Therapeutic strategies for head and neck cancer based on *p53* status (Review)

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Abstract. Squamous cell carcinomas of the head and neck (HNSCC) are one of the most common types of cancers world-wide, and despite advances in treatment, they still represent a clinical challenge. Inactivation of one or more components in the p53 signaling pathway is an extremely common event in human neoplasia, including HNSCC. The loss of p53 function is responsible for increased aggressiveness in cancers, while tumor chemoresistance and radioresistance can depend on deleted p53 expression, or on the expression of mutated-p53 proteins. Thus, consideration and manipulation of the *p53* status during HNSCC cancer therapy should be considered. This review discusses the *p53* signaling pathways activated by various cellular stresses, including exposure to cancer therapies. The recognition of the *p53* status in cancer cells is a significant factor and could provide valuable assistance during the selection of an effective therapeutic approach.

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1. Introduction

HNSCC is the sixth most common type of cancer worldwide. More than 49,000 new cases of HNSCC cancer are predicted to have occurred in the US in 2010 (1). The disease is multifactorial in its pathogenesis and is associated with smoking, alcohol and infection with the human papillomavirus (HPV) (2). However, the abrogation of p53 function is one of the most common molecular alterations in human cancer cells, including HNSCC (3-5). The prognosis for patients with tumors bearing *p53* mutations is often worse than for those with tumors lacking a wild-type *p53* (wt*p53*) gene (6). For predictive assays, which can be used to evaluate prognosis in cancer therapy, the genetic status of the *p53* gene is one of the most critical candidates among various cancer-related genes (7). In addition, the spectrum of *p53* deletions or mutations observed among tumor cells suggests that the mutations vary in their prognostic power. Disruptive *p53* mutations in tumor DNA are reported to be associated with reduced survival following surgical treatment of HNSCC (2).

It has been previously reported that the radio-, heat- and chemo-sensitivities of HNSCC cells are *p53*-dependent, and are closely correlated with induction of apoptosis *in vitro* (8-10) and *in vivo* (11-13). Consequently, the restoration of wt*p53* function and *p53*-independent therapy have been developed as therapeutic strategies to target tumors with abrogated wtp53 functions.

In this review, cancer therapies aimed at targeting signaling pathways controlled by p53 are described. These include *p53*-gene therapy, chemical chaperones, p53 C-terminal peptides and small molecules that can target p53. In addition, therapeutic strategies independent of *p53* status in cancer cells are discussed. These include high-linear energy transfer (LET) heavy-ion radiation, and enhancement of cancer therapies with other strategies, including an RNA-silencing therapy targeted at DNA repair pathways, and a molecular-targeting therapy for the survival pathway Akt-mTOR.

2. The p53 signaling pathway is activated by various cellular stresses

The p53 protein was identified in simian virus 40 (SV40) trans-formed cells where it is associated with the large T antigen (14),

and was initially considered to be an oncogene. Subsequently, the *p53* gene was revealed to be mutated in various human tumors (15), while its protein product was reported to act as a tumor suppressor (16). *p53*-deleted and *p53*-mutated cells make up approximately 50% of the cells in advanced cancers (17).

p53 is normally in 'standby' mode. The p53 protein is a powerful transcription factor and plays a pivotal role in the pathway controlling apoptosis, cell growth and cell proliferation in response to cellular stresses. These include genotoxic and non-genotoxic stresses, such as DNA damage, hypoxia, oncogene overexpression and viral infection (18-20).

The p21/WAF1 (wtp53 activated fragment 1) gene product, a p53 target gene, inactivates the proliferating cell nuclear antigen (PCNA), which can regulate DNA replication (21), and induce a *p53*-dependent G1 arrest through the inhibition of cyclin/CDK activity (22,23). During cell cycle arrest, *p53*-regulated pathways, including those involving growth arrest and DNA damage inducible 45 (*Gadd45*) and the p53 ribonucleotide reductase small subunit *2* (*p53R2*), are significant in the repair of damaged DNA (24,25). In the absence of competent repair activity, DNA damage induces apoptosis through interactions with other genes in *p53*-regulated pathways, including the Bcl-2-associated X protein (Bax) (26), p53-upregulated modulator of apoptosis (PUMA) (27), Fas/APO-1 (28) and p53-activated gene 608 (PAG608) (29). By contrast, the *p53*-regulated MDM2 (murine double minute 2) (30) functions to produce negative feedback, which regulates p53 activity (26).

In the presence of cellular stresses, p53 is subjected to a complex and diverse array of covalent post-translational modifications. These include phosphorylation (31), acetylation (32), poly(ADP-ribosyl)ation (33), ubiquitylation and sumoylation (34,35). In response to cellular stress, Ser15/Ser20 in p53 are phosphorylated and MDM2 is separated from the phosphorylated p53, leading to the stabilization and activation of p53 (36-38). Therefore, p53 can bind to the promoter of the *p21* or *p53R2* genes associated with DNA repair, and induce their expression. However, if there are numerous DNA lesions or too much cellular damage, G1 arrest and DNA repair will not be successful. In this situation, p53 can be phosphorylated at Ser46, and bind to the promoter of the p53-regulated apoptosis-inducing protein 1 (*p53AIP1*) gene, leading to apoptosis (39,40). Moreover, PUMA is reported to be required not only for p53-dependent apoptosis induced by DNA damage, hypoxia and oncogenes, but also for apoptosis induced by p53-independent stimuli, including serum withdrawal, glucocorticoids, kinase inhibitors and phorbol esters (27). By contrast, p53 molecules are inactivated and degraded by activated MDM2 molecules, which are phosphorylated at multiple sites by other protein kinases. In addition, p53 is reported to bind to other proteins, including heat shock proteins (HSPs), functioning as stress proteins (41,42), and Bcl-X (43). Consequently, these modifications of p53 molecules can regulate or affect the fate of cells following exposure to stresses, including cancer therapies.

3. p53-dependent cancer therapy via the restoration of p53 function

Recently, Poeta *et al* reported an association between a *p53* mutation in a patient with HNSCC and survival following surgical treatment (2). The results demonstrated that *p53*-deleted and *p53*-mutated HNSCC patients were significantly associated with short survival periods. These data indicate that *p53* mutations could be a useful evaluation or stratification factor in prospective clinical trials. However, in the study, chemotherapy was administered only as an adjuvant measure in combination with postoperative radiation therapy, or prior to study entry in a few cases. There are no data on tumor response to chemotherapy. It would be clinically useful to determine whether *p53* mutations are associated with a response to treatments that attack p53-specific pathways. A study described that sensitization to radiation, heat and chemical therapies was observed in cells containing wt*p53*, but not in cells containing mutated *p53* (m*p53*) *in vitro* and *in vivo* (8-10). Furthermore, in attempts to treat cancer using more than one treatment modality, a synergistic depression of tumor growth was found only in tumors containing wt*p53* (44). These findings suggest that hyperthermic enhancement of tumor growth inhibition with irradiation may result in *p53*-dependent apoptosis via caspase-3 activation in HNSCC cells. Therefore, an analysis of *p53*-gene status in cancer cells could be considered as a useful predictive assay for estimating the possible effectiveness of combined therapies involving radiation, heat and anti-cancer agents. Thus, it is very reasonable to enhance p53-dependent apoptosis pathways through the restoration of p53 function even for m*p53* HNSCC cells as a more effective therapeutic strategy. A number of approaches have been employed to achieve this outcome (illustrated in Fig. 1).

A p53 gene therapy-based approach. As previously mentioned, the activation of endogenous wt*p53* by radiation and/or chemotherapy in wt*p53* cancer cells leads to *p53*-mediated apoptosis. In recent years, the introduction of exogenous wt*p53* into cancer cells, either by gene delivery or by direct protein delivery, has been explored. Although preliminary studies in cell cultures and in animal models have indicated the effectiveness and the low toxicity of these approaches (45-47), their efficacy in clinical trials is currently controversial. Clinical studies in lung, bladder, ovarian and breast cancer revealed the absence of additional beneficial effects compared to conventional treatments (48). On the other hand, encouraging results were reported for phase II and III clinical trials on 135 patients with advanced HNSCC. In this study, patients were treated with a combination of recombinant adenovirus-*p53* (Gendicine) administration and radiotherapy. The results demonstrated that 64% of the patients achieved complete regression, and 32% achieved partial regression. No serious side effects were observed (49). Although such results are encouraging, further improvements in methods are required to accomplish the safe and effective delivery of wtp53 *in vivo* (50).

Onyx-015, an adenovirus based therapy. In the absence of wtp53 activity in cancer cells, the generation of a mutated viral vector for tumor cell lysis (Onyx-015) was exploited. McCormick *et al* from Onyx Pharmaceuticals hypothesized that an adenovirus with the *E1B* region deleted could only replicate and generate cellular lysis in cells lacking functional p53, due to the putative requirement for p53 inactivation for adenoviral replication. Accordingly, the Onyx-015 reagent, a p53-targeting oncolytic mutant adenovirus, has been
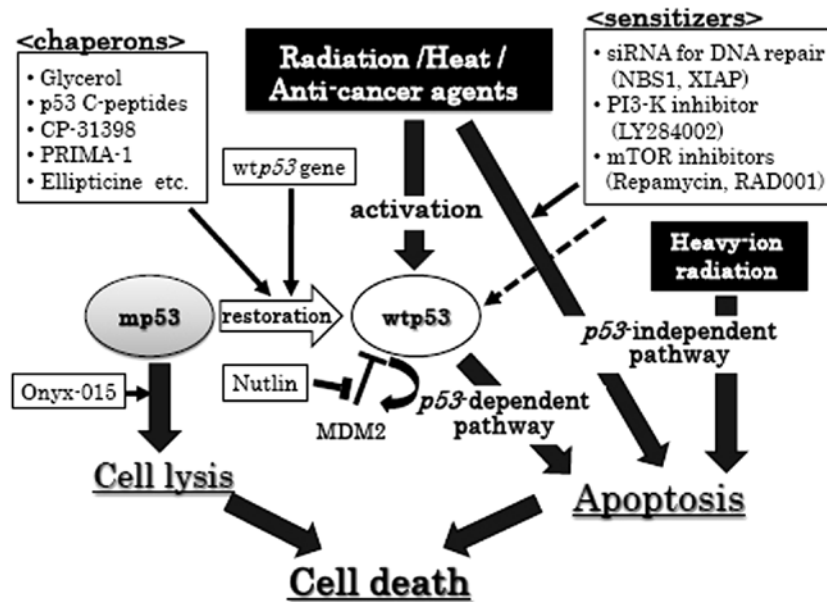
Figure 1. *p53*-dependent and -independent therapeutic strategies for cancer cells. Circles, *p53* status of cancer cells; black squares, cancer therapeutic tool; white squares, enhancer for cancer therapeutic tool; thin arrows, enhancement; dashed arrows, partial enhancement; thick arrows, therapeutic pathway. MDM2, murine double minute 2; XIAP, X-chromosome-linked inhibitor of apoptosis protein; wt*p53*, wildtype p53; mp53, mutant p53; siRNA, small interference RNA.

developed for clinical application (51). However, evaluation of numerous clinical trials performed thus far have indicated that the administration of Onyx-015 as a single agent produces only a marginal benefit, whereas its administration in combination with conventional therapy is more effective (52).

Chaperones for the restoration of the p53 molecule
Glycerol. Another approach in preclinical development involves restoring tumor-suppressing function to m*p53*. Studies have demonstrated that glycerol, as a chemical chaperone, can restore normal p53 function in mp53 HNSCC cells (53). Glycerol has been previously reported to act as a chemical chaperone due to its ability to refold proteins and restore normal activity; this type of activity was able to alter or restore a functional protein conformation from conformation forms found in a human disease state (54,55). Consistent with this observation, glycerol is capable of restoring *p53*-dependent radiosensitivity in *mp53*-HNSCC cells via Bax-mediated induction of apoptosis (53,56,57). Glycerol can also restore heat-induced *p53*-dependent apoptosis in A-172/m*p53* cells (58) and CDDP-induced tumor growth inhibition in 8305c (59) and SAS/m*p53* cells (13), by binding to p53 consensus sequences (p53CON) located upstream of *p53*-regulated genes. These results suggest that glycerol could be effective in causing conformational changes that restore wtp53 functioning to mp53, leading to enhanced results with radio-, hyperthermic- and/or chemo-therapies through the induction of apoptosis via a restored wtp53 function.

p53 C-terminal peptides. One of the significant features of p53 tumor-suppressor activity is its ability to bind to p53CON; the majority of mutations in *p53* are localized in the binding domain of p53CON (60,61). This sequence-specific DNA-binding ability of p53 is allosterically regulated by its C-terminal domain (62,63), and can be activated *in vitro*

by C-terminal truncation or anti-p53 monoclonal antibody PAb421 binding, which recognizes a C-terminal epitope (64). In addition, small peptides corresponding to the C-terminal residues 369-383 of p53 are capable of activating latent p53, and permit specific DNA-binding *in vitro* (65). Thus, the sequence-specific DNA binding activity of mp53 proteins could be rescued by PAb421 (62,66,67). The inhibition of cell proliferation (68) and the induction of apoptosis (69) were effectively induced in mp53 cancer cells transfected with C-terminal peptides following X-ray-irradiation or heat-treatment (70,71).

CP-31398 and other small molecules. In other studies evaluating binding to p53CON sequences, CP-31398 was identified as a small molecule with the ability to restore the wild-type conformation to mp53 protein by stabilizing the active conformation of the DNA binding domain (72,73). Further studies have confirmed that CP-31398 treatment causes: i) stabilization of wtp53 levels; ii) apoptosis-related changes; and iii) induction of p53 target genes. Moreover, CP-31398 was demonstrated to increase the levels of wtp53 protein by inhibiting the MDM2-mediated ubiquitylation and degradation of p53 (73). The observation that CP-31398 stabilizes wtp53 suggests that CP-31398 interacts with newly synthesized p53 molecules *in vivo* and changes its folding behavior (74). Subsequently, PRIMA-1 (75) and ellipticine (76) were also found to be capable of inducing *mp53*-dependent cell death. On the other hand, Nutlin was developed to rescue wt*p53* from degradation mediated by MDM2 (77). More recently, *p53* family members could be activated, were capable of serving as substitutes for p53 in tumor cells, and were able to induce cell death. These observations may provide a novel tool for the correction of mp53 conformation and loss of function, and may be applicable to *p53*-targeted cancer therapy.

4. *p53*-independent cancer therapy

High-LET heavy-ion radiation. High-LET charged particle radiation has several potential advantages over X-rays, including an excellent dose distribution, a higher relative biological effectiveness (RBE), a reduction in the oxygen enhancement ratio, less variation in cell cycle-related radiosensitivity, and the existence of less efficient repair mechanisms for cellular radiation injury (78-80). As a result, high-LET charged particle radiation could have serious lethal effects, even on radioresistant tumors (81). Heavy ion beams can also allow a high radiation dose to be delivered to a tumor with minimal irradiation of the surrounding normal tissues. High-LET radiation also induces apoptosis effectively regardless of the genetic status of the *p53* gene in cancer cells (82,83). Heavy ion radiotherapies consequently appear to be attractive for use in numerous types of human cancer. The lack of a *p53*-regulated pathway is a common feature in a large number of tumors, suggesting that it is a significant factor in the pathogenesis of human cancers. As previously reported, cellular sensitivity to radiation and/or heat depends on the *p53*-gene status in HNSCC cells (9,18) and other cells (82,84). Therefore, the aim of a number of studies has been to induce apoptosis by reinstating normal functioning of the m*p53* gene (58). However, it has not been practical in the clinic to monitor the status of the *p53* gene or other useful genetic markers. Thus, attention has been given to therapies using high-LET radiation, which have highly lethal effects on radioresistant tumors (81), and which can induce apoptosis effectively regardless of the *p53*-gene status (82,83). It has been suggested that high-LET radiation delivered to HNSCC cells may enhance apoptosis through the activation of caspase-3 through caspase-9, even in the presence of m*p53* (85).

RNA-silencing therapy targeting DNA repair pathway. RNA interference has become a valuable tool for the selective suppression of the expression of a target gene. The mRNAs produced by a targeted gene are cleaved by an RNA-induced silencing complex, which includes small interference RNAs (siRNAs) and a nuclease. Cell cycle signaling or DNA repair proteins, including ATM, ATR and DNA-PKcs, have become the targets of interest in investigations involving the siRNA-mediated enhancement of radiation sensitivity (86,87). Studies have demonstrated the potential use of siRNA as a novel radiation sensitizer for improving the effectiveness of radiation therapy in cancer.

The NBS1 protein is essential for the initial processing of the DNA double-strand break via the homologous recombination (HR) repair pathway (88,89). NBS1 forms a complex with MRE11 and RAD50 in the nucleus. This complex binds to ATM-phosphorylated γH2AX and is recruited to the area surrounding damaged DNA ends (90). MRE11/RAD50/NBS1 complexes can be visualized in the form of foci in an irradiated area of the nucleus (91). Studies indicate that NBS1 appears to regulate radiation sensitivity in cells through its role in the HR repair system. In addition, it has recently been demonstrated that the siRNA-mediated reduction of NBS1 appears to lead to an increase in radiation-induced mutagenesis in human cells (92).

Ionizing radiation induces a signaling pathway which activates the transcription factor NF-κB. NF-κB then regulates the transcriptional activation of a number of genes involved in cell proliferation, angiogenesis, metastasis and the suppression of apoptosis (93). Therefore, the radiation-induced activation of NF-κB could promote oncogenesis and resistance to cancer therapy (94,95). Moreover, it is possible that the NBS1 protein may play a role in the NF-κB pathway, which is activated by radiation; NBS1-deficient cells exhibit a defective activation of NF-κB following exposure to radiation (96). Thus, inhibition of NBS1 could result in depression of NF-κB activation and in the transcriptional activation of NF-κB-regulated genes. One of the proteins regulated by NF-κB, X-chromosome-linked inhibitor of apoptosis protein (XIAP), plays a pivotal role in cancer progression, and is a strong candidate among cancer therapy targets (97).

Sensitization to radiation results from *NBS1*-siRNA mediated suppression of DNA repair functions and X-ray-induced cell survival signaling pathways, which operate through NF-κB and XIAP (98). NBS1 is also involved in the heat-induced cellular responses to DNA damage, and it has been suggested that *NBS1*-siRNA is a potential candidate for a sensitizer for heat treatments, which could be effective regardless of cellular *p53*-gene status (99,100). Moreover, siRNA that can target *XIAP* can lead to an effective enhancement of X-ray-induced apoptosis in human cancer cells with m*p53* (98,101). The results described in these studies suggest that siRNA designed to target DNA repair functions could lead to novel methods that could increase radiation and/or heat sensitivity, even in human m*p53*-bearing cancer cells.

Molecular-targeting therapy for Akt-mTOR pathway. The PI3K/Akt pathway is a major cell survival pathway and plays a critical role in oncogenesis and tumor cell growth (102). Recent studies have reported that Akt activation contributes to resistance to radiation, chemotherapy and tyrosine kinase inhibitors by promoting survival signals, which protect cancer cells from undergoing apoptosis (103,104). The inhibition of PI3K/Akt through pharmacological or genetic means induces anti-proliferative effects in HNSCC cells *in vitro* and *in vivo* (105,106). Akt is activated by heat and radiation through a phosphatidylinositol-3-kinase (PI3K)-mediated phosphorylation pathway (107). Radio-sensitization induced by LY294002, a specific inhibitor of PI3K, has been reported in *in vitro* (108) and *in vivo* experiments (109). LY294002 inhibits anti-apoptosis signaling through the induction of Hsp27 and Hsp72, and cell survival signaling through Akt and survivin. LY294002 appears to be a noteworthy candidate as a *p53*-independent heat sensitizer in hyperthermic cancer therapy (110).

The mammalian target of rapamycin, mTOR, is a 289-kDa serine-threonine kinase, which acts as a downstream effector for Akt (111). It regulates key processes, including cell growth and proliferation, cell cycle progression and protein translation through two distinct pathways; one involving the ribosomal p70S6 kinase (p70S6K), and one involving the eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs) (112). Akt activation is closely associated with the upregulation of mTOR activity. It has been suggested that dysregulation of mTOR contributes to cancer progression (111), and therefore, mTOR may be a potential therapeutic target which could inhibit or block the PI3K/Akt pathway. Inhibitors

of mTOR are currently under development; rapamycin and its derivatives CCI-770, AP23573 and RAD001. The antiproliferative effects of mTOR inhibitors have been observed in various tumor cells *in vitro* and *in vivo* (113-115). These mTOR inhibitors are generally regarded as cytostatic agents, since they induce G1 cell cycle arrest, but not apoptosis (113). However, recent studies have demonstrated that mTOR inhibitors can enhance the cytotoxic effects of chemotherapeutic agents and radiation in a number of human cancers (116-118). Furthermore, a study has demonstrated that rapamycin in combination with radiation was able to augment the cytostatic effects of radiation regardless of cellular *p53*-gene status in lung cancer and HNSCC cells, suggesting that inhibition of the mTOR signaling may be a promising strategy for radiosensitization regardless of *p53*-gene status, with respect to cell lethality and cell growth depression (119).

5. Conclusion

Over the past few decades, despite the introduction of new multimodal therapies, there has been a failure to achieve a high efficacy in tumor therapy. This may be caused by a primary or acquired resistance to the DNA damaging agents used in chemotherapy and radiotherapy, and remains a formidable and poorly understood problem. This review discussed the effect of *p53*-targeting cancer therapies on *p53* signaling pathways, including *p53*-gene therapy, chemical chaperones, p53 C-terminal peptides, *p53*-targeting chemicals and inhibitors targeting several signaling pathways, in an effort to induce cell death in cancer cells. High-LET radiation can induce apoptosis effectively regardless of the genetic status of the *p53*-gene in cancer cells. Identification of the *p53* status in the target cells is imperative, and the knowledge of additional oncogenic events contributing to specific types of cancer could significantly aid in the selection of appropriate therapeutic protocols. The restoration of p53 functioning could be helpful when pathways upstream of p53 expression are defective, but not if defects are downstream of p53 signaling. The re-expression and re-activation of p53 in human cancer cells could increase tumor susceptibility to radiation or chemotherapy, enhance the efficacy of standard therapeutic protocols, and lead to individually designed therapies (52). Further investigations in this area will hopefully lead to more effective cancer treatments in the near future.

Acknowledgements

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