

LABORATORY-CLINICAL INTERFACE

# Apoptosis and non-apoptotic deaths in cancer development and treatment response

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Received 4 February 2008; received in revised form 8 July 2008; accepted 11 July 2008

#### **KEYWORDS**

Apoptosis; Autophagy; Mitotic catastrophe; Necrosis; Tumorigenesis; Treatment response; Radiotherapy **Summary** Resistance to apoptosis is closely linked to tumorigenesis, as it enables malignant cells to expand even in a stressful environment. Cells resistant to apoptosis are also assumed to be resistant to anti-cancer therapies. Apoptosis has therefore taken a central position in cell death research. However, its contribution to treatment success is highly debated for solid tumors. It becomes more and more clear that cells can also die by non-apoptotic mechanisms, such as autophagy, mitotic catastrophe and necrosis. In this review, we summarize the current knowledge regarding the molecular pathways that underlie these apoptotic and non-apoptotic death pathways, and discuss the clinical data that have now accumulated to evaluate their roles in tumor development and cancer treatment. © 2008 Elsevier Ltd. All rights reserved.

#### Introduction

The process by which a normal cell develops into a malignant cell with the capacity to form a tumor requires several cellular alterations. Evasion of apoptotic cell death is one of the proposed alterations.<sup>1</sup> Importantly, evasion of apoptosis is also recognized to result in resistance to anti-cancer therapies.<sup>2</sup> Much research has therefore focused on finding ways to circumvent this resistance to apoptosis in order to improve the treatment of cancer patients. However, the contribution of apoptosis resistance to treatment success remains a matter of debate, especially in solid tumors.<sup>3</sup> Increasing attention is being directed towards other types of cell death, such as mitotic catastrophe, autophagy and necrosis. These alternative types of cell death may compensate for the resistance to apoptosis. Understanding the regulation of apoptosis and non-apoptotic death pathways will help us to better evaluate their impact on tumor development and treatment response *in vivo*. Moreover, detailed knowledge regarding the molecular events that contribute to treatment success will facilitate a more rational approach of anti-cancer treatments.

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#### Cell death pathways and tumorigenesis

#### Apoptosis pathway

The term Apoptosis (Greek: apo – from, ptosis – falling) is based on the morphological characteristics of the dying cells, which include cellular shrinkage, membrane blebbing and eventually fragmentation into membrane bound apoptotic bodies.<sup>4</sup> During apoptosis, the cell membrane loses its asymmetry, and phosphatidylserine (PS) becomes exposed on the cell surface (illustrated in Fig. 1). This PS exposure functions as 'eat me' signal for macrophages, which can mediate the effective clearance of apoptotic cell.<sup>5</sup> This type of cell death is therefore suggested not to trigger inflammation.

Apoptosis is a tightly regulated form of cell death, which can be initiated by two different types of signals: intracellular stress signals and extracellular ligands (illustrated in Fig. 2). Intracellular stress signals, such as growth factor withdrawal, DNA damage, oxidative stress or oncogene activation, lead to permeabilization of the mitochondrial outer membrane. The consequent release of cytochrome c and other pro-apoptotic proteins propagates the apoptotic signal. Although it remains debated how cytochrome c is released from the mitochondria, the process is tightly regulated by the Bcl-2 family of proteins, which consists of pro- and anti-apoptotic proteins. The multidomain proapoptotic Bax and Bak are essential, since mitochondria deficient for Bax and Bak fail to release cytochrome c.<sup>6</sup> Bax and Bak are thought to induce permeabilization by forming pores upon oligomerization.<sup>7</sup> The pro-apoptotic BH3-only family members (such as Bid, Bim, Bad, Noxa and Puma) activate Bax and/or Bak. Two models have been proposed for this activation; several peptide studies suggest that they do so through binding of anti-apoptotic Bcl-2 proteins (such as Bcl-2, Bcl-x<sub>L</sub>, Bcl-w and Mcl-1), thereby relieving the inhibitory function of these anti-apoptotic proteins. Others propose that a subset of BH3-only proteins can directly bind and activate Bax and/or Bak.<sup>8–11</sup> The consequent release of cytochrome *c* leads to the formation of a complex – the apoptosome – which contains cytochrome *c*, Apaf-1 and initiator caspase-9. Caspase-9 is auto-activated by induced proximity in the apoptosome.<sup>12–14</sup> Active caspase-9 cleaves and thereby activates the executioner caspases.

When extracellular ligands such as Fas ligand,  $TNF\alpha$  or TRAIL (TNF-related apoptosis-inducing ligand) bind to their receptors, the intracellular death domains of these receptors recruit adaptor proteins (such as FADD and TRADD) and initiator caspase-8 and -10. Together these comprise the death-inducing signaling complex (DISC).<sup>15,16</sup> Caspase-8 and -10 are activated at the DISC, due to induced proximity of the caspases.<sup>13,17,18</sup> This activation is controlled by c-FLIP (cellular FLICE inhibitory proteins). Both the short (c-FLIPs) and long (c-FLIP) forms prevent caspase activation, although c-FLIP<sub>L</sub> is proposed to facilitate caspase binding and activation when lowly expressed.  $^{19-26}$  Once caspase-8 is active, it propagates apoptosis via direct cleavage of executioner caspases. However, the extracellular and intracellular apoptotic pathways cross at the level of the mitochondria since caspase-8 can also cleave the protein Bid into its active form tBid. Being a pro-apoptotic member of the Bcl-2 family of proteins, tBid induces



**Figure. 1** Morphological characteristics of apoptosis and non-apoptotic cell death. Apoptosis is characterized by membrane blebbing, cytoplasmic shrinkage, chromatin condensation, exposure of phosphatidylserine (PS) on the cell surface, and finally the formation of apoptotic bodies. In experimental assays, apoptotic cell death is often determined by the binding of Annexin V to the exposed PS or by detecting caspase-cleaved proteins or fragmented DNA. Death by autophagy is characterized by the double-membrane vesicles containing cytosolic organelles. The autophagic-vesicle-associated form of Atg8/LC3 is used as a marker of autophagy, since its cleavage, lipidation and recruitment to the autophagosomes results in an increased mobility with Western blot assays, and a punctate staining of the protein which can be visualized by fluorescence microscopy using green-fluorescent protein (GFP)-fused Atg8/LC3. Cells dying from mitotic catastrophe are usually large and contain uncondensed chromosomes. The main characteristic of mitotic catastrophe is the formation of multiple micronuclei, and also aberrant mitotic spindle formation can be involved. During necrosis, cells swell and loose their membrane integrity.



**Figure 2** Regulation of apoptosis. Triggering of death-receptors results in the assembly of the DISC, which results in activation of executioner caspases (e.g. caspase 8) that in turn directly activate effector caspases (e.g. caspase-3). Release of cytochrome *c* is mainly regulated by the Bcl-2 family of proteins, consisting of pro- and anti-apoptotic family members (illustration adopted from Certo et al.<sup>10</sup>). Cytochrome c together with Apaf-1 activates the executioner caspase-9 that in turn activates effector caspases. The released mitochondrial proteins Smac/DIABLO and HtrA2/Omi antagonize the inhibitors of apoptosis (IAPs). There is a cross-talk between the two pathways as caspase-8 can activate Bid, which facilitates cytochrome *c* release.

Bax/Bak-dependent permeabilization of the outer mitochondrial membrane and release of cytochrome  $c.^{27}$ 

Both apoptotic pathways lead to activation of the executioner caspases, caspase-3, -6 and -7, which are the main proteases that degrade the cell. Their activity is, at least to some extent, kept in check by IAPs (Inhibitor of Apoptosis Proteins).<sup>28,29</sup> IAPs themselves are inhibited by the proteins SMAC/DIABLO<sup>30,31</sup> and the serine protease HtrA2/Omi.<sup>32</sup> These proteins are also released from the mitochondria, possibly simultaneously with cytochrome *c* to alleviate the inhibitory signal and to enhance the apoptotic signal.

#### Apoptosis in tumorigenesis

Apoptosis is recognized as a major barrier that must be circumvented by tumor cells to allow them to survive and proliferate in such stressful conditions.<sup>1</sup> Tumors acquire resistance to apoptosis through several strategies. Loss-of-function mutations of the p53 tumor suppressor protein are, for example, frequently observed. Since p53 can promote apoptosis by activating transcription of pro-apoptotic Bcl-2 proteins in the context of DNA damage,<sup>33</sup> nonfunctional p53 can directly be linked to a failure to induce apoptosis after cellular stress. In addition, mice deficient for p53 are highly prone to develop tumors.<sup>34–36</sup> Other anti-apoptotic modifications observed in human tumors involve the Bcl-2 family of proteins, such as loss of functional pro-apoptotic

Bax and Bak<sup>37,38</sup> or high expression of anti-apoptotic proteins. In a subtype of B-cell lymphomas, Bcl-2 is highly expressed as a consequence of a Bcl-2 gene translocation next to an immunoglobulin gene.<sup>39</sup> In agreement with the hypothesis that apoptosis resistance favors tumor formation, this translocation increases the incidence of spontaneous B-cell tumors in mice.<sup>40</sup> Furthermore, modifications in the death receptor pathways can also play a role in apoptosis resistance. For example, the Fas receptor expression is high in normal colon mucosa but is reduced or even lost in colon carcinomas.<sup>41</sup> Absence of Fas allows tumor cells to evade the immune destruction mediated by cytotoxic lymphocytes via this pathway. In addition, c-FLIP is specially overexpressed in colon cancers, which has been shown to protect tumor cells against cytotoxic T cell-induced apoptosis in vivo.<sup>42,43</sup> To conclude, apoptosis resistance is the common outcome of all the different anti-apoptotic modifications found in human tumors.<sup>2</sup> It follows that resistance to apoptosis plays an important role in tumorigenesis. Nevertheless, several other cell death programs have been described that may also regulate cellular degeneration.

#### Autophagy regulation

Autophagy is defined as a process in which proteins and organelles are degraded by lysosomal proteases. The formation of autophagosomes – double-membrane vesicles

containing cytosolic organelles — that fuse with lysosomes is a major characteristic of autophagy (illustrated in Fig. 1). As reviewed by Klionsky and Emr,<sup>44</sup> autophagy is not only important for degradation, but it also provides an alternative source of nutrients. In yeast, autophagy is induced under nutrient-limiting conditions as a mechanism to survive. However, autophagic structures are formed during morphogenesis in *Drosophila melanogaster*, suggesting a role in cell death.<sup>45</sup> It has therefore been considered that autophagy might start as an adaptive response in order to enhance survival, but can result in cell death beyond a certain threshold.

Genetic studies in yeast have identified more than twenty genes involved in autophagy. Based on homology with veast, human autophagy-associated Atg proteins have been identified. These proteins are involved in two conjugation systems which play a role in autophagosome formation: Atg12 and Atg8/LC3<sup>44</sup> (illustrated in Fig. 3). Both in yeast and in mammalian cells, Atg12 and Atg8/LC3 are activated by Atg7 and conjugate to Atg5 or phosphatidylethanolamine (PE). respectively.<sup>46,47</sup> The two systems are related, since the Atg12-Atg5 complex is required for Atg8/LC3 targeting to the vesicle membranes.<sup>48,49</sup> Studies in yeast show that the kinase TOR lies upstream of all these autophagy-associated proteins. It thus follows that TOR plays an important role in the initiation of autophagy.<sup>50</sup> Such an important role has also been shown for mammalian TOR (mTOR); inhibition of mTOR enhances autophagy, while its activation suppresses autophagy.<sup>51,52</sup> Another initial step in autophagosome formation is the activation of the class III phosphatidylinositol 3-kinase, which depends on complex formation with the Atg6/Beclin1 protein.  $^{53}$ 

#### Autophagy in tumorigenesis

The double-membrane bound vesicles that are typical for autophagy can be observed in several types of human tumors, and indicate that autophagy occurs in vivo.<sup>54</sup> Autophagy has been proposed to play a tumor suppressive role in the early stages of tumorigenesis. For example, the incidence of tumor formation of MCF7 cells is lower when Beclin1 is highly expressed, which is shown to promote autophagy in these cells.<sup>55</sup> More importantly, mice with impaired autophagy, such as Beclin1/- and Atg4C-/-, are more prone to develop tumors.<sup>56,57</sup> In several human tumors, Beclin1 and DRAM are found to be lowly expressed. 55,58 Furthermore, human tumors often display mutations in the PI3-kinase pathway, leading to activation of mTOR and thus suppression of autophagy.<sup>59,60</sup> These anti-autophagic mutations altogether suggest that cells need to circumvent autophagy in order to form a tumor.

Autophagy may prevent a normal cell to become a malignant cell by degrading damaged organelles and thereby reduce cellular stress, or by degrading specific proteins that enhance tumor formation.<sup>61,62</sup> Since monoallelic loss of Beclin1 is found to be associated with chromosome gains and losses, autophagy may also limit chromosome instability and thereby limit tumor progression.<sup>63</sup> Alternatively,



**Figure 3** Regulation of autophagy. Starvation triggers autophagy by modifying mTOR signaling, which represses autophagy under growth factor (GF) conditions. Atg proteins are involved in autophagosome formation, which is positively regulated by Atg6 and class III PI3K. This autophagosome formation involved two conjugation pathways. Atg12, -5 and -16 are physically associated with the membrane, while Atg8/LC3 is conjugated to the PE that is inserted in the membrane. The autophagosome fuses with a lysosome in order to degrade its content.

autophagy may kill the developing premalignant cells and thereby prevent tumorigenesis.

Although anti-autophagic mutations are found in human tumors, its definitive role in human tumorigenesis remains unclear. The observation that the Beclin1 gene is just monoallelically lost<sup>55</sup> suggests that a certain level of Beclin1 expression is required for tumor cell survival. Furthermore, although some tumor types have a low Beclin1 expression, colorectal and gastric tumors show a higher expression of Beclin1 compared with normal cells.<sup>64</sup> Besides its potential tumor suppressive role in the early stages of tumorigenesis, autophagy has proposed to play a tumor-promoting role during the later stages of tumor growth. In this case, autophagy protects cells against stressful conditions. The hypothesis that autophagy can function as survival mechanism has been shown in growth factor-dependent cells from Bax/Bak-deficient mice. These cells activate autophagy upon growth factor withdrawal, which enables them to survive for several weeks. These cells die when autophagy is inhibited.<sup>65</sup> Importantly, experiments with established myc-induced lymphoma show that autophagy occurred in the surviving cells, whereas the other cells died through apoptosis upon activation of p53 in these cells. Inhibition of autophagy in this setting enhances tumor cell apoptosis and tumor regression. These in vivo experiments suggest a cytoprotective role for autophagy in established tumors.<sup>66</sup> Nevertheless, measuring fully functional autophagy needs to include degradation within autophagolysosomes, which cannot be determined in human tissues at this moment.<sup>67</sup> Thus, despite some connections between autophagy and tumorigenesis, the lack of good markers that can detect autophagy in vivo limits investigations in human tumor tissues at present.

#### Mitotic catastrophe

Mitotic catastrophe is defined as a type of cell death that is caused by aberrant mitosis. It is originally described in yeast, where cells die as a result of aberrant chromosome segregation.<sup>68</sup> In mammalian cells and particularly in tumor cells, mitotic catastrophe is mainly associated with deficiencies in cell cycle checkpoints.<sup>69,70</sup> To detect the occurrence of mitotic catastrophe, both morphological characteristics (such as enlarged and multinucleated cells) and the presence of mitotic defects (such as incomplete nuclear condensation, chromosome alignment defects, unequal DNA separation or mitosis in the presence of DNA damage) are used (illustrated in Fig. 1).<sup>69,71–74</sup>

Since the G2/M checkpoint is responsible for blocking mitosis in the case of damaged DNA, altered expression of proteins involved in this checkpoint is likely associated with mitotic catastrophe. Several studies indeed show a role for G2/M regulatory proteins. High expression levels of proteins that promote entry of mitosis (such as Cdk1 and cyclinB) as well as inhibition or knockout of proteins that prevent premature mitosis (such as ATR, ATM, Chk1, Chk2, Plk and 14-3- $3\sigma$ ) can induce mitotic catastrophe.<sup>75–79</sup> Since p53 induces a G2-arrest upon DNA damage via the Cdk-inhibitor p21, both p53 and p21 might play a role in preventing mitotic catastrophe as well.<sup>80</sup>

Next to defects in the G2/M checkpoint, defective mitotic spindle checkpoints have been linked to mitotic catastrophe, as such defects usually lead to missegregation of chromo-

somes. Adequate spindle functioning depends on proteins involved in the spindle formation (such as Mad and Bub), and on chromosomal passenger proteins (such as Survivin and Aurora kinases).<sup>81–84</sup> Conditional deletion of the Survivin gene indeed leads to disorganized mitotic spindles in early passage cells, and these cells finally die with morphological characteristics of mitotic catastrophe.<sup>85</sup> Also drugs that affect the mitotic spindle as well as specific downregulation of spindle checkpoint proteins or inhibition of aurora B kinase activity can result in aberrant mitosis.<sup>86,87</sup>

#### Mitotic catastrophe in tumorigenesis

Cells that survive abnormal mitosis can potentially divide asymmetrically, leading to aneuploid cells which are in general more tumorigenic. Also cells that go into mitosis with damaged DNA are more likely to acquire tumorigenic capacity as these cells are genetically unstable. Mitotic catastrophe might thus kill such cells and thereby prevent tumor formation. An observation that might support a tumor suppressive role for mitotic catastrophe comes from experiments in a colon cancer xenograft model, in which a dominant-negative mutant Survivin significantly induced mitotic catastrophe and apoptosis, and inhibited tumor growth.<sup>88</sup> High expression of Survivin is often found in tumor cells while it is rarely detected in normal tissue.<sup>89</sup>

In theory, Survivin might prevent cells to undergo mitotic catastrophe, and thereby promotes tumor development. However, the question remains whether high Survivin indeed prevents mitotic catastrophe. Survivin expression is also associated with tumor cell proliferation.<sup>90</sup> Moreover, this protein has been originally identified as a member of the Inhibitor of Apoptosis Proteins (IAPs).<sup>91</sup> Even though structural studies have shown that a direct inhibitory effect on caspases is unlikely,<sup>92</sup> a role for Survivin as apoptosis inhibitor cannot be excluded. In colorectal tumors, the expression of Survivin indeed inversely correlates with the level of apoptosis.<sup>90,93–95</sup> It is therefore difficult to distinguish the effects of Survivin on promoting proliferation and suppressing apoptosis from its effect on suppressing mitotic catastrophe.

In addition, some studies show that mitotic catastrophe can be followed by apoptosis, and it is therefore still a matter of debate whether mitotic catastrophe is a specific death process or just functions as a trigger for apoptosis.<sup>69,70</sup>

#### Necrosis

In contrast to apoptosis, necrosis has been considered as an uncontrolled form of cell death. Morphologically, necrosis is characterized by vacuolization of the cytoplasm, loss of membrane integrity and cellular swelling, as illustrated in Fig. 1. The resulting release of intracellular components into the microenvironment can provoke an inflammatory response. Although necrosis is usually a consequence of pathological traumas such as infection or ischemia, it can be induced by  $TNF\alpha$  or Fas ligand via their respective death receptors.<sup>96,97</sup> The latter observation points to the fact that necrosis may not be such an uncontrolled form of cell death as initially suggested. Indeed, growing evidence supports the idea that necrosis can be regulated. Death receptor-induced necrosis might depend on the kinase RIP1

(receptor-interacting protein 1); cells with downregulated RIP1 as well as RIP1-deficient Jurkat cells show partial resistance to Fas-induced cell death.<sup>98</sup> As reviewed by Festjens et al.<sup>99</sup> RIP1 likely targets the mitochondria resulting in excess formation of reactive oxygen species (ROS). ROS are considered to play a central role in necrosis, since the ROS scavengers efficiently prevent necrosis induced by several treatments.<sup>100–102</sup>

Besides death receptor/triggered necrosis, DNA damage (e.g. MNNG-induced) can result in necrosis as well. This necrotic death is mediated by PARP-1, a protein involved in DNA damage repair. Overactivation of this enzyme results in a drop of cellular NAD+ and ATP, hinting to a connection with the mitochondria.<sup>103,104</sup> In agreement with this hypothesis, PARP-1 activation has been shown to induce AIF translocation from the mitochondria to the nucleus, mediating a caspase-independent death.<sup>105</sup> PARP-1-mediated necrosis has been shown to depend on the proteins RIP1, TRAF2 and JNK1.<sup>106,107</sup> These observations altogether indicate that necrosis should no longer be exclusively viewed as an unregulated process. A regulated form of necrosis – also called necrosis-like programmed cell death – might be considered as a different type of cell death, besides accidental necrosis.<sup>108</sup>

#### Necrosis in tumorigenesis

Tumor cell necrosis can provoke an inflammatory response, and stimulate an immune response towards potentially malignant cells. In this case, necrosis might prevent tumor development. Experiments with  $TNF\alpha$  support this notion. As reviewed by Aggarwal et al., <sup>109</sup> TNF $\alpha$  is originally isolated as an anti-cancer cytokine, able to kill tumor cells and to induce tumor regression in mice. On the other hand. mice with impaired TNF $\alpha$  signaling, such as TNF $\alpha$ -/- and TNFR1-/mice, are less prone to develop tumors in inducible tumor mouse models.<sup>110,111</sup> It thus follows that  $TNF\alpha$  can also promote tumorigenesis. It has been proposed that chronic inflammation, in contrast to an acute inflammatory response, can promote tumor development. In agreement with the latter, patients with chronic inflammatory bowel diseases (IBD) have an increased risk of cancer development,<sup>112</sup> and patients with the familial adenomatous polyposis (FAP) syndrome show a significant reduction in the number and size of colorectal adenomas upon treatment with the anti-inflammatory drugs celecoxib. 113,114 Since necrosis can lead to inflammation and a sustained inflammatory response can stimulate tumor development, these data provide some indirect evidence for a role of necrosis in tumor development. However, due to its unregulated nature, it is almost impossible to experimentally prevent or induce necrosis in vivo without affecting other types of death. Whether necrosis plays a major role in tumorigenesis is therefore still unclear.

## The role of the cell death pathways in cancer patients' treatment

#### Apoptosis as prognostic marker

Many different anti-apoptotic modifications are found in human tumors, and resistance to apoptosis most likely is re-

quired for tumor cells to survive. This resistance could therefore be associated with a poor prognosis for cancer patients. In order to evaluate its prognostic value, several studies have scored the expression of a single apoptosisassociated protein, such as p53, Bcl-2 and/or Bax, and have correlated their expression with prognosis. As reviewed by Brown and Wilson,<sup>115</sup> studies on solid tumors present conflicting data; some show significant correlations with good or poor prognosis, whereas others describe no significant associations. A limitation in scoring a single protein is that the expression of a single protein may not reflect the level of apoptosis since apoptosis is regulated by a complex network of proteins. Indeed, the expression of Bcl-2 or p53 does not always correlate with the number of apoptotic cells.<sup>116–118</sup> Therefore, apoptosis might be more adequately determined by evaluating the exact number of apoptotic cells in tumor tissues by a TUNEL assay (detects DNA fragmentation) or by staining with the M30 antibody (recognizes caspase-cleaved cytokeratin-18).

We have summarized studies that evaluate the association between apoptosis and prognosis for colorectal cancer patients. Most of these patients are treated by surgery only, allowing evaluations regarding the predictive value of 'spontaneous' apoptosis which is not influenced by (neo-) adjuvant treatments (Table 1). Although these studies are evaluated retrospectively, the prevalent finding in rectal cancer patients is a positive correlation with higher spontaneous apoptosis favoring less local recurrence development when treated with surgery only.<sup>119,120</sup> Despite one study,<sup>121</sup> all others show no effect of apoptosis on the development of distant recurrences and survival.<sup>117–120,122</sup> For colon cancer patients, local tumor control is not a major clinical issue, and studies focus on survival. Whereas some studies found a positive association between high apoptosis and good prognosis,<sup>123,124</sup> others show no<sup>124–127</sup> or a negative association.<sup>118,128,129</sup>

The observation that high levels of apoptosis in primary rectal tumors are associated with a better local control after surgery may reflect a capacity of local effectors, such as cytotoxic T cells and natural killer cells, that can control tumor cells outside the resection margin. In this case, lowapoptotic tumors may have a less aggressive microenvironment. An alternative explanation might be that low-apoptotic tumor cells are less sensitive to apoptotic triggers, and thus more likely to survive and develop a local recurrence. A review on prognostic markers in rectal carcinoma shows that high levels of apoptosis in pre-treatment biopsies correspond with more tumor regression upon pre-operative radiochemotherapy.<sup>130</sup> This observation supports the notion that spontaneous apoptosis may reflect the cells' sensitivity to local apoptotic triggers. The fact that survival largely depends on distant recurrences rather than local control can explain the lack of consistent correlations with survival for both rectal and colon cancer patients.

Focusing on the prognostic value of apoptosis, several studies on rectal carcinoma show that patients with high-apoptotic tumors have a good prognosis, and most likely will not benefit from pre-operative radiotherapy.<sup>119,120,131</sup> Thus, for rectal cancer patients and likely for other cancer types as well, apoptosis can be of clinical use as a marker to select those patients that have a relatively high risk to develop a local recurrence and need (neo-)adjuvant to reduce this risk.

1st author	Patients	Treatment	Outcome	
Tannapfel <sup>122</sup>	32 Rectal	Neoadjuvant CRT	No correlation with recurrences	=
Schwandner <sup>117</sup>	160 Rectal	Adjuvant CRT for TNM II - III	No correlation with recurrences and survival	=
Adell <sup>119</sup>	162 Rectal	Randomized for neoadjuvant RT	High apoptosis less local recurrences, not survival	+
Rodel <sup>121</sup>	44 Rectal	Neoadjuvant CRT	High apoptosis less recurrences	+
Hilska <sup>118</sup>	124 Rectal	-	No correlation with survival	=
de Bruin <sup>120</sup>	1198 Rectal	Randomized for neoadjuvant RT	High apoptosis less local recurrences, not survival	+
Langlois <sup>123</sup>	74 Colorectal		High apoptosis better survival	+
Sinicrope <sup>124</sup>	64 Proximal colon		No correlation with survival	=
	82 Distal colon		High apoptosis better survival	+
Michael-Robinson <sup>125</sup>	100 Colorectal		No correlation with survival	=
Elkablawy <sup>126</sup>	53 Colorectal		No correlation with survival	=
Rupa <sup>128</sup>	278 Colorectal		High apoptosis worse survival	_
Noguchi <sup>127</sup>	80 Colorectal		No correlation with survival	=
Bendardaf <sup>129</sup>	49 Colorectal		High apoptosis worse survival	_
Hilska <sup>118</sup>	239 Colon		High apoptosis worse survival	_

Table 1 Prognostic value of apoptosis for (colo)rectal cancer patients

Note: in the case of neoadjuvant chemoradiotherapy (CRT), apoptosis was determined in pre-treatment biopsies.

#### Apoptosis as therapeutic target

The guestion is whether the inherent resistance to apoptosis of tumor cells influences their responses to anti-cancer therapies. It has been proposed that failure to undergo apoptosis can result in treatment resistance. Indeed, in vitro experiments often show that anti-apoptotic modifications in tumor cells, such as Bcl-2 overexpression, suppress cell death induced by radiation or chemotoxic drugs and mice with myc-driven lymphomas with impaired apoptosis fail to respond to chemotoxic drugs.<sup>36,132</sup> However, other in vivo experiments cannot find an association between apoptosis resistance and treatment failure. For example, the response of established HCT116 tumors is similar for cells overexpressing Bcl-2 or not, while these cells clearly differ in apoptosis resistance in in vitro assays.<sup>3</sup> Hence, the effect of apoptosis resistance to the overall treatment effect is likely to depend on the specific tumor type. It has been suggested that resistance towards apoptosis might be more important for therapy resistance of hematopoietic malignancies rather than solid tumors of epithelial origin.<sup>3</sup>

For solid tumors, several anti-cancer treatments have been shown to trigger apoptosis in tumor tissues. For example, 5-fluorouracil or radiotherapy increases the level of apoptosis in colorectal tumors.<sup>119,133</sup> However, the clinical outcome of the patients appears not to be influenced by this induced apoptosis, as radiotherapy reduces local recurrence rates for rectal cancer patients irrespective of the level of apoptosis after the treatment.<sup>119,120</sup> In agreement, two reviews describe a modest role for apoptosis in response to radiotherapy in several other solid tumor types.<sup>3,134</sup>

Apparently, the success of treatment does not solely depend on the induction of apoptosis. An explanation can be that radiotherapy and most chemotherapeutics are not designed to specifically induce apoptosis. Alternatively, tumor cells are intrinsically resistant to apoptosis, and other types of cell death compensate for this apoptosis block.<sup>135,136</sup> Exploring non-apoptotic types of cell death might therefore provide new opportunities for a more effective anti-cancer approach.

#### Autophagy in cancer treatment

Markers that easily detect autophagy in vivo are not available at present, limiting investigations regarding its prognostic value for cancer patients. Nevertheless, experimental settings and mouse tumor models have shown that autophagy can be induced by radiation and chemotherapy in tumor cells.<sup>69,137–140</sup> However, seemingly conflicting data have been published as to whether autophagy exerts a positive or negative effect on treatment response. Some data suggest that autophagy functions as survival mechanism. For example, inhibition of autophagy by specific drugs enhances the radiosensitivity of malignant glioma cells in an experimental setting.<sup>140</sup> Inhibition of autophagy also enhances the induction of apoptosis, increases tumor regression and results in delayed tumor outgrowth in a lymphoma mouse model.<sup>66</sup> Autophagy can thus protect cells from death. In agreement with this hypothesis, induction of autophagy by rapamycin protects various tumor cell lines against the induction of apoptosis.<sup>141,142</sup> Autophagy inhibitors as adjuvant treatment could thus enhance the effect of apoptosis-inducing anti-cancer therapies for cancer patients.

On the contrary, experiments with cells that have impaired apoptosis show that these cells are more sensitive to radiation than wild-type cells via the induction of autophagy.<sup>143–145</sup> This radiosensitivity was, however, determined using clonogenic survival assays, measuring the percentage of cells that can form a colony after radiation. Such assays are not only affected by cell death but also by a delay or stop in proliferation; effects suggested to occur during autophagy.<sup>54</sup> Autophagy can thus be a survival mechanism, which reduces but preserves the cells' clonogenic potential for a period of time. Another possible explanation for these contradictory findings can be that autophagy needs to reach a certain threshold before it results in cell death. If this is the case, induction of autophagy as adjuvant treatment could enhance the effect of anti-cancer therapies. In line with this idea is the observation that clinical application of mTOR inhibitors result in a prolonged survival of patients with metastatic renal cancer or breast cancer.<sup>146–148</sup> Obviously, these data are not fully conclusive regarding the role of autophagy in treatment response, since the anti-tumor effects of inhibiting mTOR could reflect its role in cell cycle regulation or translation as well. It is thus still unclear whether treatment-induced autophagy functions as a cell death mechanism or as a mechanism by which tumor cells are able to survive.

#### The prognostic value of Mitotic catastrophe

There is some evidence for the hypothesis that mitotic catastrophe influences prognosis. High expression of proteins involved in entering mitosis, as Plk1 and CDC25B phosphatase, is associated with poor prognosis for colorectal cancer patients.<sup>149,150</sup> *In vitro* studies show that a constitutively active mutant of Plk1 can indeed override growth arrest induced by DNA damage, resulting in aberrant mitosis.<sup>151</sup>

The association between poor prognosis and the inability of the tumor cells to induce mitotic catastrophe-associated death is also supported by studies that evaluate the expression of Survivin. For colorectal cancer patients, high Survivin expression is indeed associated with an increased tumor recurrence risk and shorter survival for colorectal cancer patients.<sup>93–95,115,152</sup> Also for multiple myeloma, high Survivin correlates with more advanced stages.<sup>153</sup> A review by Brown and Gilson addresses the role of Survivin on the outcome of surgery, and shows that increased expression of Survivin is usually associated with poor clinical outcome in a variety of human solid tumors. However, as described above, the link between Survivin expression and mitotic catastrophe is far from clear.

#### Mitotic catastrophe as therapeutic target

Tumor cells are frequently deficient in their cell cycle checkpoints. It allows cells to enter mitosis without an arrest that allows for DNA repair. This implies that tumor cells can be susceptible to mitotic catastrophe induction, particularly when treated with DNA damaging agents.<sup>154,155</sup> Indeed, mitotic catastrophe has been pointed out as an important form of death in solid tumors upon irradiation.<sup>3,69,134,156</sup> Experimental settings show, for example, that twelve of fourteen solid-tumor cell lines display mitotic catastrophe when treated with the DNA damaging drug doxorubicin, while only two lines die through apoptosis.<sup>6</sup> In addition, an increase in mitotic catastrophe can compensate for impaired apoptosis, resulting in similar overall cell death as for cells with functional apoptosis. 69,135,136 A more recent study confirms these in vitro data in an in vivo experiment. Established tumor cells with impaired cell cycle checkpoints show a mitotic catastrophe response that corresponds with enhanced tumor regression when treated with DNA damaging drugs.<sup>73</sup> Another finding favoring a role for mitotic catastrophe *in vivo* is the observation that the largest cell death effects induced by radiotherapy are not at the time of treatment, but several days later when cells re-enter the cell cycle.<sup>157</sup> This late form of death has been characterized by various abnormal mitotic characteristics, and thus might be related to mitotic catastrophe.<sup>69,115</sup>

The exact molecular mechanisms of mitotic catastrophe are largely unknown, and molecular markers have not been defined to distinguish mitotic catastrophe from other forms of cell death in tumors of cancer patients. Such markers are awaited with excitement, as these allow confirmation or rejection of the above-described hints regarding mitotic catastrophe's contribution to overall cell death *in vivo*.

#### Necrosis in cancer treatment

It is known from *in vitro* experiments that necrosis is induced by anti-cancer drugs, particularly by DNA-alkylating drugs. DNA-alkylating agents have shown to cause necrotic cell death via activation of PARP-1. This necrosis occurs with equal effectiveness in cells with or without functional apoptosis.<sup>158</sup> Interestingly, especially cells using aerobic glycolysis are shown to be sensitive for this PARP-mediated necrosis.<sup>158</sup> Since many tumor cells depend on aerobic glycolysis, this observation suggests that tumor cells in particular might be killed through necrosis upon treatment with alkylating agents.

In cancer patients, apoptotic cell death can be discriminated from necrotic cell death by measuring the size of DNA fragments, or by screening the different forms of cytokeratin-18 (caspase-cleaved versus non-cleaved) in plasma samples.<sup>159,160</sup> Interestingly, patients with endometrial tumors show predominantly the non-cleaved form of cytokeratin-18 after treatment with chemotherapy.<sup>161</sup> In agreement with this observation, chemotherapy induces more necrotic than apoptotic cell death in breast cancer patients, and this necrotic response is associated with a better survival.<sup>162</sup> At present, it is, however, not clear whether the presence of non-cleaved cytokeratin-18 in serum marks necrotic cell death specifically, or whether it measures non-apoptotic cell death in general. Nevertheless, these in vivo data show that current anti-cancer therapies are well capable of inducing non-apoptotic cell deaths.

#### **Concluding remarks**

Almost all human tumors have acquired anti-apoptotic modifications. Therefore, it is tempting to conclude that an intact apoptotic pathway is likely tumor suppressive and that inhibition of apoptosis is necessary for tumor development. In line with this assumption, researchers have speculated that defective apoptotic pathways result in therapy resistance, since many cancer therapies induce apoptosis in tumor cells.

In the case of solid tumors, this inferral may not hold very well. The large majority of colorectal tumors, for example, show a defective p53 pathway, but radiation still induces some apoptosis. However, a correlation between defective apoptosis and radiotherapy resistance is not established unambiguously. Even if the level of apoptosis after treatment is low, the irradiated rectal cancer patients still have a better prognosis than non-irradiated patients. It is therefore likely that other radiation-induced effects, leading to death or permanent growth arrest, are also involved in the overall treatment response. In the case of radiotherapy, mitotic catastrophe-associated death is thought to play a prominent role. However, at present mitotic catastrophe is difficult to discriminate from other forms of cell death, and therefore might well be accompanied with apoptosis. It will be interesting to see future research in this area especially when reliable markers for the different forms of cell death become available.

The observation that apoptosis may not be the most dominant form of cell death for solid tumors might be due to the fact that conventional therapies, such as radiotherapy, are not specifically designed to induce apoptosis. These therapies may result in apoptosis as a secondary effect, due to induced cellular damage. Enhancement of the apoptotic potential of such therapies may thus increase the overall tumor cell death.

Currently, recombinant soluble TRAIL or agonistic TRAIL antibodies are being evaluated in clinical trails, and seem promising when combined with radiation.<sup>163</sup> In addition, the recently discovered small-molecule inhibitor of antiapoptotic proteins such as the Bcl-2 family proteins (ABT-737) or IAPs sensitizes many tumor cells to cytotoxic agents *in vitro*.<sup>119,164</sup> It will be interesting to see whether the same observation can be made *in vivo* when these drugs make their way into clinical trials. Since apoptosis is an effective and tidy way to eliminate tumor cells, such a direct induction of apoptosis may have great therapeutic potential.

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