

## SHORT REVIEW

## APOPTOTIC PROTEINS AND CANCER: MANY FACES

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## INTRODUCTION

Complex genetic and epigenetic alterations that disrupt the physiological regulation of apoptosis are thought to be strategically critical for the carcinogenic process and are thought to provide survival and growth advantages for cancerous cells. Targeting and the induction of apoptosis (programmed cell death) is an attractive target for successful cytotoxic therapy for many different types of cancer, including leukaemias and lymphomas.<sup>1,2</sup>

**Bcl-2 protein**

In normal liver cells, Bcl-2 family members have essential roles in liver homeostasis. On other hand, in carcinogenesis, these proteins play a significant role by suppressing apoptotic death rather than stimulating cell proliferation. The up-regulation of either Bcl-2 or Bcl-xL in mouse liver has been shown to protect hepatocytes from Fas-induced apoptosis and, therefore, liver destruction in a dose-dependent manner.<sup>3</sup> Recent data from our work demonstrated the protein expression of cytoplasmic Bcl-2 in 16% of chronic HCV patients with no hepatocellular carcinoma (HCC) versus 8% in patients with HCC.<sup>4</sup> In HCC, Bcl-2 is significantly down-regulated while Bcl-xL is predominately expressed.<sup>5</sup> However, Fiorentino *et al* reported consistent increased level of Bcl-2 RNA in HCC which may be suggest a post-transcription down-regulation of Bcl-2 at the protein level.<sup>5</sup>

Another interesting finding was the delay of liver tumour development in concordance with Bcl-2 up-regulation in TGF $\alpha$ /Bcl-2 double transgenic mice<sup>6</sup> which appears to inhibit c-myc-induced liver carcinogenesis<sup>7</sup>. However, the in vivo electrophoretic transfer of Bcl-2 antisense oligonucleotide (ASO) into liver demonstrated inhibitory effects on HCC in rat models.<sup>8</sup> The deferential expression of Bcl-2 with the regards to tumours development could be contributed to the expression status of p53. The expression of Bcl-2 is significantly up-regulated in p53-positive HCC tissue while down-regulated in p53-negative tissues.<sup>9</sup>

Changes in p53 and Bcl-2 protein expression are a molecular hallmarks during hepatocarcinogenesis<sup>10</sup> that occur in concordance with high expression of the proliferating cell nuclear antigen (PCNA) and loss of differentiation and HCC progression.<sup>11</sup> The PCNA

expression is significantly elevated in late G1 and S phases of proliferating cells, and has been used as a biomarker for progression in different types of cancers including HCC.<sup>12</sup> The PCNA over-expression was even considered as an indicator for increased risk of HCC development in HCV-infected patients.<sup>13,14</sup>

Additionally, it was demonstrated that the cell division rate and subsequently the size of thymocytes population in vivo is significantly reduced by the expression of Bcl-2 or Bcl-xL in some reports<sup>15</sup> while these two proteins can also inhibit apoptosis of dividing cells<sup>16</sup>. Bradley *et al* demonstrated that over-expression of Bax and Bcl-2 in T-cell of transgenic mice can result in disturbing the cell cycle of dividing thymocytes. It was found that while Bax has stimulatory effects, Bcl-2 has inhibitory effects on cell cycle of cycling thymocytes. Furthermore, in activated T-cells, Bcl-2 overexpression was seen to delay the protein degradation of the tumour suppressor gene p27, whereas Bax accelerated that.<sup>17</sup>

Haemopoitic stem cells (HSC) with over-expressed Bcl-2 in Bcl-2-transgenic mice generated by Demon *et al* were reported to remain viable after growth factor withdrawal whereas HSC from WT mice did not survive in the absence of growth factor. It was demonstrated that HSC from Bcl-2-transgenic mice responded to pro-growth factors (such as IL-1, IL-3, IL-6, SCF and Flt3-ligand) with significantly faster and more extensive proliferation with more delay in the cell cycle entry when compared with that from WT mice. Interestingly, when cultured with SCF, only 20% of WT HSC remained viable after one week, while HSC from Bcl-2-transgenic mice demonstrated greater survival capabilities and more extensive proliferation. It was concluded that over-expression of Bcl-2 and SCF/c-kit signalling pathway are sufficient for HSC proliferation. However, one should note that proliferation also participated into the transformation of progenitor cells to the myeloid lineage.<sup>18,19</sup>

**p53, Fas and Apaf-1**

A study by our group pointed out that in blast crisis (BC) of chronic myeloid leukaemia (CML) the p53 expression is significantly increased when compared with the chronic phase of CML.<sup>20</sup> Interestingly, while relatively high p53 expression was in general detected

along with up-regulation of apoptosis activating factor (Apaf-1), a significant down-regulation of Apaf-1 was oddly seen when p53 had become clearly over-expressed suggesting a disturbance in the p53 pathway (11, CML paper). Data from our group suggested decreased expression level of p53 and Apaf-1 in patients with BC.<sup>21</sup> It seems that the Apaf-1 up-regulation by several oncoproteins such as E2F1 is mechanistically critical for facilitating the apoptosome assembly. Furthermore, Kannan *et al* demonstrated the presence of point mutation, deletions and other genomic rearrangements of p53 gene in 25% of BC and that p53 is an upstream regulator of Apaf-1.<sup>22</sup> In fact, we previously suggested a link between increased expression of p53, decreased expression of Apaf-1 and lack of Fas expression in one hand and progression of CML. Therefore, one should be careful towards understanding the significant up-regulation of these pro-apoptotic genes in BC transformation as well as in response to therapeutic approaches.

For any normally growing cell population molecular defects either at gene level, mRNA level or protein level for genes regulating proliferation and apoptosis during cell cycle can interfere with the balance between cell division and apoptosis for that cellular population *in vivo* and provide strategically advantageous scenario for carcinogenesis.<sup>23</sup> Our previous results indicates that apoptosis (via Fas-FasL) play a role in regulating haemopoietic progenitor cell kinetics in humans as it does in mice. It also showed that caspases activation was required for the myeloid maturation.<sup>24,25</sup>

### Cell cycle proteins

Genes regulating apoptosis have significant impact on the cell cycle. A number of studies demonstrated that cell-cycle regulators could interconnect with proliferation and apoptosis.

Both p16<sup>-/-</sup> and p21<sup>-/-</sup> mice are deficient in key cell cycle genes, while *lpr* and *gld* mice (Fas and FasL mutant mice, respectively) have a defective apoptotic mechanism.<sup>24</sup> However, Lewis *et al*<sup>26</sup> showed that p16<sup>-/-</sup> knockout mice have a higher self-replication capacity than do wild-type (WT) mice, which links the cell cycle and apoptosis. Similarly, p21<sup>-/-</sup> knockout mice have a higher self-replication capacity (i.e., cell proliferation) than do WT mice.

We showed that both *lpr* and *gld* mice have a higher self-replication (i.e., cell proliferation) capacity than do WT mice, which links apoptosis and proliferation.<sup>24</sup> Miyashita *et al*<sup>27</sup> showed that the restoration of p53 function resulted in down-regulation of Bcl-2 levels and the occurrence of apoptosis. They also showed that p53 activates the Bax promoter and induces high levels of Bax mRNA and protein. Moreover, Yin *et al*<sup>28</sup> showed that Bax is required for

50% of p53-induced apoptosis. Gomez *et al*<sup>29</sup> demonstrated a relationship between p27, cdk2 and apoptosis in thymocytes, which was modulated by p53, Bcl-2 and Bax. Thus, cdk2 activation seems to be the key point at which the cell cycle and apoptosis meet.

Janicke *et al*<sup>30</sup> showed that the retinoblastoma (RB) gene is cleaved during apoptosis, at the caspase consensus cleavage site (DEAD), resulting in a protein product of 50 kDa. Dou *et al*<sup>31</sup> showed that RB is also cleaved on an interior site, producing proteins of 48 and 68 kDa. Fattman *et al*<sup>32</sup> demonstrated that caspase-3 and caspase-7 cleave RB at the DSID cleavage site, resulting in proteins of 68 and 48 kDa.

These findings support a two-step model for RB cleavage and a promoting role in chemotherapy-mediated apoptosis. Browne *et al*<sup>33</sup> demonstrated that RB is cleaved at the carboxyl terminal, producing 43- and 30-kDa protein fragments. In addition, ZVAD was found to inhibit the cleavage of RB, poly-ADP-ribose polymerase (PARP) and apoptosis. In contrast, YVAD did not inhibit primary carboxyl terminal cleavage of RB and PARP. These results suggest that different caspases are responsible for the cleavage of different substrates during apoptosis.

In contrast, Suzuki and colleagues<sup>34</sup> demonstrated that survivin interacts with cdk4, and, as a result, p21 is released from its complex with cdk4 and interacts with pro-caspase-3 in mitochondria, resulting in inhibition of apoptosis. Cell-cycle transitions are mediated through multiple phosphorylations of cyclin-cdk complexes. RB phosphorylation releases E2F transcription factor, which activates certain genes during S phase. Activation of p21 results in negative regulation of the cell cycle. P21 interacts with cdk and PCNA, resulting in a block on DNA replication and subsequent cell-cycle progression. p21<sup>-/-</sup> mice are also unable to stop the cell cycle in G1 in response to DNA damage. As with p21, p27 inhibits cyclin E and A binding to cdk2, and both p21 and p27 are absent from non-proliferating cells.

A further relevant interaction in the regulation of apoptosis is inhibition of p53 function mediated by Mdm-2, which is cleaved by caspase-3. This implies the existence of an auto regulatory loop during p53-induced apoptosis. Activation of p53 potentially can amplify p53 apoptotic signalling in the cell by stimulating caspase-3-dependent cleavage of Mdm-2. Consistent with this interpretation, Bcl-2 can also increase Mdm-2 activity and inhibit p53-induced apoptosis.

### TOSO, Survivin, and TRAIL

When examined, leukaemic cells from patients with adult T-cell leukaemia shows significantly increased expression of Fas.<sup>35</sup> Flow cytometric analysis for Fas expression in samples from 28 multiple myeloma patients showed that about half of the patients were

positive for Fas.<sup>36</sup> Also, the expression of Fas was detected in 5–41% of CML patients.<sup>37</sup> To our knowledge, only few studies reported the expression of Fas on AML cells. Despite high expression of Fas receptors in on these AML cells, cells were responsive to the induction of apoptosis following ligation of CD95 by CH11.<sup>38,39</sup> Also, and despite of expression of Fas receptor leukaemic cells from some AML patients demonstrated resistance to Fas-mediated apoptosis *in vitro*.<sup>39</sup> In fact, additionally, resistance to apoptosis mediated by TNF receptor 1-, and TRAIL-R has been reported in leukaemic cells from AML patients as well as human leukaemia cell lines although their expression was normal.<sup>40–44</sup> More mechanisms for receptors-mediated apoptosis in AML cells are being reported.<sup>45</sup>

Abnormalities of anti-apoptotic machinery (e.g., Bcl-2 family), and abnormalities in adaptor molecules (e.g., down-regulation or absence of FADD expression) predicts resistance therapeutic strategies of Death Receptors-mediated apoptosis and, subsequently, demonstrate poor prognosis.<sup>46</sup> One molecule may be involved in creating resistance to Fas-mediated apoptosis is TOSO has been shown to be expressed in the spleen, lymph nodes, thymus and peripheral white blood cells.<sup>47</sup> In previous work, we assessed the expression of TOSO protein in normal cells, CD43<sup>+</sup> leukaemic cells, and some AML cell lines and found it constitutively expressed in all samples despite variable expression levels which further suggested TOSO involvement.<sup>48</sup>

A recently described member of inhibitor of apoptosis proteins (IAP) family, survivin, has been shown to be expressed broadly in myeloid leukaemia cell lines and almost in all primary AML samples examined.<sup>49,50</sup> Indeed, we reported similar findings in myeloid leukaemia cell lines and primary AML blasts, while it was not detected in normal CD34<sup>+</sup>.<sup>51</sup> Another report also demonstrated the lack of survivin expression in haematopoietic progenitors suggesting the absence of survivin expression in normal bone marrow progenitors.<sup>52</sup> Due to its inhibitory effects on cell death induced by different stimuli *in vitro* survivin has been suggested to play a role in weakening the apoptotic indices and poor therapeutic response in solid tumours *in vivo*.<sup>53,54</sup>

Earlier reports demonstrated the activation of ERK pathway by TRAIL in resistant and normal CD34<sup>+</sup> cells.<sup>55</sup> This observation supporting earlier studies that reported the transmission of cell proliferation and survival, instead of death signals, by Fas ligation through activating ERK pathway.<sup>56</sup> Recent data came from two groups demonstrated that phosphorylated ERK and/or PI3 kinase pathways resulted in the up-regulation of survivin gene.<sup>57,58</sup> It seems the diversity of TRAIL receptors contribute to the variable levels of sensitivity to TRAIL-induced apoptotic signals in

primary AML cells and the up-regulation of survivin in some AML samples but not all which reflects the heterogeneity in sensitivity to TRAIL in primary AML cases.<sup>59</sup> While therapeutic strategies targeting survivin will require mechanistic validation in a large-size scale studies to determine the usefulness of this approach, survivin has been suggested to be an attractive target for gene therapy for cancer.<sup>60</sup>

### Apoptosis stimulating proteins of p53 (ASPP)

Apoptosis stimulating proteins of p53 (ASPP-1 and ASPP-2) are a novel family of proteins that have been found to co-stimulate p53 activation of Bax (Bcl-2 associated protein X) inducing caspase-mediated apoptosis. ASPP, a recently described family of apoptosis-regulating proteins, are tumour suppressor genes that particularly enhance the binding and trans activation of p53 during the transcription of pro-apoptotic genes.<sup>61,62</sup> Members of the ASPP family have been reported to be abnormally expressed in various cancers.<sup>63,64</sup>

We determined if ASPP-2 has a role in differentiation by examining the ASPP-2 expression in three cancerous tissues with graded differentiation patterns: bladder, prostate and breast cancers along with cervical pre-malignant intraepithelial neoplasia. Data obtained suggested that the ASPP-2 expression is increased as the differentiation degree increased. The correlation was even clear in the cervix which had two very distinctive patterns of staining within the stratified squamous epithelium.<sup>65</sup> In two mouse models, it have been shown that the haplo-insufficiency (i.e., being expressed from one single functional copy of a gene) of ASPP-2 underlines its role the carcinogenic process.<sup>66</sup>

### CONCLUSION

The presence of fully functional genes that regulate both the cell cycle and apoptosis will maintain the balance between the rate of cell division and apoptosis in any population *in vivo*. Therefore, malfunction in, or loss of, any of these genes may lead to an increase in their self-replication.

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