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Current Gene Therapy, **2015,** *15,* **000-000**

Combined Therapy for Gastrointestinal Carcinomas: Exploiting Synergies Between Gene Therapy and Classical Chemo-Radiotherapy

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Abstract: Surgical resection is the only curative option for patients with gastrointestinal carcinomas. Unfortunately, the majority of patients are diagnosed in advanced stages when surgery is not possible. Moreover, the incidence and mortality for certain type of tumors such as hepatocellular carcinoma or pancreatic cancer are steadily increasing worldwide. In spite of the advances in the development of molecular targeted therapies for cancer, the impact on patient survival has been rather limited. It is unlikely that individual agents would be ultimately successful as monotherapy. There is a growing area of research focused on the combination of classical chemotherapy (e.g. cyclophosphamide, gemcitabine, paclitaxel and doxorubicin) with radiotherapy and/or gene therapy strategies. Combined approaches seem to be required due to multiple resistance mechanisms that tumors utilize to limit the activity of chemotherapeutic agents (e.g. the occurrence of multidrug resistance or epigenetic alterations), evade immune responses (e.g. induction of regulatory T cells or myeloid-derived suppressor cells) and to generate resistance against anti-angiogenesis or to radiotherapy by, for example, the induction of hypoxia-inducible factor 1. In addition, new studies suggest that combination of low dose of conventional chemotherapy and gene therapy could allow the development of synergic mechanisms able to achieve significant therapeutic effects against diverse tumors. Although cancer gene therapy is not yet available in clinical practice, advances being recently made look promising, especially when it was applied in combination with standard chemo- or radiotherapy protocols.

Keywords: ??????????????????????????????

INTRODUCTION

In spite of advances in molecular diagnostics and therapeutics a low percentage of patients with advanced gastrointestinal carcinomas (GIC) are amenable to curative therapies [1-3]. Classical anticancer approaches include chemotherapeutic cocktails and radiation therapy. The novel strategies include molecular targeted therapies such as: sorafenib, for hepatocellular carcinoma (HCC); monoclonal antibodies against epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF), for metastatic colorectal cancer (CRC); or the recently approved nab-paclitaxel, for metastatic pancreatic cancer (PC) [4-7] . Despite advances made in the development of molecular targeted therapies for cancer, their impact on patient survival has been rather limited. Therefore, there is an important need for new therapeutic agents in gastrointestinal oncology. In the last two decades, a growing area of research was focused in the combination of classical chemo- and radiotherapy-based strategies with gene therapy tools. Such strategies were designed on the basis of our current knowledge regarding the development of multiple resistance mechanisms in tumors which more often limit the efficacy of conventional treatments. In this review we describe a variety of resistance mechanisms to chemo- and radiotherapy strategies and discuss combined therapies based on gene transfer in the experimental treatment of GIC.

TUMORS ACQUIRE MULTIPLE MECHANISMS TO RESIST ANTI-CANCER THERAPY

Why Does Chemotherapy Fail?

Many reasons can account for failure of cancer chemotherapy. Chemotherapeutic drugs can induce DNA damage or cellular apoptosis by acting as alkylating drugs [8] (e.g. cisplatin, cyclophosphamide, oxaliplatin), anti-mitotic agents [9] (e.g. taxol, paclitaxel, vinblastine), topoisomerase inhibitors [10] (e.g. etoposide, irinotecan), anti-metabolites [11] (e.g. 5-fluorouracil, gemcitabine), and/or anti-tumor antibiotics [12] (e.g. anthracyclines such as doxorubicin). Regardless of the chemotherapeutic compound used, cancer cells were found to develop mechanisms able to resist chemotherapy ultimately resulting in treatment failure [13]. Tumor resistance occurs in multiple ways: i) through multidrug resistance (MDR); ii) due to epigenetic changes, i.e. by DNA-methylation; or iii) as a result of the induction of cellular stress, such as hypoxia. In addition, all these mechanisms can act in synergy [13].

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1566-5232/15 $58.00+.00

A variety of drug transporter proteins localized in the cellular membrane are responsible for the generation of MDR in tumors [14]. Examples of these proteins are the multidrug resistance–associated protein (MRP) and P-glycoprotein, being both of them members of the ATP-binding cassette (ABC) transporter genes superfamily [15]. The enhanced activity of these transporters is involved in tumor resistance to chemotherapy [16]. In fact, an upregulation in the expression levels of ABC transporters has been shown in HCC murine xenograft models as well as in patients [17-18]. Moreover, it was recently described that mRNA levels of the transcription factor Octamer 4 (Oct4) were increased in doxorubicin/5-fluorouracil-resistant HCC cells due to DNA demethylation of Oct4. In this case, Oct4 overexpression was found to enhance cancer cell resistance to chemotherapy by increasing the expression of ABCG2 transporter [18]. Furthermore, IL-8 secretion shown to be induced by chemotherapy could in turn increase the expression of ABC transporters thus promoting HCC growth *in vitro* [19]. ABCG2 was also involved in resistance to SN38, the active metabolite of irinotecan, in CRC [20]. Exposure to increasing concentrations of SN38 induces the appearance of resistant clones in a human CRC cell line (HCT116 cells) without affecting the expression of topoisomerase I. This phenomenon was associated with a higher ABCG2 expression, supporting the role of ABCG2 in the development of irinotecan resistance in CRC cells [20]. Additionally, the use of a topoisomerase inhibitor found to be less toxic than irinotecan in another human CRC cell line devoid of ABCG2 expression (SW1116 cells) was shown to induce an upregulation in ABCG2 gene expression levels, further involving them in CRC resistance [21]. The increased expression of ABC family was also observed in patients with advanced PC and it correlated with worse tumor prognosis [22].

On the other hand, autophagy, a homeostatic cellular recycling process involving lysis of damaged proteins or organelles, is being considered as a key player in adaptive responses to metabolic and therapeutic induced stress. It was found implicated in both pro- or anti-tumoral mechanisms[23]. For example, autophagy was able to protect tumor cells against chemotherapy, by diminishing their DNA damage and metabolic stress [24]. Metabolic stress is frequently observed in tumor cells under hypoxia and/or starvation and it was shown induced by uncontrolled proliferation and vascular collapse as well as by the use of therapeutic agents [25]. Thus, autophagy can be seen as a relevant tumor cell survival mechanism, playing a key role in tumor resistance to chemotherapy. This process has been recently involved in primary and metastatic human HCC [26]. Consistently, autophagy inhibition in a mouse model of HCC pulmonary metastases established with primary or metastatic patient tumor samples, was shown to suppress lung metastasis [27]. This effect was associated to a decrease in resistance to cell death by anoikis, a form of apoptosis induced by the loss of attachment capacity to extracellular matrix [28], a key step required for cancer cells to invade and metastasize. Autophagy was also reported to mediate survival of pancreatic tumors exposed to hypoxia [29]. On the contrary, an association between autophagy and p53 was recently described in PC; mice lacking p53 showed loss of autophagy which was associated with increased glucose uptake and tumor progression [30]. Both hypoxia and oxaliplatin were shown to be able to induce autophagy in many human CRC cell lines such as HT29, HCT116, HCT15, SW620, KM12, WiDr, and LoVo [31]. However, the use of the autophagy flux inhibitor chloroquine was reported to increase sensitivity to oxaliplatin therapy[32]. Oxaliplatin was also found to induce cell death and autophagy in Caco2 CRC cells. Similarly, the use of autophagy inhibitors such as bafilomicyn A1 was shown to increase cell death and suppress cell proliferation with involvement of reactive oxygen species [32]. These findings were also described in a murine model of HCC [33], and suggest a role for autophagy in cancer development as well as implications of autophagy inhibition in enhanced efficiency of cancer chemotherapy.

A hypoxic microenvironment could induce resistance to chemotherapy by the activation of the mammalian target of rapamycin (mTOR) pathway [34]. Although molecular mechanisms involved in mTOR-mediated hypoxia-induced chemoresistance remains unclear, it was recently reported that the mTOR target N-myc downstream regulated gene 1 (NDRG1) promotes alkylating chemotherapy resistance via distinct molecular pathways involving HIF-1α, p53, and the mTOR complex 2 (mTORC2)/serum glucocorticoid-induced protein kinase 1 (SGK1) [34]. As a likely separate mechanism, the ubiquitin ligase subunit 1 (WSB-1) was shown to be involved in DNA damage response by targeting homeodomain-interacting protein kinase 2 (HIPK2) for ubiquitination and degradation. Hypoxia and HIF-1 significantly up-regulate the expression of WSB-1 in human HCC cells. Interestingly, a knockdown in hypoxia-induced HIF-1α expression was shown to inhibit WSB-1 expression and this in turn resulted in etoposide-induced HCC cell death under hypoxic conditions [35]. Taken together, previous data suggest that different strategies can be employed to overcome tumor chemoresistance and support the use of combinatorial strategies to increase therapeutic benefits for patients.

Surpassing Radiotherapy Effects

Diverse radiation therapy schemes are being applied in many types of tumors including advanced unresectable HCC [36], hepatic metastases of CRC [37] and advanced PC [38]. Radiation induces tumor cell death by causing DNA damage through single and double-strand breaks, and DNA–protein cross-linking [39]. In addition, it was also described that radiation induces reactive oxygen species (ROS) generation promoting alterations in cellular macromolecules. These events were shown to trigger several signal transduction pathways many of them involved in cell survival [40]. In this context, tumor cells develop adaptative mechanisms allowing them to resist radiation therapy, which include: initiation of DNA repair machinery; activation of phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR signaling pathway; up-regulation of ion transporter channels; induction of autophagy; stimulation of epithelial to mesenchymal transition, as well as microenvironment factors such as hypoxia [39, 41-43].

Interestingly, survivin was found to be over-expressed in PC and this was found involved in the development and progression of PC tumors, through apoptosis inhibition. Survivin exogenous expression in PC cells was associated to

cancer cell resistance to radiotherapy [44]. Radiosensitive MIAPaCa-2 cells transduced with wild-type survivin gene proliferated more rapidly and were significantly less radiosensitive than cells transduced with a control vector. On the other hand, transduction of a dominant-negative mutant survivin gene into radioresistant PANC-1 cells augmented their radiosensitivity [45]. A reduced response to radiation induced by overexpression of survivin was also shown in human CRC cells [46].

As described for chemotherapy resistance, autophagy appears to be a crucial mechanism to overcome radiotherapy in PC [47]. Thus, reduced levels of the microRNA (miRNA) miR-23b and increased autophagy were found in radiotherapy resistant PC cells when compared to radiosensitive cells. In such study, an enhanced expression of miR-23b was shown to inhibit radiation-induced autophagy, whereas inhibition of miR-23b was able to promote autophagy in tumor cells [47].

Finally, resistance to radiotherapy has also been observed in HCC. It is worth noting that human apurinic/apyrimidinic endonuclease (APE1), involved in DNA repair and regulation of transcription factors activity, shown to be strongly expressed in human HCC cells and that could be induced after irradiation, was shown to be able to limit radiation therapy effects [48]. Silencing of APE1 by using the adenoviral vector Ad5/F35 in xenograft models significantly potentiated both growth inhibition and apoptosis induction of irradiation both *in vitro* and *in vivo* [48].

Tumors Resist Immunotherapy Approaches

Cancer immunotherapy aims to control the growth and dissemination of malignant tumors by the activation of specific immune responses [49]. To this end, a variety of strategies are being investigated in pre-clinical and clinical settings including: i) nonspecific activation of the immune system by cytokines; ii) cancer vaccination using autologous/allogeneic antigen-preloaded DCs; iii) adoptive T-cell therapy targeting of tumor-associated antigens, iv) and immunostimulatory monoclonal antibodies, among others. Although many of these strategies have demonstrated to be potent in animal models, it was not until a few years ago with the use of DCs in hormone refractory prostate cancer or with immunostimulatory monoclonal antibodies (such as ipilimumab, tremelimumab, daclizumab, 4-1BB and OX40) that clinical results were more satisfactory [50-52]. Such frustrating clinical results can be partially explained by immunosuppressive mechanisms developed by tumors cells to escape from the host immune system. Cancer tumor cells are able to induce a reduction in the stability of the mRNA of certain antigen-presenting machinery proteins, such as transporter-associated with antigen processing 1 (TAP-1), resulting in abnormal tumor-associated antigens (TAAs) presentation [53]. In addition, it was extensively demonstrated that tumors are able to promote a selective recruitment and expansion of a variety of regulatory cells such as tolerogenic dendritic cells (DCs), natural and inducible regulatory T cells (Tregs), myeloid derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and natural killer-T (NKT) [54]. Sakaguchi *et al.* were the first to identify Tregs as a subtype of $CD4^+$ T cells that constitutively express the CD25 molecule and the transcription factor forkhead box P3 (Foxp3), essential for their suppressive activity [55]. Tregs block the effector responses of $CD4^+$ and $CD8^+$ T cells by releasing anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β), or by inducing negative signals through cell-cell contact [56]. MDSCs constitute a heterogeneous population of immature cells in early stages of differentiation that exert immunosuppressive activity, present a higher arginase activity and can release nitric oxide (NO) and peroxynitrite, thus inhibiting T cell activation. They also induce expansion of regulatory T cells $CD4^+$ $CD25^+$ $Foxp3^+$ cells and could lead to T cell anergy after antigen processing and presentation [57]. Finally, TAMs could suppress both arms of the immune system by blocking DC maturation and inhibiting cytotoxic T cell responses [58].

Presence of such immunosuppressive cells has been reported in blood and tumor microenvironment in different types of cancers, and a correlation between the presence of these cells and the suppression of both innate and adaptive responses has also been demonstrated [59]. In the case of HCC, many studies explored immunotherapy as a promising option [60-61]. It has been shown that adoptive T-cell therapy was associated with decreased HCC recurrence, although no other trials using this strategy were reported so far [62]. The lack of response in many immunotherapy protocols for HCC could be associated with the expansion of suppressive cells since an increase in numbers of peripheral and tumor-infiltrating Tregs has been observed in HCC patients [63]. Hoechst *et al.* observed an increase in a subset of MDSCs ($CD14^+$/HLA $DR^{+/-}$) in peripheral blood and tumors of HCC patients; these cells showed an enhanced arginase expression and when they were isolated *ex vivo*, were able to induce the expansion of Tregs with suppressive activity *in vitro* [64].

Tregs and MDSCs have also been shown to get accumulated in advanced CRC patients [65]. Several studies have attempted to determine the prognostic significance of the elevated frequency of such immunosuppressive cells [65-69]. Microarrays and immunohistochemistry analysis revealed that tissue localization and the ratio of $CD3^+$ tumor infiltrating lymphocytes/ $Foxp3^+$ have a predictive value [70]. Several studies demonstrated a link between the presence of high levels of Tregs in tumor stroma with poor prognosis [71]. However, clinical outcome not only depend on effector T cells/Tregs density but also on the association with chemotherapy or radiotherapy and/or the CRC stage [70-71].

Due to the poor response of PC patients to chemo- or radiation therapy, strategies aimed to induce immunity have been explored [72]. For instance, over-expression of the cell-surface enzyme α-Enolase (ENO1) in pancreatic cancer cells was shown to induce a specific immune response in patients [73] . Amedei *et al.* have recently demonstrated elevated levels of ENO1-specific CD4+Foxp3+ T cells in PC patients, which likely inhibit antigen-specific effector T cells, pointing at a role for Tregs in promoting PC progression [73]. In addition, increased levels of peripheral blood Treg and MDSCs were also observed in patients with metastatic pancreatic carcinoma [74-75]. Overall, these evidences suggest that HCC, CRC and PC cells may recruit, activate and expand Tregs and MDSCs as a mechanism of immune evasion. From the therapeutic point of view, modulation or removal

of these cells or their functional inactivation might contribute to tumor growth control or eradication.

How Do Tumors Fight Against Anti-Angiogenic Therapies?

Tumor growth, progression and dissemination depend on the concomitant new blood vessel formation and many strategies are being focused on blocking this process[76]. The most important players in tumor angiogenesis are endothelial cells, pericytes, and vascular endothelial growth factor (VEGF) [77]. Antiangiogenic strategies include the use of monoclonal antibody anti-VEGF (bevacizumab), multiple kinase inhibitors targeting vascular endothelial growth factor receptor (VEGFR) and/or platelet-derived growth factor receptors (PDGFRs) such as sunitinib or sorafenib (indicated for patients with advanced HCC) [77]. The majority of these molecules have showed limited therapeutic benefit, which can be partially explained by the development of tumor resistance [78]. A number of mechanisms account for anti-angiogenic resistance such as: i) activation of pro-angiogenic signaling pathways; ii) compensatory upregulation of growth factors (e.g VEGF, PDGF, granulocyte colony-stimulating factor, and stromal cell–derived factor 1-alpha (SDF-1); iii) presence of mature tumor vessels that may be resistant to VEGF-targeted therapy; iv) up regulation of MET oncogene promoting tumor cell growth and invasion, and v) up regulation of PI3K/Akt pathway [79-81].

Gene Therapy Strategies Combined with Classical Chemotherapy

Several immunotherapy strategies against cancer have been developed so far, with promising results [49]. One of the most extensively used cytokine is interleukin-12 (IL-12), a heterodimeric pro-inflammatory cytokine mainly produced by antigen presenting cells in response to different pathogens [82]. IL-12 demonstrated to have potent antitumor activity in many pre-clinical animal tumor models [83]. Different strategies were used so fa to transfer therapeutic genes into tumors including gastrointestinal carcinomas such as plasmid vectors, electroporation [84-85], adenovirus [86], and non-viral vectors [87]. Nonetheless, their efficacy is hindered by tumor-intrinsic and tumor-extrinsic mechanisms that inhibit effector immune responses [88]. It has been clearly demonstrated that the presence of potent immunosuppressive mechanisms that are active during tumor progression lead to the escape of tumors from the immune system and act limiting the success of immunotherapy approaches [89]. In this scenario, the combination of immunomodulatory approaches with classical chemotherapeutic agents, one of the pillars of conventional cancer treatment, to overcome the tolerance induced by tumors appears seems to be attractive. For instance, cyclophosphamide (Cy) is widely used to treat cancer as well as autoimmune diseases. High doses of Cy are required to kill cancer cells and its use is consistently associated with immunosuppression. Contrarily, low doses of Cy, alone or combined with other immunotherapies have been shown to generate immunity against cancer [90]. We have previously observed that the combination of low doses of Cy and intra-tumoral injection of an adenovirus encoding for IL12 genes (AdIL-12) was able to induce a potent and synergistic antitumor effects against advanced CRC and PC in mice. This approach induced the eradication of over 50% of primary and, more importantly, metastatic tumors [91-92]. Mechanisms behind such synergistic effects likely involve reduction of myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs), activation of dendritic cells (DCs) and generation of effector interferon-γ (IFNγ)-secreting $CD4^+$ T lymphocytes.

Antitumoral activity of Cy can be observed when this drug is used as a single dose and also upon metronomic schedules. Metronomic chemotherapy means that low doses of a cytotoxic agent are administered over a prolonged period of time; this strategy was found effective at inducing anti-angiogenic and/or immunostimulatory activity [93-94]. However, we showed that when combined with AdIL-12 a single dose of Cy is superior than a metronomic schedule in generating tumor-specific cytotoxic activity against CRC in mice [95]. In an attempt to increase the duration of IL-12 transgene expression, Gonzalez-Aparicio et al. administered a gutless adenoviral vector carrying a liver-specific, mifepristone (Mif)-inducible system for the expression of IL-12, directly into the liver of mice with metastatic CRC. As a result, they observed a progressive inhibition of IL-12 gene expression and concomitant reduced antitumoral effect that was solved increasing the dose and duration of the inducer mifepristone. Only with the addition of oxaliplatin the system was able to generate immunity-against-tumor rechallenge. Interestingly, these effects were not seen when 5-FU, irinotecan or gemcitabine were used in combination with gene therapy, but mechanisms involved in the specific effect obtained with oxaliplatin were not fully explored [96]. It is important to stress that the immune system plays a key role not only during tumor progression but also in the response of tumors to therapy. Some cytotoxic drugs, such as Cy, oxaliplatin, doxorubicin and mitoxantrone, can induce immunogenic cell death, a type of apoptosis that can stimulates tumor-specific immune responses. The key role of the immune system in the mechanisms of action mediating the effect of some chemotherapeutic drugs, such is the case of anthracyclines and oxaliplatin, requires a functional immune system [97]. It is worth noting that Zitvogel et al. recently demonstrated that autophagy is essential for immunogenic cell death [98].

Transcatheter arterial chemoembolization (TACE) is the current standard in the care of patients with unresectable HCC (BCLC-B) [99]. Nevertheless, median overall survival time for patients treated with TACE is around 20 months. Therefore, efforts to increase the efficacy of TACE in this patient population are needed. Xia *et al.* recently studied the combined effect of gene therapy of IL-12 and TACE in a rabbit model of HCC using VX2 cells. Such combination resulted in a potent antitumoral activity in this particular animal model although the mechanisms involved were not deeply studied [100].

It has been widely reported that the loss of p53-encoding tumor suppressor gene is responsible for apoptosis resistance of tumor cells. In addition, p53 deficiency is associated with resistance to radiotherapy and chemotherapy [101]. It was reported that p53 mutation correlates with HCC aggressiveness [102]. Guan *et al.* showed the safety and efficacy of combined therapy using a recombinant adenovirus encoding for p53 gen (Adp53) and TACE for advanced HCC. A total

of 68 patients were treated with TACE in combination with Adp53 administration. Survival rates were significantly higher for p53 treated patients than for the control group and the combination resulted was well tolerated with only minor adverse effects [103].

TACE was also used combined with gene therapy consisting in the intra-arterial injection of the vector H101, an E1B55k-deleted adenovirus. This pilot study demonstrated that the approach is feasible and it might have potential applications in future clinical trials [104]. The use of TACE for multifocal HCC patients in combination with gene therapy vectors is promising but there is a need for randomized controlled trials to really identify if this strategy will have a role in the clinical.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can selectively induce apoptosis in cancer cells, but not in healthy cells. However, the majority of cancer cells including HCC show TRAIL resistance. Zhang *et al.* used an interesting combination of mesenchymal stem cells (MSCs) genetically engineered to produce TRAIL with cisplatin-based chemotherapy in mice with MHCC97-H tumors. They observed tumor growth inhibition and a reduction in HCC vessel density after therapy with cisplatin and TRAIL-MSCs [105]. In another work, an AAV-2 vector was used to deliver TRAIL gene under the promoter of human telomerase reverse transcriptase (hTERT) in mice carrying BEL7404 HCC tumors. In this study, a more pronounced apoptosis was induced *in vivo* when animals received cisplatin in adjuvancy to gene therapy [106]. Although the mechanisms were not clearly dissected in this model, it has been reported elsewhere that cisplatin could induce apoptosis by enhancing HCC sensitivity to TRAIL [107] . A different and complex approach was explored by Han L *et al.* in a human HCC model in mice. They observed that co-delivery of hTRAIL conjugated with the transferrin receptor-specific peptide T7-conjugated PEG-modified polyamidoamine dendrimer and doxorubicin resulted in a specific tumoral uptake and the inhibition of tumor growth *in vivo* [108]. These results support the concept of using low and therapeutic doses of doxorubicin in a combined approach.

It has been largely demonstrated that conventional cancer treatments often have little impact on the course of advanced PC. A metastatic PC model was used to assess the efficacy of a combined strategy consisting in the systemic administration of liposomal nanoparticles targeted by a single-chain antibody fragment to the transferrin receptor delivering wild-type p53. Such combination demonstrated prolonged median survival in one week in comparison with individual therapies. It seems that p53 gene transfer induces sensitization of PC cells to conventional chemotherapy, but further studies are needed to validate this strategy [109].

One of the limitations of using adenoviral vectors is the generation of immune responses against viral proteins. In an attempt to protect adenoviruses from the immune system, Kangasniemi *et al.* used silica sol-gel implants to shield the oncolytic adenovirus (Ad5/3-D24). Silica implants were inserted into the peritoneal cavity and mediated the delivery of Ad5/3-D24. As a result, the implants were able to prolong viral presence and to increase the efficacy of Ad5/3-D24 when vectors were combined with gemcitabine [110].

As we mentioned before, autophagy might facilitate tumor progression. However, in certain types of tumors such as HCC, the role of autophagy, and in particular the effect of some chemotherapeutic agents such as oxaliplatin, is not fully understood. Ding *et al.* explored the role of autophagic activation after treatment of HCC with oxaliplatin [33]. Inhibition of autophagy using RNA interference showed enhanced HCC cell death. Interestingly, the combination of oxaliplatin with autophagy inhibitor chloroquine resulted in a more pronounced antitumoral effect. Therefore, it seems that for the case of HCC, manipulation of autophagy should be considered with caution.

GENE THERAPY APPROACHES IN COMBINATION WITH RADIOTHERAPY

The resistance of hypoxic cells to radiotherapy is a major obstacle for a successful treatment due to, at least in part, a reduced capacity of free radicals to generate water radiolysis [111].

Twelve patients with progressive CEA^+ liver metastatic CRC were treated with a replication-competent recombinant vaccinia (rV)-CEA/TRICOM vector and boosted with a replication-defective recombinant fowlpox (rF)-CEA/TRICOM together with radiotherapy. The vaccinia was derived from the New York City Board of Health vaccine and it was shown to be replication-competent while the fowlpox was replication-defective in mammalians. The vaccine contains the CEA transgenes for B7-1, ICAM-1, and LFA-3 human T-cell costimulatory molecules. In this pilot study radiotherapy was administered one day after each vaccine boost. In general, this multimodal therapy was well tolerated and no severe side effects were observed. Regarding antitumoral activity, no objective responses were observed and given the low number of patients enrolled it is unclear if this therapy has an impact on tumor behavior; therefore, more information is needed in order reach a conclusion regarding its biological activity. In addition, in recent years a key role has been attributed to hypoxia-inducible factor 1 (HIF-1) that has been associated with resistance to radiotherapy and poor prognosis in patients with cancer [112]. A specific set of microRNAs (miRNAs) are upregulated by hypoxia. Among these hypoxia-regulated miRNAs, the HIF-1-responsive miR-210 is one of the key miRNAs [113]. In line with this, Yang *et al.* demonstrated that knockdown of miR-210, which resulted in a reduced HIF-1α expression, in combination with radiotherapy showed an increased antitumoral effect on human HCC growth *in vivo* in nude mice [114].

Sodium iodide symporter (NIS) gene transfer may confer tumor sensitivity to radioiodine treatment. Grünwald *et al.* treated HCC using a replication-selective adenovirus under the control of AFP promoter encoding NIS *in vivo*. This combination resulted superior in terms of tumor growth inhibition and overall survival than the use of the vector alone [115]. Mesenchymal stem cells were also used as carriers of NIS for the treatment of experimental HCC [116-117].

It is known that the tumor suppressor gene p53 plays a key role in radiation-induced cell death and that it is negatively regulated by the protein MDM2 [118]. To inhibit p53-MDM2 binding and to increase HCC radiosensitivity in highly MDM-2-expressing cells a replication-deficient ade-

noviral vector containing human p53 was used by Koom *et al.* Enhanced anti-tumor effects were observed using this strategy both *in vitro* and *in vivo* [119].

γ-radiation has the ability to facilitate immune mediated response against cancer cells. In a pilot trial low doses of radiotherapy were combined with a vector-based vaccine targeting CEA in patients with advanced gastrointestinal carcinomas; the majority of them having liver metastases. Although the treatment was well tolerated, the low number of patients included precludes reaching a firm conclusion about the efficacy of treatment [120].

Raf proteins participate in cancer cell resistance to radiation therapy and antisense oligonucleotides to c-raf-1 showed capacity to sensitize cancer cells to radiation therapy. A dose escalation study was carried out by Dritschilo *et al.* in 17 patients with advanced malignancies including CRC and PC, using liposomes complexed with *raf* antisense oligonucleotide (LErafAON) and radiation therapy. Serious adverse events occurred in 5 patients, although liposomal therapy did not increase radiation-related toxicity. Twelve of 17 patients were evaluable for tumor response; 4 showed partial response, 4 showed stable disease, and 4 progressed. This is an attractive strategy but new studies are needed in order to fully characterize the toxicity profile of the liposomal formulation [121].

Suicide gene therapy has shown be effective to induce antitumor and antiangiogenic effects in murine models of HCC and CRC [122-123]. Also the gene-direct enzyme/prodrug therapy (GEPT) was used in human colon carcinoma xenografts, showing the capability of induce tumor apoptosis and significant bystander effects [124]. Moreover, Long *et al.* recently described a fusion suicide gene therapy with anti-angiogenesis gene therapy for pancreatic carcinoma [125].Interestingly, it has recently demonstrated that radio-inducible herpes simplex virus thymidine kinase (HSV-TK) gene therapy enhance local tumor growth after radiation therapy, and also limit transgene expression in the irradiated tumor tissues in an ectopic model of HCC. Moreover, the combination of radio-inducible HSV-TK gene therapy with the stimulation of endogenous dendritic cell proliferation using systemic adenovirus Flt3 ligand (Adeno-Flt3L) gene therapy, results in complete tumor regression for 40% of trimodal treated mice [126]. The study was completed with the addition of sequential administration of recombinant adenovirus-expressing CD40L (Adeno-CD40L) in order to potentiate the efficacy of RT + HSV-TK + Adeno-Flt3L therapy in an orthotopic model of HCC. Then, the use of Flt3L to induce dendritic cell proliferation and CD40L to enhance their maturation in combination with radio-inducible suicide gene therapy allowed an increase in survival of mice and enhance HCC tumor control, becoming a good strategy for improve cancer immunotherapy [127].

Table **1** summarizes therapeutic combinations between gene therapy and chemo or radiotherapy described above.

Table 1. Summary of combined therapy for gastrointestinal carcinomas based on gene therapy.

Gene Therapy + Chemotherapy			
Combination	**Tumor**	**Therapeutic Effects**	**Reference**
AdIL-12+ cyclophosphamide	CRC Metastatic CRC PC	Decreased levels of Tregs INF-γ secreting T CD4+ lymphocytes Tumor complete regressions	[93-94]
AdIL-12/MIF + oxaliplatin	Metastatic CRC	Tumor growth inhibition Immunity against tumor rechallenge	[96]
pIL12 +TACE	HCC	Tumor growth inhibition	[100]
Adp53+ TACE	HCC	Increased survival	[103]
MSC-TRAIL + cisplatin	HCC	Tumor growth inhibition Antiangiogenic effects	[105]
AAV-TRAIL + cisplatin	HCC	Induction of tumor cell apoptosis	[106]
Ad5/3 D24 + gemcitabine	PC	Tumor growth inhibition	[110]
Atg5siRNA+oxaliplatin	HCC	Enhanced tumor cell death	[33]
Gene Therapy + Radiotherapy			
Knockdown of hypoxia-regulated miRNA-210 + X-ray	HCC	Tumor growth inhibition	[114]
Ad-NIS + ^{123}I	HCC	Tumor growth inhibition	[115]
Adp53+ gamma-ray	HCC	Tumor growth inhibition	[119]
Raf antisense oligonucleotides + X-ray	CRC PC	Partial response/tumor stabilization in patients	[121]

AdIL-12: Adenovirus carrying Interleukin-12 genes; AdIL-12/MIF: gutless adenoviral vector carrying a liver-specific, mifepristone (Mif)-inducible system for the expression of IL-12; pIL12: recombinant plasmid expressing IL-12; Adp53: Adenovirus carrying p53 gen; MSC-TRAIL: mesenchymal stem cells carrying Tumor necrosis factor-related apoptosis-inducing ligand; AAV: Adeno-Associated vector; Atg5siRNA: Autophagy protein 5 RNA interference; miRNA: microRNA; Ad-NIS: Adenovirus carrying Sodium iodide symporter; CRC: colorectal carcinoma; HCC: hepatocellular carcinoma; PC: pancreatic carcinoma. TACE: trans-arterial chemoembolization.

Immunostimulatory monoclonal antibodies are aimed to unleash disease-destroying cellular immunity that negatively influences the outcome of patients with cancer. One of them (anti-CTLA-4) demonstrated clinical benefit for metastatic melanoma and metastatic castration-resistant prostate cancer [51, 128]). Therefore, it would be reasonable to consider in a near future the use of immunostimulatory monoclonal antibodies in combination with gene therapy tools for the treatment of gastrointestinal carcinomas.

CONCLUDING REMARKS

The increasing number of newly diagnosed cases of gastrointestinal carcinomas and the lack of a really effective treatment for those patients marks the need for novel therapeutics. In this sense, gene therapy constitutes a widely explored modality *in vivo* both using animal models as well as ~~in~~ phase I/II clinical trials. With the help of gene therapy it is possible to repair cancer cell mutations (e.g. mutation of p53) or to sensitize these cells to chemotherapy-induced apoptosis. We have discussed in this review many ways in which tumors are capable of evading the effects of conventional cancer therapies. Thus, new approaches using gene therapy in combination with radiotherapy and chemotherapy must be explored in order to overcome the resistance mechanisms developed by tumors and furthermore to enhance the clinical outcome of patients. Evidences herein discussed give hope for that by applying such kind of therapeutic combina-tions it; would eventually be feasible to increase the effi-ciency of individual treatments with low or minimal adverse effects.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

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Received: April 23, 2014 Revised: August 11, 2014 Accepted: August 13, 2014