The relevance of molecular biomarkers in cervical cancer patients treated with radiotherapy

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Background: Radiotherapy (RT) plays an integral role in the combined-modality management of cervical cancer. Various molecular mechanisms have been implicated in the adaptive cellular response to RT. Identification of these molecular processes may permit the prediction of treatment outcome and enhanced radiation-induced cancer cell killing through tailoring of the management approach, and/or the employment of selective inhibitors of these pathways.

Methods: PubMed was searched for studies presenting biomarkers of cervical cancer radioresistance validated in patient studies or in laboratory experimentation.

Results: Several biomarkers of cervical cancer radioresistance are validated by patient survival or recurrence data. These biomarkers fall into categories of biological function including hypoxia, cell proliferation, cell-cell adhesion, and evasion of apoptosis. Additional radioresistance biomarkers have been identified in exploratory experiments.

Conclusions: Biomarkers of radioresistance in cervical cancer may allow molecular profiling of individual tumors, leading to tailored therapies and better prognostication and prediction of outcomes.

Keywords: Cervical cancer; biomarker; radiation therapy; radioresistance

Introduction

Cervical cancer is a significant health problem for women around the world: it is the second most common female cancer (1), and the leading cause of cancer death in women in most developing countries (2). Human papillomavirus (HPV), the most common sexually transmitted infection worldwide, plays a major role in cervical cancer carcinogenesis, with HPV types 16 and 18 together accounting for about 70% of all cervical cancers (3). While early disease can be treated by radical hysterectomy, the standard treatment for advanced disease is surgery followed by radiotherapy (RT) with or without concurrent cisplatin chemotherapy (1) or radical concurrent chemoradiotherapy (CCRT) (4). Five-year survival rates are high for early stages, but decrease to 50-70% for stage IIB and only 30-50% and 5-15% for stage III and IV, respectively (5). Locoregional treatment failure is ascribed primarily to radioresistance: in stage IIB-III tumors, even high doses of 85 Gy result in 35-50% local failure (6). Moreover, radiation treatment failure is commonly associated with the development of metastases (7). Therefore, radioresistance is a clinically relevant problem in the management of cervical cancer.

The evaluation of biomarker status in cancers yields information about prognosis, treatment response, and vulnerability to targeted therapies. Historically, biomarker status in oncology was used in the differential diagnosis to differentiate cancer from normal tissue (8). However, rapid advances in genetic/genomic and histologic/proteomic technologies have led to a rapid expansion in biomarker functionality, ranging from estimating cancer risk [i.e., the breast and ovarian cancer mutation *BRCA1* (9)] to predicting response to therapy [i.e., *KRAS* mutations in colorectal cancer (10); *HER2* expression in breast cancer (11)].

The importance of radioresistance in cervical cancer treatment failure indicates that certain biomarkers may be useful for cervical cancer treatment individualization. Indeed, both translational and clinical research articles investigating this topic have increased dramatically in the past few years. However, the literature currently lacks a review of biomarker relevance in cervical cancer patients treated with RT. Therefore, we aimed to perform a review of the available literature to identify both clinically-validated intrinsic, non-secreted tumor cell protein biomarkers of radioresistance in cervical cancers, as well as putative biomarkers identified by laboratory experimentation in order to identify salient targets for further clinical investigation. This review aims to present validated tumor protein biomarkers that have pragmatic relevance to RT, and to discuss the biologic mechanisms of these biomarkers to better elucidate the cellular processes implicated in RT resistance.

Methods

From December 1983 to August 2015, PubMed was searched using the following queries:

(I) "Cervical carcinoma AND radioresistance" (85 results);
(II) "Cervical carcinoma AND biomarker radioresistance" (11 results);
(III) "Cervical carcinoma AND biomarker radiosensitivity" (35 results).

These queries yielded 131 results. Abstracts were screened for relevance. The full text of selected articles was read to assess for relevance and presentation of desired data. Studies were divided into clinical or basic science categories as defined below.

For the clinical studies reviewed, the following criteria were used for selection. Selected studies had to present evidence of a biomarker of radioresistance validated by immunohistochemistry for evaluation of expression levels in primary patient tumor samples. Radioresistance in these studies was defined as "poor or incomplete response to radiation therapy", by "greater (than expected) rate of recurrence" after radiation therapy, or by poor overall survival (OS), metastasis-free survival (MFS), or disease-free survival (DFS) after radiation therapy compared to a reference radiosensitive group. Studies also needed to present patient survival or recurrence outcomes in biomarker *vs.* non-biomarker groups for a minimum of 3 years of follow-up. Studies also needed to include statistical significance of the association of the biomarker with a measure of poor prognosis.

The following criteria were used for the selection of basic science studies: studies could be carried out in either immortalized cervical cancer cell lines or primary cervical cancer cells. However, only those studies not presenting any patient outcome data were selected. Studies fulfilling these criteria were selected, and screened for quality.

Results

Studies presenting biomarkers validated in patient tumor data

Full-text screening and assessment for inclusion criteria yielded 19 publications that presented a biomarker of cervical cancer radioresistance validated using patient data (*Table 1*). Of these, 11 met the selection criteria. Eight studies presented survival data without univariate analysis (UVA) or hazard ratio (HR), and one included UVA and HR without survival outcome, and so were excluded.

Cohort size ranged from 27 to 300. Seventeen of the 19 studies assessed radioresistance in patients treated with radiation alone, or presented data that distinguished patients treated with radiation alone separately from CCRT treated patients. All studies evaluated biomarker expression in patient samples via immunohistochemistry, except for Haensgen *et al.*, which evaluated hypoxia via Eppendorf histograph rather than by a protein marker (23).

These 19 studies identified a total of 23 biomarkers (22 gene products and the 23rd, hypoxia, a general characteristic of the tumor microenvironment) that can be separated into six biologic subgroups by the function of the protein: apoptosis, cell adhesion, DNA repair, hypoxia, metabolism, pluripotency, and proliferation. While the facile nature of these subgroups do not reflect the complex nature of some of these markers (for example, the complex roles of Akt and c-Erb-B2 in cell migration, metabolism,

Table 1 Biomarkers of cervical cancer radioresistance identified in patient studies

Year	Author	N	Biomarker	Biological function	End point with significant P value[§]	OS hazard ratio (95% CI)	DFS hazard ratio (95% CI)
2000	Loncaster et al. (12)	100	VEGF(+)	Angiogenesis	4Y OS and 4Y DFS	NR	NR
2013	Cao et al. (13)	44	iASPP(+)	Apoptosis	5Y OS, DFS	NR	8.4 (5.1-11.7)[U]
2006	Liu et al. (14)	84	ΔNp73(+)	Apoptosis	5Y LR/DM/OS	4.9 (1.8-12.9)[U]	NR
2004	Wootipoom et al. (15)	174	Bcl2(+)Bax(+)	Apoptosis	5Y DFS*	NR	3.6 (1.3-9.7)[U]
2003	Jain et al. (16)	76	p53(+)Bcl2(+)	Apoptosis	5Y DFS	NR	NR
2015	Huang et al. (17)	111	Opn(+)E-cad(−)	Cell adhesion	5Y PFS	NR	7.3 (3.2-16.8)[U]
2014	Zhang et al. (18)	59	β-cat(+)	Cell adhesion	5Y OS/DFS	4.1 (1.3-12.6)[M]	1.3 (0.4-4.6)[M]
2012	Roossink et al. (19)[€]	300	ATM(+)	DNA repair	4Y DFS	NR	1.4 (1.0-2.0)[U]
2013	Huang et al. (20)	154	Lgals1(+)	Hypoxia	10Y CSS	CSS: 1.9 (1.2-3.2)[M]	LR: 2.6 (1.5-4.5)[M]
2011	Kim et al. (21)	36	HIF2α(+)	Hypoxia	4Y PFS	NR	1.5 (1.1-2.0)[NS]
2004	Ishikawa et al. (22)	38	HIF1α(+)	Hypoxia	10Y LR/DM, 10Y MFS	NR	NR
2001	Haensgen et al. (23)	70	Hypoxia(+)tp53(+)	Hypoxia, apoptosis	3Y OS (P=0.07)	NR	NR
2014	Shen et al. (24)[€]	92	Stc-2(+)	Metabolism	5Y OS, DFS	6.1 (2.5-9.3)[M]	5.4 (2.3-7.2)[M]
2014	Shen et al. (25)	132	Sox2(+)Oct4(+)	Pluripotency	5Y PFS	NR	3.3 (1.8-5.9)[U]
2005	Gaffney et al. (26)	55	EGFR(+)	Proliferation	10Y OS	NR	NR
2006	Kim et al. (27)	27	pAkt(+)	Proliferation	5Y PFS	NR	NR
1999	Nishioka et al. (28)	107	c-erb-B2(+)	Proliferation	4Y OS and 4Y MFS	NR	NR
2012	Moreno-Acosta et al. (29)	38	IGF1R(+)	Proliferation	3-month CR	NR	26.8 (1.7-41.2)[M]
2012	Schwarz et al. (30)	62	PI3K/Akt pwy(+)	Proliferation	5.5Y CSS, 5.5Y PFS	NR	NR

Biomarkers are indicated as overexpression (+) or under-expression (−) status of each marker. [§], endpoints are derived from comparison between patients with differential expression vs. normal expression of biomarkers, P<0.05 except where indicated; [U], univariate analysis; *, text of paper stated HR is significant compared to least anti-apoptotic signature, but P value not specified; [M], multivariate analysis; [€], concurrent chemoradiation therapy was used in these studies; [NS], not specified whether analysis was univariate or multivariate. CR, complete response; CSS, cause-specific survival; DFS, disease-free survival; DM, distant metastasis; DSS, disease-specific survival, LACSCC, locally advanced cervical squamous cell carcinoma; LR, local recurrence; MFS, metastasis-free survival; NR, not reported; OS, overall survival; PFS, progression-free survival; pwy, pathway; Y, year (e.g., 10Y, 10-year).

and apoptosis in addition to proliferation), these divisions are useful for a functional understanding of how the native protein product may be involved at the cellular level in the induction of radioresistance (*Figure 1*). Subgroups with the greatest representation and clinical relevance are discussed below.

Apoptosis evasion biomarkers

The ability to evade apoptosis is a hallmark of cancer cells of every origin (31). Of specific relevance to radiation therapy, radiation-induced apoptosis is a main mechanism

of cancer cell killing in radiotherapeutic techniques (32,33). Expectedly, multiple biomarkers identified in this literature review are involved in the inhibition of apoptosis.

In response to various cellular stresses, including irradiation, pro-apoptotic Bax induces porosity of the outer mitochondrial membrane, and pro-apoptotic factors, notably cytochrome *c* and SMAC (an inhibitor of caspase inhibitors), invade the cytosol (34). These factors initiate, via activation of Casp9, proteolytic activation of a caspase cascade that cleave essential cellular proteins and thereby induce controlled cell death via the mitochondrial apoptotic pathway. p53, a master regulator of apoptosis, promotes apoptosis in response to DNA damage,
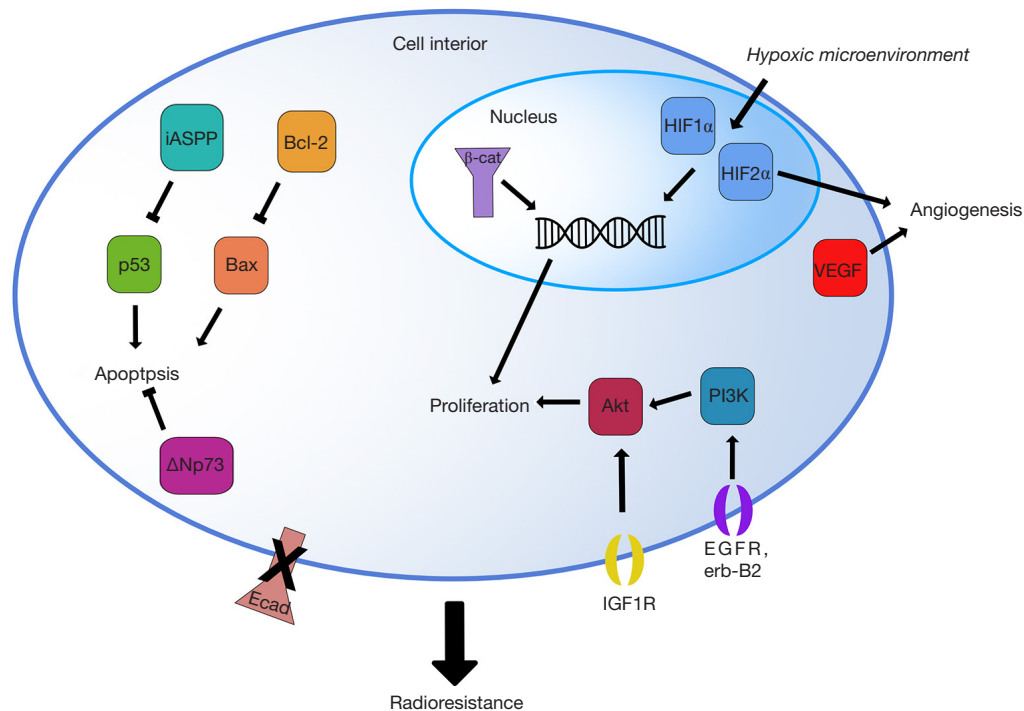
Figure 1 Visualization of the major biomarkers of radioresistance in cervical cancer, their biological functions, and their interrelationships.

UV exposure, stress, and other cytotoxic agents by several mechanisms including upregulation of Casp9 coactivators, transactivation of several death receptors including Fas, and shifting of the Bax/Bcl-2 balance to favor apoptosis (35).

Bcl-2

Bcl-2 controls cellular commitment to apoptosis by inhibiting the mitochondrial pathway. Bcl-2 interacts with targets such as Bax via its functional fold, defined by seven alpha-helices burying a central hydrophobic alpha-helix. Bcl-2 fold binding to Bax at Bax's hydrophobic BH domains physically inhibits Bax signaling, blocking mitochondrial rupture and preventing caspase activation (34).

Several studies have identified Bcl-2 as an *in vitro* and *in vivo* marker of radiosensitivity in numerous malignancies. Recent work from the Haffty group has demonstrated a role for Bcl-2 in breast cancer radioresistance *in vitro* (36) and *in vivo* (37). Using the small-molecule Bcl-2 inhibitor ABT-737, a BH domain mimetic that binds Bcl-2 and prevents its binding and inactivation of Bax, in breast cancer cell lines MCF-7, ZR-75-1, and MDA-MB-231, Wu *et al.* (36) showed that Bcl-2 inhibition strongly sensitized cells to radiation, and that the mechanism of radiosensitization is via the down-regulation of pro-survival Mcl-1 and

induction of the apoptotic Bak pathway. In a study of 116 patients receiving RT for early-stage breast cancer, Yang *et al.* demonstrated that Bcl-2 is an independent prognostic marker and a marker of radioresistance: Bcl-2 expression was associated with a significantly increased rate of local recurrence after RT (P=0.03) (37). Bcl-2 is also a clinically validated biomarker of radioresistance in small-cell lung cancer, multiple myeloma, several types of leukemia (38), laryngeal squamous cell carcinoma ,and prostate cancer (39). Laboratory studies suggest a role in the radioresistance of additional malignancies: work from other groups further confirms that in breast cancer cells, treatment with ABT-737 reverses acquired radioresistance (40); and Bcl-2 overexpression in the hematopoietic system protects mice from normally lethal levels of radiation (41). This validation as a radioresistance biomarker in multiple cancer types coupled with the existence of ABT-737 and other inhibitors such as the orally bioavailable ABT-199 suggests that Bcl-2 status should be evaluated in cervical cancers and that Bcl-2 inhibition should be investigated for potential radiosensitization in these lesions (42).

Two studies identified Bcl-2 as a biomarker of radioresistance in cervical cancers (*Table 1*) (15,16). In 174 cervical cancer patients treated with radiation, Wootipoom *et al.*
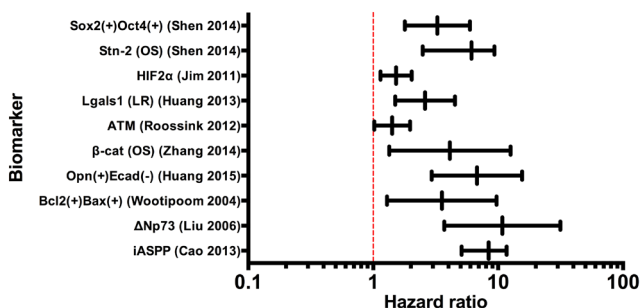
Figure 2 Hazard ratio and 95% confidence interval for association of biomarkers with radioresistance. For studies that provided hazard ratios for multiple outcomes, outcome is indicated in parentheses after biomarker.

demonstrated that tumors with the Bcl-2⁺/Bax⁺ signature had the worst DFS (HR =3.55; 95% CI, 1.29-9.72) (15) (*Table 1* and *Figure 2*). Jain *et al*. (16) found that in 76 patients treated with RT, tumors with the p53(+)/Bcl-2(+) signature had poorer DFS (*Table 1*). Given that a major tumoricidal mechanism of radiation is the induction of apoptosis, the observation that tumors with increased Bcl-2 expression are resistant to radiation is not surprising. Although both of these studies demonstrated that Bcl-2 is not an independent marker of prognosis nor radioresistance, it remains a significant prognostic and radioresistance marker when evaluated in conjunction with Bax (15) or with tumor suppressor p53 (16). These observations likely reflect the fact that activation of the mitochondrial pathway is a multifactorial process dependent on the balance of diverse upstream inputs.

p73

p73 is a tumor suppressor homologue of p53, with a more complex role in oncogenesis. While some studies identify p73 as a tumor suppressor, others suggest that it functions as an oncogene (43); these contradictory observations are reconciled by the existence of several antagonistic splice variants of p73. Variant TAp73 drives apoptosis via activation of Bax in the mitochondrial apoptosis pathway (described above), whereas dominant-negative ΔNp73 inhibits both TAp73 and p53 to strongly repress Bax and inhibit apoptosis, analogous to Bcl-2 (44,45). Liu *et al*. demonstrated that upregulation of ΔNp73 in cervical tumors strongly predicts the likelihood of radioresistance (HR =10.8; 95% CI, 3.704-31.492), and predicts local recurrence (HR =4.857; 95% CI, 1.823-12.943) (14)

(*Table 1* and *Figure 2*). Moreover, ΔNp73 expression is associated with poorer 5-year OS (90% *vs*. 46% in ΔNp73 negative *vs*. n ΔNp73 positive cases, P<0.001). ΔNp73 may therefore impart radioresistance via inhibition of apoptosis through down-regulation of p53, TAp53, and Bax signaling.

iASPP

Compared to the well-characterized Bcl-2 and p73, the p53 inhibitor iASPP is less well-studied and understood. Genetic and biochemical studies in *C. elegans* and the human U2OS cell line demonstrate that iASPP inhibits p53-mediated apoptosis and that a physical interaction between iASPP and p53 exists, suggesting that iASPP exerts its oncogenic role by physically inhibiting p53 activity (46). Cao *et al*. showed that after resection and subsequent radiation, cervical cancer patients with high nuclear iASPP expression had shorter 5-year OS (66% *vs*. 100%, P=0.01) and 5-year DFS (53% *vs*. 90%, P=0.013) compared to patients with low nuclear iASPP (13) (*Table 1* and *Figure 2*). Although a relationship between iASPP and radioresistance in other malignancies has not been studied, iASPP is associated with poor prognosis in melanoma (47), and drives proliferation of gastric (48), prostate (49), and tongue squamous carcinoma (50) cells *in vitro*, suggesting that iASPP should be further investigated in cervical and other cancers for prognostic and radiotherapeutic implications.

Hypoxia biomarkers

Inadequate oxygen supply, or hypoxia, is a characteristic of most tumors (51). Although malignant cells are defined by their ability to induce angiogenesis, the microvascular topology of a tumor is usually abnormal, with larger than normal intercapillary distances that are greater than the diffusion distance of oxygen (51). Hypoxia thus acts as a selective pressure that favors malignant pro-survival cell phenotypes such as migration, angiogenesis, and suppression of apoptosis, and also promotes genetic instability due to increased mitochondrial release of reactive oxygen species (ROS) (51,52). Furthermore, hypoxia directly opposes the efficacy of RT and yields radioresistance in tumor cells. Ionizing radiation is cytotoxic due to the induction of DNA double-strand breaks (DSBs); in hypoxic cells, the number of DSBs incurred upon irradiation is reduced, with anoxic cells displaying only 1/2 to 1/3 the DSBs of normoxic cells at a given dose, and the expression of DNA repair pathway components is also altered (53,54). Hypoxic conditions also induce expression of hypoxia-inducible factors (HIFs),

transcription factors instrumental for survival in hypoxic conditions via inducing genes for angiogenesis, metabolism, invasion, proliferation, and metastasis, among other malignant phenotypes (55). Moreover, HIFs are essential to radioresistance in hypoxic lesions. Irradiation induces HIF expression, propagating the HIF survival phenotype and rendering further radioresistance (56); indeed, radiosensitization requires the presence of oxygen at the time of irradiation (53). Therefore, the innate hypoxic state in many tumors confers radioresistance, which is further aggravated by the upregulation of HIFs. Further confirming the well-demonstrated relationship between hypoxia and radioresistance is the identification of multiple molecular markers of hypoxia in the complement of radioresistance biomarkers in cervical cancer.

HIF1α and HIF2α

As discussed above, HIFs are up-regulated in response to both hypoxia and irradiation and induce multiple neoplastic attributes upon cancer cell. Ishikawa *et al.* identified HIF1α as a biomarker of poor prognosis after radiation therapy in cervical cancer patients; the 10-year recurrence-free survival (RFS) was only 22% in HIF1α positive tumors compared to 68.7% in HIF1α negative tumors (P=0.04) (22) (*Table 1*). Similar findings were reported by Kim *et al.* where HIF2α was significantly associated with a higher recurrence risk (HR =1.525; 95% CI, 1.144-2.033) (21) (*Table 1*).

Lgals1

While HIFs are well-characterized for their role in radioresistance, a relatively novel marker of hypoxia, Lgals1 (Galectin-1) is also implicated in cervical cancer radioresistance (57). Galectin-1 is a downstream transcriptional target of HIF1α, but also may participate dynamically in positive feedback upregulation of HIF1α via interactions with H-ras (57-59). In patients who received RT for cervical cancer, increased galectin-1 level was associated with a higher 10-year LR (HR =2.60; 95% CI, 1.50-4.52; P=0.0001) (*Table 1* and *Figure 2*). This high galectin-1 expression patterns was similarly observed in relatively radioresistant tumors such as prostate cancer, melanoma, and glioma cells (57). In contrast, galectin-1 levels in Hodgkins lymphoma, a radiosensitive tumor, are decreased, but even within Hodgkins lymphoma subtypes, galectin-1 is lowest in patients with best prognosis (nodular lymphocyte type), and higher in patients with poorer prognosis markers (Reed Sternberg cells and anaplastic large-cell lymphomas) (57). These observations suggest that

galectin-1 may drive radioresistance in resistant cancers, and that its synergistic relationship with HIF1α may perpetuate hypoxia-induced radioresistance.

Proliferation biomarkers

Uncontrolled proliferation is another hallmark of cancers of all types (31). Broadly, pro-proliferative cell surface receptors, cytosolic receptors, and intracellular signaling factors converge on or act directly as transcription factors to repress quiescence genes and/or stimulate proliferative genes. Indeed, many common oncogenes, such as the Ras superfamily, Myc, Abl, Cdks/cyclins, and numerous growth factor receptors such as the epidermal growth factor receptor (EGFR) and RET, are proliferative factors that allow cell division to proceed in an uncontrolled fashion. Accordingly, several of the biomarkers identified in this review are pro-proliferative factors up-regulated in radioresistant tumors. The strongest representatives of this class of biomarkers were members of the EGFR family and the Akt signaling pathway. Reinforcing the significance of these biomarkers being identified separately is that there is significant crosstalk between these pathways, affirming their role in radioresistance.

EGF and erbB receptors

EGFR and c-Erb-b2 are members of the large erbB family of receptor tyrosine kinases that drive proliferation through hormone/growth factor binding, activation, and a subsequent proliferative signal cascade. ErbB-family receptors exist as inactive monomers at the cell membrane; binding of their ligand, epidermal growth factor (EGF), initiates receptor dimerization, auto- and co-phosphorylation, and ultimately the activation of at least one downstream proliferative signaling pathway (60). Canonically, these pathways include the Ras-Raf-MAP kinase cascade and the PI3K/Akt pathways, both of which drive proliferation via upregulation of transcription and translation of pro-proliferative proteins (60). Furthermore, components of the ErbB family have been identified as oncogenes in numerous tumor types. HER2/neu (erb-B2/EGFR2) overexpression in breast cancer is perhaps the most well-known example, but EGF receptor dysregulation has also been identified in head and neck, ovarian, and colon carcinomas, among many others (61). The role of EGFR in cervical carcinoma is less well-defined. EGFR is expressed in the basal layer of normal cervical epithelium and in 50% to 70% of cervical squamous cell carcinomas, but studies of its association with progression, prognosis, and response to therapy are mixed, and its utility as

a biomarker remains undefined for clinical use (62). However, an investigation of the predictive value of EGFR specifically for radioresistance in cervical cancer has not been performed until this review. Several studies demonstrate convincing clinical evidence that ErbB family proteins are associated with radioresistance in cervical cancer. Gaffney *et al.* demonstrated EGFR as a marker of radioresistance in primary cervical tumors (26). In 55 patients who received definitive RT for stage IB-IVA cervical carcinoma, patients with high EGFR staining had significantly poorer OS compared to patients with low-EGFR tumors (P=0.037) (*Table 1*). Multivariate analysis demonstrated a hazard ratio of 2.50 (95% CI, 1.24-5.05; P=0.011) predicting poorer OS in high-EGFR patients (*Figure 2*). Nishioka *et al.* characterized another EGFR isoform, c-erbB-2, for its role in radioresistance in cervical cancer (28). In 107 cervical carcinoma patients treated with RT, patients with c-erbB-2-positive tumors had significantly poorer OS in comparison to patients with low c-erbB-2 tumors (P=0.019), as well as significantly poorer MFS (70% *vs.* 33%; P<0.01) (*Table 1*). Identification of EGFR family members in two separate studies strongly supports the role of EGFR overexpression in cervical cancer radioresistance.

PI3K/Akt signaling

The PI3K/Akt signaling pathway is a major pro-proliferative, pro-survival, anti-apoptotic signaling pathway with upstream regulation by numerous growth and proliferation receptors. The activation of numerous growth factor receptors (including EGFR), insulin receptors, cytokine receptors, synaptic receptors, and immune cell receptors induces signaling through the PI3K/Akt pathway (63). This signaling is usually via direct activation of phosphoinositide-3 kinase (PI3K), which catalyzes the conversion of membrane phospholipids to PIP_3, promoting phosphorylation and activation of Akt, also known as protein kinase B, through its kinase Pdk1. The kinase activity of Akt itself modulates the activity of the diverse targets mentioned above. Akt is a well-known oncogene; Akt itself is frequently mutated in ovarian (63,64), prostate, and breast cancers (65), and other components of the Akt pathway, including the tumor suppressor and opponent of PI3K, PTEN, are often mutated as well (66). The role of Akt signaling in cervical cancer has been largely unexplored. Several preliminary studies using small-molecule inhibitors of Akt have demonstrated that the inhibition of Akt signaling inhibits cervical cancer cell proliferation or promotes cancer cell death (67,68). Furthermore, Akt signaling is implicated in tumorigenesis in 3q-amplification,

the most common chromosomal aberration in cervical cancers; this region of the long arm of chromosome 3 bears the catalytic subunit of Akt activator PI3K, and treatment of cervical cancer cell lines bearing this amplification with PI3K inhibitors decreases cell growth and promotes apoptosis (69).

Kim *et al.* explored the role of Akt activity in cervical cancer radioresistance (27). A total of 27 cervical carcinoma samples from patients who received RT were examined for Akt phosphorylation status (a direct indicator of Akt activation status). pAkt levels were significantly higher (P=0.004), and 5-year progression-free survival was significantly shorter (P=0.008), in radioresistant tumors compared to radiosensitive tumors (*Table 1*). These observations suggest that increased Akt activity can enhance the radioresistance of cervical tumors.

Work in the nascent field of gene signature profiling has also identified signaling activity in the PI3K/Akt pathway as a marker of cervical cancer radioresistance. Schwarz *et al.* used microarray-based gene expression quantification and gene set enrichment analysis to investigate the status of the PI3K/Akt pathway in radiosensitive *vs.* radioresistant cervical cancers (30). Components of the pathway, including Akt, PI3K, and nine others, were significantly overexpressed in radioresistant tumors (P=0.006) (*Table 1*). These observations were confirmed by immunohistochemistry for Akt expression levels in the corresponding tumors, and this measure of Akt pathway activity at the protein level independently confirmed that Akt is a marker of poorer progression-free survival and increased rate of recurrence. These observations further confirm that the Akt pathway is involved in cervical cancer radioresistance, making this pathway a salient target for further characterization. Furthermore, the complex relationship between EGFR signaling and the Akt pathway (*Figure 1*) supports a role for these pathways in radioresistance.

Cell adhesion biomarkers

Another defining characteristic of malignant cells is the ability to migrate from their niche and become invasive and metastatic, and a critical step in this process is the loss of cell-cell adhesion (31). A prime example in human carcinomas is the loss of epithelial adherens junction transmembrane protein E-cadherin (70). In normal epithelia, E-cadherin forms adherens junctions between adjacent epithelial cells, maintaining the integrity of the mucosal lining, preventing cell migration, and forbidding

cells from exiting G0; predictably, the inhibition of E-cadherin promotes cellular migration, and E-cadherin loss promotes the epithelial-to-mesenchymal transition (EMT) (31). Accordingly, loss of E-cadherin has been demonstrated in carcinomas of the breast, gastrointestinal tract, pancreas, and numerous others (70), as well as in cervical squamous cell carcinoma (71-73). Another adherens junction protein, β-catenin, is an important component of cell adhesion. A unique membrane protein involved in both physical maintenance of cell adhesion as well as cytosolic signaling, β-catenin in its transmembrane form tethers E-cadherin's intracellular domain to the actin cytoskeleton, promoting adhesion and quiescence in similar fashion to E-cadherin (74). However, translocation of β-catenin to the nucleus, where it acts directly as a transcription factor, promotes proliferation, and therefore β-catenin is an oncogene. Research on the role of β-catenin in cervical cancer is scarce, although work in transgenic mice has demonstrated that activated, nuclear β-catenin accelerates carcinogenesis in premalignant HPV-positive cervical lesions (75). In other cancer types, the EMT, to which E-cadherin loss contributes, has been implicated in radioresistance; examples include non-small cell lung cancer (17), prostate cancer (76), and others.

Two studies in human cervical patients implicate components of cell adhesion in radioresistance. In a study of 111 patients treated with radiation, Huang *et al.* demonstrated that concurrent loss of E-cadherin and overexpression of the epithelial cell transformation marker osteopontin is associated with significantly poorer progression-free survival at 5-year follow-up (*Table 1* and *Figure 2*) (17). In another study, Zhang *et al.* identified an increase in nuclear β-catenin (i.e., upregulation of its oncogenic transcription factor function) as a biomarker of radioresistance (18). In 59 patients treated with RT, increased nuclear β-catenin was associated with poorer DFS (HR =1.3; 95% CI, 0.4-4.6), as well as poorer OS (HR =4.1; 95% CI, 1.3-12.6) (*Table 1* and *Figure 2*). These studies implicate a role for aberrant cell adhesion in cervical cancer radioresistance.

Studies of putative biomarkers in laboratory experimentation

Investigation of potential biomarkers using cancer cell lines or primary cancer cells is a useful method of identifying potential biomarkers for further characterization in a clinical setting. Immortalized cancer cell lines are particularly useful

because putative targets can be identified at less expense, and more rapidly. Subsequent clinical studies can then be targeted toward clinically relevant biomarkers. Laboratory studies are therefore essential for driving progress in the identification of clinically significant biomarkers. Clinicians interested in characterizing biomarkers of radioresistance in their cervical cancer patient populations should refer to the existing basic science literature to guide their selection of targets. To this end, we also present the biomarkers of radioresistance in cervical cancer identified in laboratory studies, highlighting those supported by the most salient and convincing data that clinicians may wish to characterize in a clinical study.

Screening for papers that presented high-quality laboratory evidence for a biomarker of radioresistance in cervical cancer not yet studied in patients yielded 16 results (*Table 2*). Five studies used post-radiation primary cervical cancer cells (from either post-radiation residual tumor, or from tumors otherwise demonstrated to be radioresistant). The remainder used either pre-treatment primary cervical cancer cells or one or more of the following immortalized: the cervical carcinoma lines C33A, CaSki, ME180, QG-U, SKG-I, SKG-II, or SiHa; and/or the cervical adenocarcinoma cell line HeLa. All studies that used pretreatment primary cervical cancer cells or immortalized cell lines used repeated irradiation to generate radioresistant subclones. These studies identified a total of 23 biomarkers of radioresistance in cervical cancer cells (*Table 2*). While, several of these studies identified radioresistance multigene signature, others demonstrated miRNA, epigenetic signature or protein product as key players in acquiring radioresistant features. Similarly to the clinical studies, these biomarkers can be categorized into eight groups by their general function: apoptosis, cell adhesion, cell cycle progression, cell motility, cytokine signaling, hypoxia, immunity, and proliferation or proliferation and inhibition of apoptosis.

Most studies used similar techniques, directly evaluating biomarker expression status qualitatively (IHC) or quantitatively (qPCR, microarray, flow cytometry) after repeated irradiation. Several studies further validated biomarker status by using various knockdown techniques (shRNA or siRNA) to verify reduction of radioresistant phenotype after biomarker knockdown. Several studies also performed additional assays of radioresistance, including apoptosis assays, clonogenic survival assays, and various other cell cycle assays. All 16 studies provide convincing evidence for their biomarker's role in radioresistance in cervical cancer, and we recommend that any of them be

Table 2 Putative biomarkers of cervical radioresistance identified in laboratory studies

Year	Author	Biomarker	Biological function	Methods	Source of cells
2015	Liu et al. (77)	miR-21(+)	Apoptosis	qPCR, WB	Primary cervical cancer cells from radioresistant tumors
2012	Zhao et al. (78)	MTDH(+)	Cell adhesion	shRNA, WB, IF, cell cycle/viability assays	SiHa
2009	Li et al. (79)	UHRF1(+)	Cell cycle progression	CSA, MTT assay, FC analysis of apoptosis and cell cycle distribution, WB, siRNA	HeLa
2013	Zhang et al. (80)	miR-630(+), miR-1246(+), miR-1290(+), miR-3138(+)	Cell motility (miR-630 and miR-1246), apoptosis (miR-1290), unknown (miR-3138)	qPCR	HeLa, SiHa
2015	Kim et al. (81)	SOCS(+)	Cytokine signaling	RT-qPCR, IHC, methylation-specific PCR, bisulfite sequencing	HeLa, CaSki, ME-180
2005	Tanaka et al. (82)	CD40(+)	Cytokine signaling	FC	ME180
2009	Beskow et al. (83)	DNA-PKcs(+), Ku70(+), Ku86(+)	DNA repair	IHC	Primary cervical cancer cells from post-radiation residual tumors
2014	Yuan et al. (84)	miR-218(−)	Hypoxia	CSA, apoptosis analysis, WB, IHC	CaSki, SiHa; primary cervical cancer cells repeatedly irradiated
2010	Liu et al. (85)	NDRG2(+)	Hypoxia	Hypoxia induction, irradiation, colony-forming assay, WB, qPCR, apoptosis FC	HeLa
2013	Ke et al. (86)	miR-181a(+)	Immunity	Microarray, miRNA expression in cell culture, apoptosis assay	Primary cervical cancer cells from radioresistant tumors
2005	Kubota et al. (87)	GRP94(+)	Immunity	WB, survival assay, siRNA	HeLa, CaSki, SiHa
2015	Muthusami et al. (88)	FTS(+)	Proliferation	WB, IF, IP	ME180
2011	Cinghu et al. (89)	FTS(+)	Proliferation	shRNA, IF, IHC, cell cycle analysis, WB, apoptosis FC, apoptosis assay	HeLa, CaSki, ME-180, SiHa; primary cervical cancer cells from patients with recurrence after radiation
2012	Su et al. (90)	K-Ras(+), c-Raf(+), p38(+)	Proliferation, inhibition of apoptosis	siRNA, WB, IHC	C33A, CaSki, HeLa
2001	Mukherjee et al. (91)	p53, p21, Bcl2(+), Bax(−), MCM2(+)	Proliferation, inhibition of apoptosis	IHC	Primary cervical cancer cells from post-radiation residual tumors
1998	Britten et al. (92)	Raf-1(+)	Proliferation, inhibition of apoptosis	CSA, kinase activity assay	Primary cervical cancer cells repeatedly irradiated

Biomarkers are indicated as overexpression (+) or under-expression (−) status of each marker. CSA, clonogenic survival assay; FC, flow cytometry; IHC, immunohistochemistry; IP, immunoprecipitation; MTT, dimethylthiazol (cell viability assay); WB, Western blot.

further investigated with prospective or retrospective clinical correlation. However, two studies of four biomarkers were exceptional in techniques used and the level of evidence presented for the biomarker's role in radioresistance.

miR-181a

Ke *et al*. used both cell culture and mouse experiments to define the microRNA miR-181a as a biomarker of radioresistance in cervical cancer and to elucidate the mechanism of this radioresistance (86). miRNAs are endogenous small RNAs that reduce translation and decrease mRNA stability of their target genes by binding the 3' untranslated region of their target mRNAs and inhibiting translation (93). Given that 30% of all genes may be regulated by miRNAs (94), they are likely relevant to many oncogenic and tumor suppressor processes. The miR-181 family of miRNAs includes miRNA-181b, miRNA-181c, and miRNA-181d in addition to miR-181a and it is involved in various endogenous functions, including T cell maturation (95) and vascular development (96). More importantly, miR-181 miRNAs are implicated in a number of cancers: miR-181a is a biomarker of multiple myeloma (97), and miR-181 family expression is associated with tamoxifen resistance in breast cancer (98). In this study, Ke *et al*. identified miR-181a as a potential radioresistance biomarker via microarray comparison of miRNA expression in primary cervical cancer samples obtained from radioresistant and radiosensitive tumors (86). The overexpression of miR-181a in SiHa and ME180 cell lines decreased their radiosensitivity, whereas treatment with miR-181a inhibitor increased cells radiosensitivity. To test the effect of miR-181a on actual tumor radioresistance *in vivo*, ME180 cell lines with the miR-181a overexpression vector were xenografted into nude mice, and mice were subjected to radiation treatment: mice with miR-181a tumors had significantly larger tumors after irradiation than mice with vector-control tumors. The group further defined the mechanism of this radioresistance, demonstrating that miR-181a inhibits apoptotic caspase activity in irradiated cells, promotes bypass of the G2/M block, and via genetic and biochemical approaches, showed that miR-181a inhibits apoptosis by silencing the protein kinase C isoform PRKCD; indeed, re-expression of PRKCD in the miR-181a overexpression background restored radiosensitivity to ME180 cells. The strong evidence for miR-181a as a biomarker of radioresistance in cervical cancer suggests that clinicians should consider pursuing investigations of its prognostic value and clinical implications of radioresistance.

EGFR, K-Ras, c-Raf, and p38

The Ras family of GTPases are the most commonly mutated oncogenes in human cancers (99); they drive proliferation via a mitogen-activated protein kinase (MAPK) cascade starting with their effectors, oncogenic Raf kinases (100). Another member of the MAPK family, p38, promotes cell migration (90). Upstream of the kinase cascade, EGFR activates Ras and itself is overexpressed in a plethora of human cancer types (101). Thus, EGFR signaling through the Ras/MAPK pathway is important in tumorigenesis, and correspondingly, these factors have been previously demonstrated as mediators of radioresistance in several mouse and rat cell lines (102,103), but their role specifically in cervical cancer radioresistance in human cases was not previously elucidated.

Su *et al*. investigated the role of the K-Ras pathway in radioresistance using experiments in cell culture and mice (90). Su *et al*. repeatedly irradiated CaSki, HeLa, and C33A cells to generate radioresistant subclones, and observed that migration was increased in the C33A and CaSki lines, measured by migration assays in culture as well as lung metastasis potential when injected into nude mice. This enhanced migration was dependent on K-ras, which was massively up-regulated in the radioresistant subclones and activated c-Raf in these clones. siRNA knockdown of either K-ras or c-raf separately obliterated the increased migration phenotype. siRNA or selective small-molecule inhibition of p38 similarly decreased radioresistant migration, suggesting that p38 is the downstream effector of Ras/Raf-mediated migration in radioresistant tumors. Evaluation of K-ras status in a small number of post-radiation distant metastases from cervical tumors paired with pre-radiation primary tumor showed that K-ras expression is much more strongly expressed in post-radiation distant metastases compared to primary tumor. These observations not only implicate Ras, Raf, and p38 in cervical cancer radioresistance, but provide a potential mechanism for the observation that many patients who fail radiation treatment exhibit distant metastases (7). Clinical validation of Ras, Raf, and p38 as biomarkers of cervical cancer radioresistance, to complement existing evidence for EGFR as a biomarker of radioresistance, and investigation of their association with treatment failure and poor prognosis would provide strong support for this theory, and should be pursued by clinicians.

Discussion

With the rapid expansion of the cervical cancer

molecular literature, we examined the available clinical and laboratory studies to provide an updated review of radioresistance biomarkers in cervical cancer. Here we present a comprehensive analysis of the available high-quality literature investigating clinically-validated and putative biomarkers of radioresistance in cervical cancer. We identified 19 clinical studies describing 23 biomarkers of radioresistance validated by patient outcome data. We additionally identified 16 high-quality laboratory studies that identified 23 potential biomarkers of radioresistance that should be investigated in patient populations for outcome data. Biomarkers of radioresistance validated in patient studies included gene products functioning in apoptosis, cell adhesion, DNA repair, hypoxia, metabolism, pluripotency, and proliferation.

From our review, the most promising targets from clinical studies performed to date indicate that apoptosis proteins iASPP, ΔNp73, and Bcl-2, and hypoxia protein Lgals1 and HIF1α, induce radioresistance and are associated with poorer prognosis and merit further study. Select candidate biomarkers identified in laboratory studies should be further characterized in patient studies, particularly the miRNA miR-181a and proliferation/migration genes K-ras, c-Raf, and p38.

Additionally, further work is needed in specific areas to further our mechanistic understanding and our resultant treatment approach of biomarker-associated radioprotection.

Does HPV infection directly lend radiosensitivity to tumors, or is radiosensitivity conferred indirectly via modulation of other markers?

One accepted marker of cervical cancer radiation response is HPV status: low-risk HPV subtype positivity is associated with better OS, DFS, and local control (104-107). Interestingly, a similar association between response to radiation and HPV status has also been demonstrated in head and neck cancers, which can also be caused by HPV infection (108). HPV-positive head and neck squamous cell carcinomas (HNSCCs) have a favorable prognosis compared to HPV-negative HNSCCs when treated with radiation; the increased radiosensitivity in HPV-positive tumors is likely due to defective DNA DSB repair in HPV-positive tumors (109). However, the precise nature of a putative HPV-radiosensitivity relationship is not yet understood, whether in cervical malignancies or otherwise.

Several studies have suggested a relationship between

HPV status and expression levels of potential biomarkers. Moreno-Acosta *et al*. identified insulin-like growth factor 1 receptor (IGF1R) as a biomarker of radioresistance in patients with HPV-16-positive cervical cancer (29). Interestingly, IGF1R has previously been identified as a marker of radioresistance in lung cancer (110), breast cancer (111), and osteosarcoma (112), as well as in cervical carcinoma of unknown HPV status (113). Moreno-Acosta's observations suggest that interplay between HPV-16 and IGF1R may alter the radioresistance status of cervical tumors. Adding further complexity to the IGF1R narrative, Luo *et al*. showed that IGF1R levels are higher in cervical cancer tissues compared to paired normal tissues, and that increasing IGF1R expression in HPV-positive cervical cancer cell lines is inversely correlated with the expression of microRNA miR-497 (114). The loss of miR-497 has been implicated previously in the progression of HPV-positive HNSCC (115), so taken together; these observations suggest that HPV may drive radioresistance in cervical cancers by promoting IGF1R signaling, perhaps via suppression of miR-497 expression. Although these observations may seem to contradict the established relationship of HPV with favorable prognosis, the relationship is likely multifaceted; for instance, different isoforms of insulin-like growth factor, the ligand of IGF1R, have different downstream effects in different cancer types (116). Therefore, IGF1R is likely a significant biomarker of radiation response in HPV-positive cervical cancers, and its role in radioresistance or radiosensitivity should be further examined in future studies.

Seiki *et al*. showed that in HPV-16-positive cervical tumors, the protein phosphatase PP1γ is strongly mobilized from the nucleus, where it resides in HPV-negative tumors, to the cytosol, suggesting it is functionally inactive, and *in vitro* experiments in HPV-16-positive CaSki and SiHa cells demonstrated that HPV-16 (specifically, viral oncoproteins E6 and E7) is responsible for this relocalization and that E6/E7 mediate down-regulation of PP1γ expression (117). PP1 is of interest in that it is a major mediator of the DNA damage response and of appropriate mitotic exit, both pathways that are aberrant in cancers and particularly in radioresistant cancers (118). Furthermore, PP1 has been demonstrated to mediate radioresistance in non-small-cell lung cancer (119), making salient the model that HPV may modulate radioresistance through PP1γ. It is likely that a complex interplay between the HPV genome and endogenous factors conferring radiosensitivity or radioresistance exists; further exploration of the mechanism

of HPV-induced radiosensitivity may reveal native radioresistance factors that are altered by HPV infection.

Conclusions

Advances in molecular profiling techniques have allowed for the identification and validation of biomarkers of numerous biological characteristics in tumor cells. Biomarkers of resistance or sensitivity to chemotherapeutic agents and RT are of interest for their potential role in designing customized therapeutic strategies targeting the vulnerabilities of individual tumors. We have discussed biomarkers of radioresistance in cervical carcinoma that have been validated in patient studies; further studies should better characterize the prognostic and predictive value of these markers to determine regimens for predicting response to RT. Potential biomarkers identified in laboratory studies should be further evaluated by clinicians in patient samples to identify additional markers of radioresistance *in vivo*.

Given that radiation is an integral component of the definitive treatment plan for cervical cancer (120), and that radiation treatment failure is common (121-123) and ascribed primarily to radioresistance (5), prediction of tumor response to radiation before the initiation of treatment would be invaluable for treatment planning. Traditional plans could be modified according to radioresistance status: patients with radioresistant tumors could be treated with protocols emphasizing surgery and chemotherapy, and radiosensitization strategies could be preferentially employed in radioresistant patients. To improve patient outcomes, clinicians should consider pretreatment evaluation of radioresistance biomarker status, and may also consider developing further studies investigation of the well-validated candidate biomarkers presented here.

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Footnote

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