore a systems biology approach incorporating these interactions may have a better ability to predict responses. Apoptosis is a complex process in which the balance of pro- and anti-apoptotic proteins is tightly regulated in determining the fate of the cell; a balance which is believed to be dysregulated in cancer. For instance, this review highlights that in colorectal cancer some pro-apoptotic proteins (Bid, PUMA) are overexpressed in colorectal tumors compared to normal tissue. Letai has proposed that such tumors may be 'primed' for death [171], that is pro-apoptotic proteins may be being inhibited through interactions with anti-apoptotic proteins resulting in cell survival and therapy resistance. Therefore it is very likely that differences in a number of apoptosis proteins and their relationship to one another will have clinical use as prognostic markers for cancers. In addition, elucidation of the relationships between the various pro- and anti-apoptotic proteins is important in that these interactions represent novel therapeutic targets.

We have recently developed a systems biology based, mathematical model, APOPTO-CELL, [172,173] which allows to predict the susceptibility of cells to undergo caspase activation based on the input of quantitatively determined protein levels of apoptosis related family members (in particular Caspase 3, Caspase 9, APAF-1, SMAC and XIAP). This model is advantageous in that it has incorporated a biological interaction network based on protein interaction data and hence represents a novel approach to identifying and utilizing apoptosis related proteins by looking at a system rather than a random combination of proteins. It enables the identification of proteins which are critical in the determination of a systems fate, or more precisely when a protein or combination of proteins becomes important. For example, in a system in which the anti-apoptotic protein XIAP is highly expressed, SMAC has little effect on cell fate. SMAC only becomes important when both XIAP and SMAC protein levels are high [173]. The identification of proteins which are only important in certain scenarios may have clinical significance in the development of targeted therapeutics such as SMAC mimetics. While the APOPTO-CELL model was established in cervical cancer cells, there is the potential for this tool to be translated into the clinical setting such that it would be able to assess the probability a patient will respond to chemotherapy. On the basis of quantitative protein analysis, concentrations of apoptotic proteins (Caspase 3, Caspase 9, APAF-1, SMAC and XIAP) would be determined followed by input into the

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APOPTO-CELL model, with the prediction of caspase substrate cleavage indicating whether the system is likely to undergo apoptosis. That is increases in substrate cleavage indicate that apoptosis can be executed, while no increases in substrate cleavage indicate that apoptosis could be inhibited or severely impaired in the tumor sample and therefore may serve as a surrogate marker for response. Fig. 2 represents how this model may be used in predicting patient outcome.

Modeling of apoptosis of both the extrinsic apoptotic pathway and of mitochondrial mediated apoptosis at the level of Bcl-2 family proteins has also been and continues to be developed. Recently, a model aimed at predicting for effector caspase cleavage following activation of the extrinsic pathway by TRAIL has been developed [174]. Computational modeling of the intrinsic pathway upstream of the mitochondria is difficult and these models are limited in that there are too many unknowns or 'black boxes' involved in this pathway. In particular, the mechanisms by which Bax and Bak are activated are not fully understood. However, initial modeling of Bcl-2 family member interactions is clearly a feasible approach which will need to be further developed and clinically applied in the future. This model can then be improved upon as the black boxes of this portion of the pathway are filled in.

9. Limitations and conclusion

The potential of apoptosis associated biomarkers for the prognosis in colorectal cancer is vast, but not without limitation. Drug resistance and tumor growth are also influenced by defects in apoptosis, but other confounding variable such as angiogenesis, increased drug metabolism and drug detoxification also contribute to resistance [175]. Cells also undergo alternative mechanisms of cell death such as necrosis, mitotic catastrophe and autophagy. While necrosis and autophagy can be controlled by Bcl-2 family members these cell death pathways also occur independent of apoptosis related proteins, therefore the usefulness of apoptotic proteins as prognostic biomarkers may be limited further.

The recent identification of colorectal cancer 'stem cells' is an important discovery in the colorectal cancer research [176–178]. Cancer stem cells only represent a small portion of the tumor and may be inherently resistant to current chemotherapy, thereby presenting a definite dilemma in how to effectively treat this disease [179,180]. While defects in apoptosis may play a role in a patient's chemotherapy response, a differential dysregulation of apoptosis signaling in colorectal cancer stem cells compared to non-stem cells within the tumor

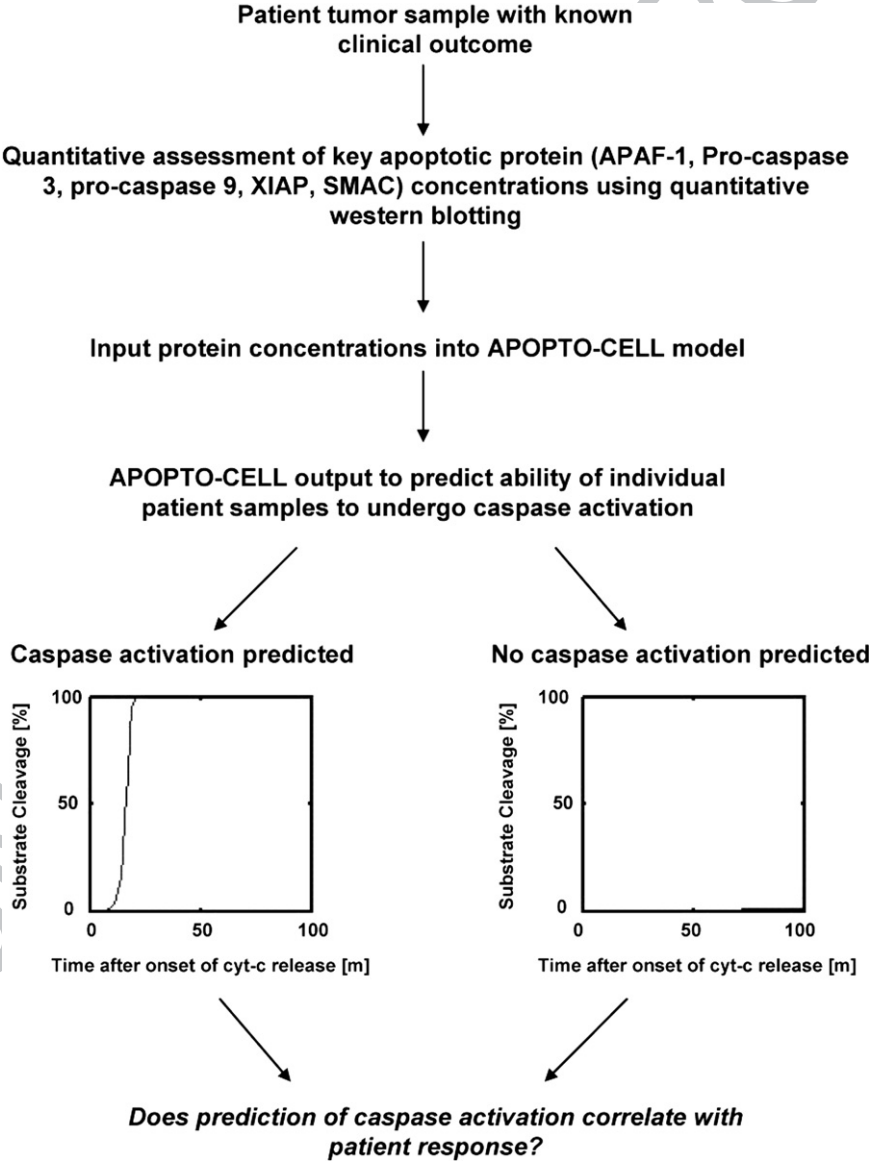

Fig. 2. Flow chart representing how the APOPTO-CELL systems biology model may aid in assessing patient prognosis.

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may limit the use of apoptotic biomarkers for use in disease prognosis and response to treatment.

Stem cells therefore represent an important target in effectively treating colon and other types of malignancies, and a definitive colon cancer stem cell marker needs to be identified to truly isolate and characterize this population of cells with respect to apoptosis sensitivity. Previous studies have used different markers to isolate and identify potential colon cancer 'stem cells', including CD133 [176,177] and CD44/EpCAM [178], which were chosen based on studies in other malignancies. A recent study has questioned the use of CD133 as a marker for the identification of colorectal cancer stem cells [181] and there may be other more specific markers that warrant examination in isolating this cell population [182]. Clearly, the importance of the apoptotic pathway in colorectal cancer stem cells in patient response warrants further investigation.

In conclusion, determination of the tumors ability to undergo apoptosis holds immense potential as a new prognostic marker for colorectal cancer and to identify patients who will respond to chemotherapy. Ultimately this could lead to more personalized and effective cancer care.

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