MINI-REVIEW

Targeted Silencing of Inhibitors of Apoptosis Proteins with siRNAs: A Potential Anti-cancer Strategy for Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies, with a very poor prognosis. Despite significant improvements in diagnosis and treatment in recent years, the long-term therapeutic efficacy is poor, partially due to tumor metastasis, recurrence, and resistance to chemo- or radio-therapy. Recently, it was found that a major feature of tumors is a combination of unrestrained cell proliferation and impaired apoptosis. There are now 8 recognized members of the IAP-family: NAIP, c-IAP1, c-IAP2, XIAP, Survivin, Bruce, Livin and ILP-2. These proteins all contribute to inhibition of apoptosis, and provide new potential avenues of cancer treatment. As a powerful tool to suppress gene expression in mammalian cells, RNAi species for inhibiting IAP genes can be directed against cancers. This review will provide a brief introduction to recent developments of the application IAP-siRNA in tumor studies, with the aim of inspiring future treatment of HCC.

Keywords: Hepatocellular carcinoma - IAP - caspase - siRNA - therapy

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide (Bosch et al., 2004). While earlier diagnosis and more effective treatments mean that the prognosis of HCC has significantly improved in recent years, tumor recurrence and metastasis are still major obstacles to long-term survival. Although liver transplantation is considered a curative treatment for HCC, the overall recurrence rate after the procedure, attributable to distance metastasis or intrahepatic reappearance, could be as high as 65% and 43% , respectively (Wong, 2002).

Apoptosis is a form of programmed cell death that plays a crucial biological function in development and homeostasis in both vertebrates and invertebrates. It is also considered to be the major method of eradicating tumors. Many regulators of apoptosis have been identified, and one of these, inhibitor of apoptosis protein (IAP), is documented to have abnormal expression in tumor cells. The IAP gene, encoded by a viral gene, was first identified in insect SF-21 cells infected by baculovirus, could inhibit infected SF-21 cells from undergoing apoptosis (Crook et al., 1993; Wei et al., 2008). Up to now, 8 IAP-family members have been identified in human cells: NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), XIAP (BIRC4), Survivin (BIRC5), Bruce (BIRC6), Livin (BIRC7), and ILP-2 (BIRC8) (Vucic and Fairbrother, 2007). Members of the IAP family are defined by the presence of a baculovirus IAP repeat (BIR) protein domain (LaCasse et al., 2008), but some members of this family (XIAP, c-IAP1, c-IAP2, and Livin) also have a RING domain that allows these proteins to act as E3 ubiquitin ligases (Eckelman et al., 2006). Although the main role of IAP is to act as an endogenous inhibitor of caspases (the main executioners of apoptosis) by binding to their BIR domains, the E3 ubiquitin ligase activity gives IAPs the ability to promote the ubiquitination and subsequent proteasomal degradation of caspases, TRAF2, and additional partners (Vucic and Fairbrother, 2007).

Caspases, the primary mediators of apoptosis, are cytosolic cysteine proteases that, once activated, initiate an irreversible cascade of events that culminates in rapid cell death (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998). Caspase inhibition occurs mainly by binding to the unique BIR domains of IAP. NAIP, c-IAP1, c-IAP2, and XIAP have three BIR domains, while other family members possess only one BIR domain. In IAPs with three BIR domains, the third BIR mediates the binding to, and inhibition of caspase-9, an initiator caspase capable of processing and thereby activating other caspases (Srinivasula et al., 2001; Shiozaki et al., 2003). Interaction of caspase-9 with the third BIR domain of IAPs prevents its homodimerization, inhibiting its activity (Shiozaki et al., 2003). The second BIR domain and the preceding linker region cooperatively mediate the interaction of IAPs with caspase-3 and -7, the effector caspases that are activated by initiator caspases. Effector caspases are the common downstream caspase involved in the

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activation of apoptotic pathways, and upon activation they proteolytically cleave the cellular substrates with which they interact. The binding of IAPs to caspases prevents the interaction between caspases and their cellular substrates, and thus inhibits the proteolytic degradation of the cell that marks the final execution stage of apoptosis (Chai et al., 2001; Huang et al., 2001).

One of the most efficient ways to inhibit specific genes is by RNA interference (RNAi) technology, coined the "Science Breakthrough of the Year" for 2002 by the Journal of Science (Couzin, 2002). RNAi refers to a group of related gene-silencing mechanisms, in which the terminal effector molecule is a short antisense RNA (Izquierdo, 2005). It is already a valuable, and widely used, research tool for helping to understand genetic abnormalities and the molecular mechanisms of disease by silencing targeted genes, especially in cancer research (Chang, 2007). RNAi is a post-transcriptional gene-silencing tool that works by cleaving specific sequences of mRNA to which the designed dsRNA is complementary. These dsRNAs are processed and cut by the enzyme Dicer, a member of the RNase β family of ribonucleases (Bernstein et al., 2001), into 21-to-23-nucleotide short interfering RNAs (siRNAs). The siRNAs are bound to an RNA-induced silencing complex (RISC), through which the double-stranded siRNA is unwound (Chang, 2007). Meanwhile RISC, to which the antisense strand remains incorporated, guides the complex to cleave and degrade the perfectly complementary target mRNA (Karagiannis and El-Osta, 2005; Shankar et al., 2005).

Hepatocellular Carcinoma (HCC) is a major health problem, being the sixth most common cancer worldwide (Llovet and Bruix, 2008). The survival rate of patients is low, due in part to aberrant tumor metastasis, recurrence, and resistance to chemo- and radiotherapy. The combination of unrestrained cell proliferation and impaired apoptosis plays a major role in the progression of tumorigenesis. Along with the identification of the IAP family rendering tumor cells resistant to apoptosis in HCC (Augello et al., 2009; Hung et al., 2012; Liu et al., 2013; Wei et al., 2013) and the development of siRNA-producing expression vectors, stable RNAi usage is now considered a powerful tool for silencing IAP genes in HCC research. The following section will review the recent development of novel IAP-siRNA tools (particularly XIAP, Survivin, and Livin) in tumor studies, and the promising future of siRNA as a therapy for hepatocellular carcinoma.

Targeted silencing of XIAP by siRNA

Of all IAPs, XIAP (also known as ILP-1, MIHA or BIRC4 (Vucic and Fairbrother, 2007)) is the most potent inhibitor of caspases. XIAP can directly interact with the initiator caspase, caspase-9, through its BIR2 and BIR3 domains, suppressing its activation and hence the activation downstream effector caspases, (Mansouri et al., 2003)caspase-3 and-7 (Shi, 2002). Structure-function analysis of XIAP showed that it uses the second BIR domain, together with the immediately preceding linker region, to bind to and inhibit caspase-3 and -7, and its third BIR domain to specifically inhibit caspase-9 (Salvesen and Duckett, 2002). Since the effector caspases are the executors of apoptosis, XIAP can potently block these apoptotic pathways.

Increased XIAP expression has been reported in variety of human tumors, including HCC (Shiraki et al., 2003; Notarbartolo et al., 2004; Shi et al., 2008; Fabregat, 2009; Che et al., 2012), esophageal carcinoma (Zhang et al., 2007), clear cell renal carcinoma (Ramp et al., 2004; Bilim et al., 2008; Kempkensteffen et al., 2009), ovarian carcinoma (Mansouri et al., 2003; Ma et al., 2009), and lymphoma (Akyurek et al., 2006; Cillessen et al., 2008). Shiraki K et al. reported that 14 out of 20 (70%) HCC tissues demonstrated moderate or strong cytoplasmic staining for XIAP by immunohistochemistry (Shiraki et al., 2003). In addition, the median survival time for patients with high XIAP expression is short compared with patients with low or zero XIAP expression. Ramp et al. also reported that XIAP expression was found in 137 of 145 (95%) clear-cell renal cell carcinomas cases (RCCs), assessed by immunohistochemistry and western-blotting. In the same patient population, multivariate stepwise Cox regression analysis showed that, in addition to tumor staging (p=0.00005) and grading (p=0.00004), XIAP expression (p=0.018) is an independent prognostic factor indicating a poor prognosis in clear-cell RCC (Ramp et al., 2004).

Furthermore, Shi et al. established an HCC cell line, HCCLM3, stably expressing an siRNA construct against XIAP, in which more than 80% of XIAP was inhibited by 120 hours. These XIAP-knock-down HCCLM3 cells, or cells with high XIAP expression as a negative control, were injected into the flank of nude mice. Necropsy on the 42nd day indicated that the average tumor volume at the injection sites was not statistically different between the control and XIAP-knock-down group (0.69±0.15 cm^3 versus 0.66±0.26 cm^3, p=0.865, one-way analysis of variance). Although pulmonary metastasis could be detected in both groups at a 100% rate (5/5 in each group), confirmed by histological examination, analysis of the number of metastatic foci revealed a significantly reduced ability of HCCLM3 cells to establish individual metastatic foci (approximately a 40% reduction). These observations therefore suggest that altered expression of XIAP did not significantly affect the growth of tumor cells at the primary site, but instead affected the prevalence of metastasis, as revealed by reduced metastatic foci in the lung (Shi et al., 2008). Over-expression of XIAP was also associated with resistance to apoptosis, an enhanced invasiveness in vitro, which could contribute to increased metastatic foci in vivo and a subsequently reduced survival time. XIAP expression could therefore be an independent prognostic factor, and novel therapeutic target for the genetic treatment of HCC patients.

Prospectively, the discovery of siRNA brings a new hope for HCC therapy. Chen et al. revealed that the cell line HepG2 exhibits increased sensitivity to spontaneous apoptosis or anti-tumor agent-stimulated apoptosis when XIAP was silenced by siRNA. After successful transfection, XIAP expression was down regulated at both the mRNA and protein levels. They found that the non-silenced control group showed significantly elevated

apoptosis rates at 24 and 48 hours compared with the XIAP-siRNA transfected group. They also observed different apoptosis rates after treatment with Methotrexate (MTX). Flow cytometry analysis confirmed these observations: after adding 5 or 10 mg/ml MTX to cells for 24 hours, the level of apoptosis in cells transfected with XIAP-siRNA was 26.7% and 32.2%, respectively, compared with levels of 4.6% and 17.5% in the non-silenced control group. Forty-eight hours after adding MTX, the corresponding levels of apoptosis were 38.4% and 44.6% compared with levels in the control group of 6.3% and 21.1%, respectively ($p< 0.05$). This demonstrates that siRNA can specially and efficiently knockdown XIAP resulting in enhanced apoptosis, sensitizing HCC cells to chemotherapy (Chen et al., 2006). Similar results were also found in human pancreatic carcinoma (Shrikhande et al., 2006; Giagkousiklidis et al., 2007; Ruckert et al., 2010; Buneker et al., 2012), glioma (Hatano et al., 2004), breast cancer (Lima et al., 2004; Wang et al., 2012), ovarian carcinoma (Ma et al., 2009), melanoma (Hiscutt et al., 2010) and renal cell cancer (Bilim et al., 2008). XIAP can therefore be molecularly targeted by siRNA, which may ultimately result in a novel cancer therapy. However not all clinical samples from HCC patients are XIAP-positive (Shi et al., 2008), so it may not be an effective therapeutic tool for all cases of HCC.

Targeted silencing of Survivin by siRNA

Survivin (TIAP or BIRC5 (Vucic and Fairbrother, 2007)) is the smallest human IAP protein, consisting of a single BIR domain, and a COOH-terminal α-helical coiled-coil domain. It is found associated with polymerized microtubules through its coiled-coil domain. Survivin has four variants (Survivin-ΔExon3, Survivin-2B, Survivin-2α and Survivin-3B) that are formed by alternative splicing of Survivin pre-mRNA, and each isoform has diverse cellular localization.

Over-expression of survivin has been detected in numerous human tumors, including colorectal cancer (Sprenger et al., 2011; Ge et al., 2013), oral squamous cell carcinoma (Li et al., 2012), leukemia (Yahya et al., 2012; Yang et al., 2013), and HCC (Augello et al., 2009; Liu et al., 2013; Wei et al., 2013). Claudia et al. found that Survivin mRNAs were significantly overexpressed in hepatocellular carcinoma tissue compared with non-neoplastic tissue (FC = 6.86, $p< 0.001$). They also revealed that an elevated Survivin mRNA level has a significant correlation with high tumor stage (pT3 and pT4, p=0.03), high tumor grade (III and IV, p= 0.01), and vascular invasion (p=0.001). Although Survivin over-expression showed no correlation with overall disease outcome, there was a trend for decreased survival in patients with high survivin expression (p=0.09). Interestingly, they found that Survivin immunoreactivity was elevated in the cytoplasm of cirrhotic liver cells, while HCC cells generally showed weak cytoplasmic staining, and nuclear Survivin staining was found only in five HCC samples (12.5%). Although samples of HCC with high levels of Survivin were correlated with a shorter overall survival, Survivin immunoreactivity was not associated with clinical pathological type and survival time, since this trend did not reach statistical significance (p= 0.39) (Augello et al., 2009).

This discrepancy may be explained by the diverse variants (Li, 2005), their localization, and the multi-functional role of Survivin, which is an apoptosis-related and cell cycle protein (Altieri, 2003). There is growing evidence to suggest an additional role of Survivin in non-cancerous tissues. Takashima et al. reported that levels of Survivin mRNA increased during the progression of chronic liver injury, suggesting that Survivin might be an important factor for the survival of hepatocytes (Takashima et al., 2005). In addition, Mengjie et al reported that Survivin is unmethylated in cancer, but selectively methylated in normal tissues. This suggests that the targeting of Survivin for cancer treatment showed no obvious toxicity to normal tissues and cells (Bhattacharyya and Lemoine, 2006).

Survivin, similar to other IAPs, is also involved in the resistance of tumor cells to anti-cancer agents including chemotherapy drugs, and ionizing radiation (Zaffaroni et al., 2005; Capalbo et al., 2007). Capalbo et al. reviewed the role of Survivin for radio- or chemotherapy, and found the value of Survivin as a predictive marker for the treatment response to radiotherapy or chemotherapy is not reliable, even though they saw a trend towards high expression of Survivin correlating with an enhanced resistance to radio- or chemotherapy (Capalbo et al., 2007). In contrast, the silencing of Survivin by siRNA can increase apoptosis and sensitize tumor cells to chemo- and radiotherapy (Fuessel et al., 2006; Wu et al., 2007; Yang et al., 2008; Liu et al., 2009b; Song et al., 2009; Zhang et al., 2010; Liu et al., 2013). Wu et al. reported that the expression of Survivin in HepG2 cells is significantly reduced after transfection with Survivin siRNA groups (especially in 200 nmol/L siRNA transfection group) compared with control groups, as assessed by western blotting. The apoptotic index of HepG2 cells transfected with Survivin siRNA was significantly increased (most significantly in the 200 nmol/L siRNA groups), analyzed by flow cytometry. Reduced expression of Survivin could also up regulate the activity of caspase-3 (53.4±1.31 in control groups, versus 89.3±2.87 in 200 nmol/L siRNA groups), and sensitize HCC cells to Cis-Diaminedichloroplatinum (DDP) (Wu et al., 2007). Song et al. reported that the silencing of Survivin in the HCC cell line SMMC-7721/ADM by siRNA led to reduced expression of the lung resistance related protein (LRP), a protein linked to chemo-resistance in HCC, and reversed resistance to chemotherapy in hepatocellular carcinoma. They injected SMMC-7721/ADM cells into nude mice to establish xenograft tumors, and then treated with Survivin-specific siRNA combined with low-dose adriamycin (ADM). Tumor growth was significantly inhibited, and no obvious signs of toxicity were observed. Specifically, the food intake, reaction to environmental stimuli, circulating white blood cell count, and transaminase levels were all comparable between the siRNA-treated and control mice. In contrast, the group treated with high-dose ADM displayed obvious systemic toxicity, myelosuppression, and liver damage (Song et al., 2009). These data not only reveal that depletion of

Survivin by siRNA could increase the sensitivity of tumor cells to chemotherapy in vivo, but also allays concerns that Survivin-siRNA is toxic to normal human tissues or cells expressing Survivin.

Targeted silencing of Livin by siRNA

Livin, also known as ML-IAP, KIAP, or BIRC7 (Liu et al., 2007), contains a single BIR domain, and a C-terminal RING finger domain. Two splice variants (Livin-α and Livin-β) have been identified, caused by alternative splicing of Livin mRNA. Livin-α has an additional 18 amino acids between the BIR and RING domains than the Livin-β, allowing the formation of a linker α-helix.

However, the mechanism of action for the anti-apoptotic effects of Livin remains controversial. Most IAPs function by inhibiting the activity of caspases through direct interactions via their BIR domains. In support of this mechanism of action, Vucic et al. reported that Livin inhibited caspase-9 directly through binding to its BIR domain (Vucic et al., 2000; Vucic et al., 2002). Although Livin has a RING domain, allowing it to act as E3 ubiquitin ligase, the importance of the RING domain for the anti-apoptotic actions of Livin is unclear (Liu et al., 2007). Kasof et al. reported that the C-terminal RING domain of Livin might mediate its subcellular localization (Kasof and Gomes, 2001). However, subsequent work carried out in the Vucic laboratory found that the inhibitory effect of Livin on caspase-3 and caspase-9 is much weaker than that of XIAP (Vucic et al., 2005). This left an open question of how Livin inhibits apoptosis. Its mechanism of action was revealed after the identification of SMAC/DIABLO, a mitochondrial protein that can specially bind to BIR domains and block the anti-apoptotic effects of IAPs (Chai et al., 2000; Liu et al., 2000; Wu et al., 2000). Studies revealed that there is a very high-affinity interaction between Livin and SMAC, which competes with the XIAP-SMAC interaction and inhibits apoptosis by sequestering SMAC, preventing it from antagonizing XIAP-mediated inhibition of caspases (Vucic et al., 2005). Livin has therefore been described as blocking the inhibition of XIAP, rather than as a direct suppressor of caspases (Liu et al., 2007).

Although Livin can be detected in the placenta, normal testis, spinal cord and lymph nodes, it is not expressed in most normal differentiated tissues and cells (Lin et al., 2000; Vucic et al., 2000; Ashhab et al., 2001; Kasof and Gomes, 2001; Liu et al., 2007). However, it is highly expressed in several cancers, such as melanoma, cervical cancer, gastric cancer, pancreatic cancer, leukemia and lymphoma, non-small cell lung cancer (NSCLC), and carcinoma of the breast, prostate, bladder, and liver (Gazzaniga et al., 2003; Augello et al., 2009; Liu et al., 2009a; Yuan et al., 2009; Wang et al., 2010; El-Mesallamy et al., 2011; Lazar et al., 2012). For example, Claudia et al. found that Livin mRNA was significantly over-expressed in liver cancer tissue compared with non-neoplastic tissue (FC=2.33, $p<0.001$), although Livin expression showed no correlation with disease outcome (Augello et al., 2009). Studies of Livin expression in patients with nasopharyngeal cancer (Xiang et al., 2006), colon cancer (Takeuchi et al., 2005a), metastatic melanoma (Takeuchi et al., 2005b), and lung cancer (Tanabe et al., 2004) also showed no correlation with clinical outcome. However in neuroblastoma and bladder cancer, Livin was reported to be a prognostic factor. Gazzaniga et al. used reverse transcription-PCR (RT-PCR) to measure Livin-α and Livin-β expression in 30 bladder cancer patients. They found that 23% (7/30) of the cancerous tissues showed expression of Livin-α, but not Livin-β, while none of the assayed normal tissue samples exhibited expression of either Livin isoform. In the 30 cancer patients, median relapse-free duration of patients with increased Livin-α expression was 3.5, compared with 27.2 months in patients without increased Livin-α ($p<0.0001$). Elevated Livin-α expression was therefore risk factor for relapse in patients with bladder cancer. However, Livin-α expression did not correlate with the known prognostic variables of stage, grade, and multi-centricity of the tumor (Gazzaniga et al., 2003). In additional studies, Wang et al. reported that, out of 40 gastric carcinoma tissues evaluated by RT-PCR and western blot, 19 patients (47.5%) showed elevated expression of both mRNA and protein Livin-α and Livin-β. However, in paracancerous tissues and benign lesions of gastric mucosa, there was no detectable expression of either mRNA isoform. Their data also showed that Livin expression was positively correlated with lymph node metastases and histologic grade ($p<0.05$), but not with age, gender, or the extent of tumor infiltration (Wang et al., 2010).

Livin expression in neoplasms has also been linked with increased aggressive behavior of tumor cells, including decreased spontaneous apoptosis, and reduced sensitivity to chemotherapeutic agents and ionizing radiation. These observations could be reversed by treatment with Livin siRNA both in vitro and in vivo. For example, Liu et al. constructed a siRNA expression vector targeting Livin, named pU-siLivin, and the human HCC cell line SMMC-7721 was transfected with this plasmid. After successful transfection, RT-PCR and western blotting were used to evaluate Livin expression at the mRNA and protein level, respectively. They observed that mRNA expression of Livin-α and Livin-β in the pU-siLivin transfected cells were reduced by >50 and 73%, respectively, and that protein levels of Livin-α and Livin-β were decreased by 76 and 80%, respectively. Notably, the silencing of Livin significantly reduced the resistance to apoptotic stimuli, proliferation, and invasive capacity of SMMC-7721 cells. Specifically, the number of apoptotic cells induced by UV-irradiation increased approximately 8-fold in the pU-siLivin transfectants compared with the control cells ($p<0.05$), while the cell growth index of the transfected group was significantly decreased by days 3 and 4 after plating compared with the two control groups ($P<0.05$). In addition, flow cytometric analysis revealed that pU-siLivin induced cell cycle arrest in SMMC-7721 cells at the G1 phase of the cell cycle, and that the number of cells in the G1 phase increased from 40.1 to 70.72%, while the number of cells in the S phase decreased from 44.96 to 9.7%. Finally, Livin siRNA-transfected cells showed approximately 9-fold reduced ability to penetrate through matrigel-coated membranes compared with

control ($P<0.05$) (Liu et al., 2010).

In SGC-7901 gastric cancer cells, Wang et al. found a significantly decreased cell number at 72 and 96 h after plating in the siRNA-Livin transfected group compared with the negative control and parental cell group ($p<0.01$). In addition, the spontaneous apoptotic rate was significantly increased in the transfected group ($p<0.05$). When the sensitivity of these cells to 5-fluorouracil and cisplatin was tested, MTT assays revealed that the siRNA-Livin transfectants were more sensitive to cytotoxic drugs than the negative control group. The number of apoptotic cells induced by 5-fluorouracil and cisplatin in the transfected group was 2.5-3-fold elevated compared with control ($p<0.001$) (Wang et al., 2010). Similar data were reported after down-regulation of Livin by siRNA in NSCLC (Crnkovic-Mertens et al., 2006; Yuan et al., 2009), neuroblastom (Dasgupta et al., 2010), malignant melanoma LiBr (Wang et al., 2007), human Bladder Cancer T24 (Yang et al., 2010), human glioma (Yuan et al., 2012) and colon cancer cell lines (Oh et al., 2011). Specific siRNA targeting of Livin can therefore increase spontaneous apoptosis, and the sensitivity of tumor cells to chemotherapy in vitro. In a xenograft model, Oh et al. reported that siRNA targeting of Livin could significantly decrease the tumor volume. After palpable tumors had been created by injection of Viable HCT116 colon cancer cells (2.0 × in 100 μL PBS) into the right flank of 4-week-old male nude mice, tumors were treated weekly for 4 weeks by direct intra-tumoral injection with varying doses (10, 20, or 50 μmol/L) of siRNA manufactured with atelocollagen to achieve effective delivery (Oh et al., 2011). The mean tumor volume was significantly decreased in the group treated with siRNA compared with the control group in a dose-dependent fashion. There were no significant alterations in body weight in the siRNA-treated group, and no toxic reaction was detected in the kidney, liver, or brain of the mice.

Targeted silencing of c-IAPs and other members of IAP family by siRNA

Cellular IAP1 and IAP2 (c-IAP1 and c-IAP2) are closely related (in terms of amino acid composition, structure, and function) members of the IAP family, consisting of three BIR domains at their N-terminii, one RING domain near the C-terminus, and a CARD (caspase activation recruitment) domain between the BIR and RING domains. Through binding to TNFR-associated factor-2 (TRAF2), c-IAP1 and c-IAP2 directly interact with TNF-α signal transduction (Rothe et al., 1995). The c-IAPs inhibit TNF-α-induced apoptosis mainly via the TNF-α receptor-2, which is comprised of the "TNFR2-TRAF Signal Complex". Inhibition of this complex requires co-expression of TRAF1, TRAF2, and both c-IAPs, which activates NF-ϰB signaling. NF-ϰB is a transcription factor that inhibits the apoptotic response induced by TNF-α and other stimuli by blocking the activation of the initiator caspase, caspase-8 (Tartaglia and Goeddel, 1992b; Tartaglia and Goeddel, 1992a; Smith et al., 1994; Rothe et al., 1995; Wang et al., 1998). Varfolomeev et al. reported that c-IAP1 and c-IAP2 were critical mediators of TNF-α-induced NF-ϰB activation, and found that the absence of both c-IAP1 and c-IAP2 sensitized cells to TNF-α-induced cell death (Varfolomeev et al., 2008).

Bruce, also known as Appollon or BIRC6, is the largest member of the IAP family and was first identified in the mouse. It is a large, 530 kDa membrane-associated protein that contains a BIR domain at its N-terminus, and an E2 ubiquitination motif (Ubc) at its C-terminus (Hauser et al., 1998; Chen et al., 1999). Although Bruce is upregulated in certain cancers (such as gliomas) that are resistant to specific DNA-damaging agents, treatment with antisense oligonucleotides against Bruce (Chen et al., 1999) and Bruce-siRNA (Qiu et al., 2004) re-sensitized cells to the apoptosis induced by DNA-damaging agents, it remains unclear how Bruce inhibits apoptosis. Some studies have reported that Bruce acts downstream of Bcl-2/Bcl-xl (Qiu et al., 2004; Ren et al., 2005), which inhibit the release of caspase activators from the mitochondria (Merry and Korsmeyer, 1997; Kroemer and Reed, 2000). It is therefore possible that Bruce, like other IAPs, inhibits caspases. Additional lines of evidence have revealed that Bruce gene silencing induces apoptosis by stabilizing p53, and activating caspase-3 (Ren et al., 2005; Lopergolo et al., 2009).

Neuronal apoptosis inhibitory protein (NAIP), previously linked to spinal muscular atrophy (SMA) (Kesari et al., 2005), Alzheimer's disease (Cotman, 1998), and Parkinson's disease (Hartmann et al., 2000), was the first member of the IAP family to be cloned (BIRC1) in 1995. NAIP is a 160 kDa protein that contains a cluster of three BIR domains at N-terminus, a central nucleotide-binding oligomerization domain (NOD), and a C-terminal leucine-rich repeat (LRR) domain (Davoodi et al., 2004). The NOD and LRR domains make NAIP unique among the IAPs, and suggest that NAIP activity is regulated differently from other members of the family (Davoodi et al., 2004). NAIP directly inhibits the cell death effector proteases, caspase-3 and caspase-7, through its BIR domains (Maier et al., 2002), and associates with the initiator caspase, caspase-9 to inhibit apoptosis.

Inhibitor of apoptosis protein (IAP)-like protein-2 (ILP-2), also known as Ts-IAP or BIRC8, is the most recently identified member of the IAP family, and is closely related in sequence to ILP-1, or XIAP. Despite its high homology to ILP-1, ILP-2 is encoded by a distinct gene, and, under normal circumstances, is only expressed in the testis (Richter et al., 2001). In contrast to ILP-1, ILP-2 contains only one BIR domain at its N-terminal region, and a RING finger domain at its C-terminus. Although ILP-2 is structurally similar to ILP-1, ILP-2 has no protective effect on Fas or TNF-α-mediated apoptosis. Bettina et al. (Richter et al., 2001)reported that ILP-2 could potently inhibit apoptosis by stimulating over-expression of Bax to prevent cytochrome c release, and thus inhibit formation of the Apaf-1-caspase-9 holoenzyme, which induces the activation of the downstream effector caspase, caspase-3. ILP-2 therefore blocks the intrinsic apoptotic pathway, and represents a novel therapeutic target that would sensitize tumor cells to apoptosis when silenced.

These members of the IAP family all have a

documented association with malignancies, including squamous cell carcinoma, breast cancer, leukemia, colon cancer, prostate cancer, and hepatocellular carcinoma (Smith et al., 1994; Notarbartolo et al., 2002; Endo et al., 2004; Zender et al., 2006; Choi et al., 2007; Lopergolo et al., 2009). Endo et al. reported that NAIP is expressed in colon cancer, and that its expression is higher young patients compared with the elderly ($p<0.05$) (Endo et al., 2004). Ki et al. detected Bruce expression in patients with leukemia, and revealed that its over-expression was associated with resistance to chemotherapy and poor prognosis in childhood acute myeloid leukemia (Sung et al., 2007).

Some family members have been studied in additional cell lines. For example, Bruce has been silenced by siRNA in the human lung cancer cell line H460 (p53+/+) (Ren et al., 2005), HeLa cells (Qiu et al., 2004; Chu et al., 2008), and HT-1080 cells (Chu et al., 2008), leading to reduced resistance to apoptosis and sensitization of tumor cells to anti-tumor agents. Importantly, much of the research in to the newer IAP family members mentioned here (c-IAPs, Bruce, NAIP, and ILP-2) has been carried out in studying a combination with XIAP, Survivin or Livin, so understanding their independent roles in tumorigenesis requires additional studies.

Co-expression of IAPs in Hepatocellular Carcinoma

Although previous reports have documented the expression of IAP family members, including XIAP, Survivin, Livin, and c-IAPs, in hepatocellular carcinoma, most studies focus on the expression of a single IAP and its significance in HCC. To better understand the family as a whole, Augello et al. set out to evaluate the expression of all IAP family members in human HCC. They found that the mRNA of NAIP, c-IAP1, c-IAP2, XIAP, Survivin, Bruce, and Livin were all expressed at detectable levels in each HCC case tested, and that only NAIP was expressed at a slightly lower level in cancerous tissue compared with non-neoplastic tissue (Augello et al., 2009). They concluded that Kupffer cells, which are abundant in non-neoplastic liver tissue, express NAIP, accounting for this unexpected observation. The significance of the elevated expression of IAP family members in HCC differs dramatically. For example, high Survivin mRNA levels significantly correlate with advanced tumor stage, high tumor grade and vascular invasion, while high c-IAP2 mRNA levels are significantly correlated with the absence of paraneoplastic capsules ($p=0.02$). In contrast, high NAIP mRNA levels correlate with a pseudoglandular histotype ($p=0.03$), but high XIAP expression correlates with significantly shorter overall patient survival. This research may therefore provide useful information to allow combination hepatocellular carcinoma siRNA therapy.

Conclusion and future directions

It is now widely accepted that dysregulated apoptosis plays a major role in the development of cancer. The dysfunction of apoptotic pathways, and dysregulated cellular proliferation, will ultimately lead to tumorigenesis. Although there are many different regulators or apoptosis that can render tumor cells resistant to apoptosis both in vivo and in vitro, the IAP family remains one of the most promising therapeutic candidates. Moreover, cancer cells that over-express IAP family members will become increasingly resistant to standard chemo- or radiotherapy based treatments. In this context, targeting IAPs by siRNA brings new potential for cancer treatment because of its verified association with enhancing the sensitivity of tumor cells to apoptosis, and reduced resistance to anti-tumor agents. The identification of IAPs in hepatocellular carcinoma tissue provides the foundation of siRNA as a HCC genetic therapy, while the subsequent siRNA targeting of IAPs in HCC presents a promising strategy for HCC research.

However, not all clinical samples from HCC patients test positive for a single IAP protein, and different members of the IAP family act via different mechanisms. Moreover, some members of the IAP family can interact, and/or have a coordinated mechanism of action. For instance, Claudia et al. reported that all measured IAPs (NAIP, c-IAP1, c-IAP2, XIAP, Survivin, Bruce and Livin) were expressed at detectable levels in liver cancer tissues (Augello et al., 2009). This may be an obstacle for HCC genetic therapy using single IAP-siRNA. However, one way of improving the effectiveness of siRNA as a HCC treatment may be to simultaneously block several members of IAP family in liver cancer tissues, which can be achieved by constructing siRNAs to directly target multiple IAP genes. It may tåherefore be necessary to construct an siRNA cocktail, which is a vector containing different siRNAs for specifically knocking down different members of the IAP family (Caldas et al., 2006).

Additionally, recent studies revealed that certain members of the IAP family are expressed in normal tissues, such as Survivin, Livin, and c-IAPs. Esposito et al. reported that c-IAP1 was important in the regulation of apoptosis in the normal pancreas, but that its role in the neoplastic pancreas depended on its sub-cellular localization (Esposito et al., 2007). This brings concerns over the use of siRNA against IAPs as a cancer therapy, since their use may be toxic to normal tissues expressing IAPs. Although the treatment of xenograft tumors in nude mice with siRNA revealed no obvious whole-body toxicity (Song et al., 2009; Oh et al., 2011), the potential effect of IAP-siRNAs on normal tissues remains unclear. However, tissue-specific targeted delivery of the siRNA may be helpful in potentially toxic effects of IAP-siRNAs in normal tissues. This is now possible, since the identification of polymerase II promoters, some of which have been tested clinically, and have been proven to be tissue-specific (Dickins et al., 2007; Giering et al., 2008). Polymerase II promoter–driven siRNA to silence HBV gene expression was proven to be an effective and safe therapeutic in transgenic HBV mice, while control mice receiving the same hairpin siRNA under the control of a U6 promoter died of liver toxicity. It may therefore be challenging to provide a more efficient delivery system in the future application of IAP-targeting siRNA in HCC, requiring more tumor-specific delivery mechanisms.

Previous reports suggested that strategies using combination therapy with current modalities are likely to be the basis of future successful cancer therapy (Bhattacharyya and Lemoine, 2006). Expression of the IAP family is regulated by various stimuli. For example, growth factors, cytokines, hormones, anticancer agents, and kinases inhibitors can all regulate the expression of Survivin (Zhang et al., 2006). Specifically, the anti-apoptotic properties of VEGF and IL-11 appear to mediate the induction of Survivin in endothelial cells (Li, 2003), since it has been shown that VEGF is strongly associated with the expression of Survivin in hepatocellular cancer (Zhu et al., 2005), and breast cancer (Ryan et al., 2006). Another reasonable strategy to facilitate the siRNA targeting of IAPs in HCC may therefore be to block the interaction of multiple stimuli and IAP members, either subsequently or simultaneously.

In conclusion, we believe there the clinical application of IAP-siRNA shows great potential for use as a hepatocellular carcinoma therapy. Improving the efficiency of siRNA transfection, the construction of a more tumor-specific siRNA delivery system, and the use of combination therapy are all likely to further enhance the potential of this therapeutic intervention.

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