IGF1R Gene Expression as a Predictive Marker of Response to Ionizing Radiation for Patients with Locally Advanced HPV16-positive Cervical Cancer

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Abstract. *Aim: The aim of this study was to evaluate the predictive utility of Insulin-like growth factor-1 receptor (IGF1R), IGF1, IGF2, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and of hemoglobin levels for tumor response to exclusive radiotherapy, in patients with locally advanced Human papillomavirus (HPV) 16-positive cervical cancer. Patients and Methods: From 102 patients treated at our institutes, 38 patients with histologically-proven HPV16-positive cervical cancer were included in this prospective case-controlled study. All patients underwent exclusive radiotherapy-only. Complete response was defined as an absence of residual disease at clinical examination and radiological imaging, three months after the completion of treatment. Gene expression levels, assessed before radiotherapy, were compared between responders and non-responders. Controls consisted of normal cervical tissue samples from 30 patients with non-oncological indications. Results: Twenty patients (52.6%) showed a complete response. Gene expressions of IGF1R (34%), IGF2 (24%), and GAPDH (median=3.26 versus 2.12) were increased in cancer patients, in comparison with the control group. Higher levels of expression of GAPDH were observed in patients co-expressing IGF2 and IGF1R, who had a hemoglobin level ≤11g/dl (p=0.05). Clinical characteristics in the responder and in the non-responder groups were similar. In bi-variate and multi-variate analyses, IGF1R expression was the only factor predictive of response to radiotherapy (p=0.018). Accordingly, patients with IGF1R expression had a 28.6-fold greater risk of treatment failure. Conclusion: In our study, IGF1R was a strong predictive marker of lack of response to radiotherapy. Larger prospective trials are needed to validate IGF1R as a biomarker of radiation response for patients with HPV16-positive cervical cancer.*

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Key Words: Cervical cancer, IGF1R, IGF1, IGF2, GAPDH, predictive biomarkers, ionizing radiation, therapy response.

Cervical cancer of the uterine cervix is the second most common cancer in women worldwide and the fifth leading cause of cancer-related deaths (1). Focusing on Colombia, cervical carcinoma is the first representative cancer and the leading cause of cancer-related death in women of reproductive age (2, 3). Moreover, most cases are diagnosed at advanced stages and infection with high risk human papillomavirus (HPV), mainly HPV16 (57% of cases) and

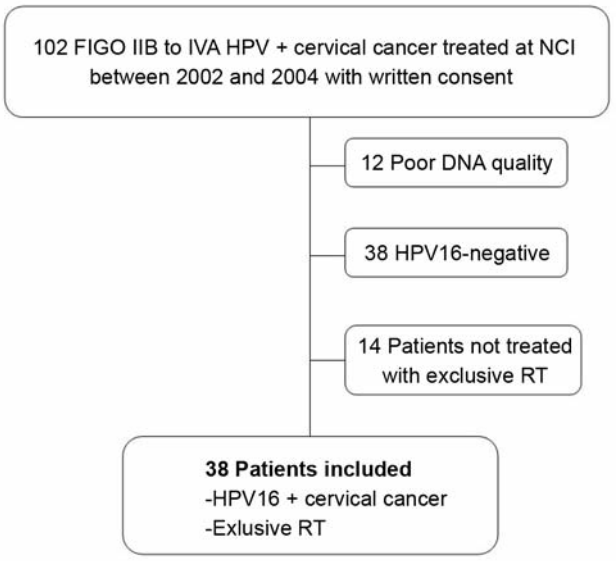

Figure 1. *Flow-chart of study. HPV: Human papilloma virus; FIGO: Federation of Gynecology and Obstetrics; NCI, National Cancer Institute; RT, radiotherapy.*

Table I. *Patients' characteristics.*

Characteristic	Median (range)	n	%
Age (years)	46 (33-70)		
FIGO stage			
IIB		7	18.4
IIIB		29	76.3
IVA		2	5.3
Histological type			
SCC		38	100
Histological diferentiation			
Well		7	18.5
Moderate		21	55.3
Poor		4	10.5
Not specified		6	15.7
Tumor size			
<4 cm		1	2.7
>4 cm		37	97.3
Hemoglobin (g/dl)			
Before RT	11.4 (6.9-15.8)		
≤11 g/d		17	44.74
≥11 g/d		21	55.26
HPV status			
HPV16 (+)		38	100

FIGO: International Federation of Gynecology and Obstetrics; HPV: human papilloma virus; SSC: squamous cell carcinoma; RT: radiotherapy.

HPV18, has been associated with the development of high-grade squamous intraepithelial lesions and cervical cancer (4). Conventional treatment includes external beam radiotherapy (EBRT) plus brachytherapy with or without concomitant chemotherapy. However, 30 to 40% of patients with similar prognosis factors do not respond similarly to a comparable standard treatment. This variation may be attributed to resistance to radiotherapy response, which depends on different molecular factors, including tissue oxygenation, oncogene activation, loss of tumor-suppressor genes and activated aberrant molecular signaling (5-8).

The insulin-like growth factor I receptor (IGF1R) is a ubiquitous growth receptor that may convey signals associated with radiation resistance. Through autocrine or paracrine stimulation with its ligands IGF1, IGF2 and insulin, IGF1R induces autophosphorylation and activation of specific tyrosine kinase residues, initiating signaling cascades such as Ras/Raf/mitogen-activated protein kinases (MAPK) and Phosphoinositide 3-kinase (PI3K), which are downstream oncoproteins involved both in cell survival and resistance (9). Moreover, previous studies have suggested that increased expression of IGF1R in mouse fibroblasts, in primary breast tumors and in cell lines of prostate and cervical cancer may confer relative resistance to ionizing radiation. The mechanism underlying this radioresistance may implicate DNA repair and anti-apoptotic pathways (10-17). Finally, an increase in the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in hypoxic tissues, as the tissues of cervical cancer, has also been reported to increase resistance to ionizing radiation (18). In fact, high GAPDH levels may enhance glycolysis and meet the metabolic requirements of the tumor cells (19, 20).

The aim of the present study was to evaluate whether the expression of IGF1R, IGF1, IGF2, GAPDH, and hemoglobin levels may predict radiotherapy response in patients with HPV16-positive locally advanced cervical cancer.

Patients and Methods

Study design, selection and patients' characteristics. This was a prospective case control study. Patient selection and characteristics are shown Figure 1 and Table I. The study included a group of 38 patients out of 102 patients with invasive locally advanced [International Federation of Gynecology and Obstetrics (FIGO) IIB to IVA] squamous cell carcinoma of the uterus treated at the National Cancer Institute (NCI) in Bogotá, Colombia. The protocols followed in this study were consistent with medical standards of practice and administrative techniques for health research from the Ministry of Health of Colombia. Each patient was first informed of the objectives of the study and voluntarily agreed to take part by signing the informed consent, previously approved by the Ethics Committee of INC.

Treatment. All 38 patients included in the study underwent radiotherapy-only. Treatment consisted of pelvic EBRT using 6 to 18 MV photons with a standard four-field technique delivering a total dose of 45 Gray (Gy) in 25 fractions (1.8 Gy per fraction, five

Table II. *Primer sequences*.

Gene symbol		Position – nucleotide 5'>3'	Primer sequence 5'>3'	Product
IGF1	S	339 (Exon 2)	GGG AAT GGA GTG CTG TAT G	120 bp
	A	442 (Exon 3)	CCA CCT CCC ACT CAT CA	
IGF2	S	489 (Exon 1)	CTG TTC GGT TTG CGA CA	97 bp
	A	568 (Exon 2)	AGC ACC AGC ATC GAC TTC	
IGF1R	S	2948 (Exon 15, subunit β)	GGG AAT GGA GTG CTG TAT G	82 bp
	A	3013 (Exon 16, subunit β)	CCA CCT CCC ACT CAT CA	
GAPDH	S	556	TGC ACC ACC AAC TGC TTA GC	86 bp
	A	622	GGC ATG GAC TGT GGT CAT GAG	

IGF1: Insulin-like Growth Factor; *IGF1R*: insulin-like growth factor receptor; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; S: sense. A: antisense; Product: size of PCR product; bp: base pair.

consecutive days per week, overall EBRT treatment time of five weeks). After initial EBRT, low-dose rate or high-dose rate intracavitary brachytherapy-boost was delivered using cesium or iridium sources, respectively, for an equivalent low-dose rate dose of 35 Gy at point A, according to the International Commission on Radiation Units and Measurements (ICRU). The total doses of radiation ranged from 80 Gy and 60 to 66 Gy to the ICRU points A and B, respectively (21).

Assessment of response. Follow-up was scheduled six weeks after completion of EBRT, then every three months during the subsequent five years. Complete response was defined as an absence of residual disease at clinical examination and radiological imaging three months after the completion of treatment. The responder group was defined as the group of patients that presented complete response, and the non-responder group, as the patients that presented partial response, stable disease or tumor progression.

Molecular techniques. Tumor and blood samples. For each patient, biopsy-proven carcinoma of the uterine cervical and hemoglobin levels (g/dl) were available before the start of treatment. In addition, 30 samples of normal cervical tissues from patients with non-oncological indications that had had a hysterectomy were used as a control group. Blood samplings were performed to analyze hemoglobin levels in cancer patients.

HPV detection: The detection of HPV DNA and the typing of HPV16 from uterine biopsies of the uterus was performed according to the method of Moreno-Acosta *et al*. (4).

Gene expression: To determine the gene expressions of *IGF1R, IGF1, IGF2* and *GAPDH*, absolute quantification of mRNA transcription by real-time PCR was used. Total RNA was isolated from tumor samples by Trizol LS reagent (Gibco Life Technologies, Burlington, Ontario, Canada), which is based on the method of Chomczynski and Saadi (22). The RNA concentration was established by reading the absorbance (Abs) at 260 nm and the purity by the ratio of 260/280 Abs. The integrity of RNA was determined by agarose electrophoresis. Total-RNA was treated with RNAse-free DNAse to avoid amplification of genomic DNA contamination.

To obtain complementary DNA (cDNA) from total RNA, the reagent kit from Promega (Madison, WI, USA), as recommended by the manufacturer was used. Briefly, 1 μg of total RNA was digested with DNase in a total volume of 11 μl. Then, 2 μl of reaction buffer, 2 μl of the mixture of NTPs, 0.5 μl of RNasin, 0.625 μl of the enzyme AMV, 1 μl of oligodT, 4 μl of MgCl-2 and 0.9 μl of water-(DEPC) were added to total volume of 22 μl. Reactions without the enzyme AMV were included as negative controls. The reverse-transcription (RT) was carried out in triplicate. Taking into account that *GAPDH* RNA is present in most tissues, we amplified a fragment of this sequence as an internal control of RT to indirectly confirm the integrity of RNA.

The respective primers (Invitrogen, NY, USA) were designed following the recommendations given by Bustin *et al*. and the design of the respective PCR was performed according to the information obtained through the program OLIGO 5 software (Molecular Biology Insights, Cascade, CO, USA) (23-24). Table II shows the nucleotide location on the mRNA and the primer sequence designed for this study.

The real-time PCR amplification was done with an MJ Research-thermocycler. Briefly, the reaction was carried out in 20 μl of PCR solution containing 10x SYBR Green Buffer, 25 mM MgCl-2, dNTPs (2.5 mM dATP, dCTP, dGTP and 5.0 mM dUTP), AmpliTaq Gold (5 U/μl), AmpErasa UNG (1 U/μl), 0.2 μM of each primer, and 5.0 μl of cDNA. To determine the expression of *IGF1R, IGF1, IGF2* and *GAPDH* genes, we also constructed a standard curve formed by nine serial dilutions of each DNA gene. These dilutions were made from 1 μl of plasmid DNA (DNA standard) from positive control cell lines AGS (human gastric cancer) and SiHa (human cervical cancer). The dilutions for the construction of standard curves and samples were analyzed in triplicate in each real-time PCR. The quantification of transcript mRNA is expressed by the average of triplicates. For each studied gene, values that were included in the 90% confidence interval on a logarithm distribution were considered positive.

Statistical analysis. For the statistical analysis variables of interest were age, hemoglobin level, clinical stage, incomplete radiotherapy, tumor size, degree of tumor differentiation, expression of GAPDH, IGF1R, IGF1 and IGF2. *t*-Test for independent samples was used to compare mean variables with normal distribution. The Mann-Whitney test was used to compare mean variables with non-normal distribution. The chi-square test was used for association between categorical variables or the Fisher's test, when assumptions were not met for the chi-square test. Gene expression levels were compared between complete responders *versus* non-responders using the chi-square test. For univariate and multivariate analyses, a logistic regression was performed. A two-sided *p*-value of <0.05 was considered significant. Statistical analyses were carried out using the Statistical Package for the Social Sciences (version18; SPSS Inc., Chicago, IL, USA).

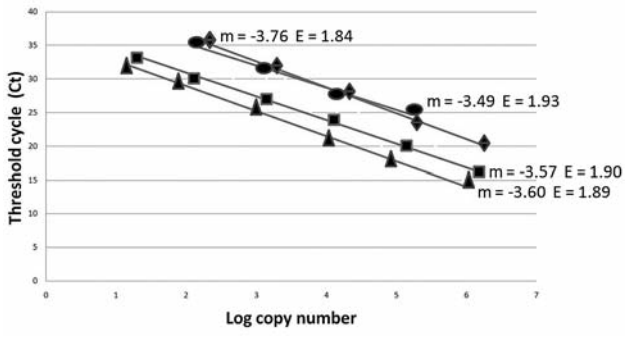

Figure 2. *Standard curves for Insulin-like growth factor-1 (IGF1) (♦), IGF2 (●), IGF1R (■) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (▲). The plot of the log of initial target copy number versus Ct was a straight line. E, efficiency; m, slope.*

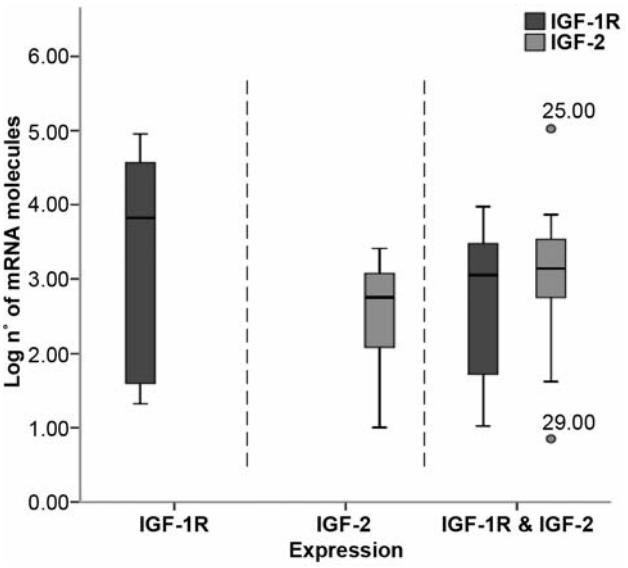

Figure 3. *Expression profiles of Insulin-like growth factor-1 receptor (IGFIR), IGF2 and co-expression of IGF1R and IGF2 in cancer biopsies. Values in the control group are not depicted because the mRNA level was below of the detection limit. IGF1R: Insulin-like growth factor receptor.*

Results

Response to radiotherapy. Twenty patients (52.6%) presented a complete response at the time of assessment. Thirty-four out of 38 patients (89.4%) underwent the entire planned treatment; out of the four patients who did not, one had a complete response.

Quantification of gene expression of IGF1R, IGF1, IGF2 and GAPDH mRNA. The standard curves made from linear DNA standards showed good linearity, as well an adequate amplification efficiency for *IGF1, IGF2, IGF1R* and *GAPDH*, that ranged from 10 to 10^9 molecules (Figure 2). Through the dissociation curves generated by real-time PCR and from transcripts obtained from biopsies isolated from the group of cancer and normal cervical samples, a single-peak was observed demonstrating that the SYBR GreenI detected only one specific product, each for *IGF1, IGF2, IGF1R* and *GAPDH* with the correct corresponding temperature. There were no primer or dimer artefacts, or signs of contamination.

The expression of *IGF1* was detected in AGS cells (used as positive controls) with a limit of sensitivity of 21 mRNA molecules, but it was not detected in the cancer group, nor in the samples from non-oncological patients. *IGF2* and *IGF1R* gene expression was detected in SiHa cells, with a limit of sensitivity of 16 and 23 mRNA molecules, respectively. An increase in the expression of *IGF1R* (34%) and *IGF2* (24%) was observed in patients samples in comparison with the normal cervical samples (Figure 3).

GAPDH expression was also detected in AGS and SiHa cells, with a limit of sensitivity of 14 mRNA molecules. The samples from cervical cancer had higher levels of *GAPDH* expression compared with control group samples (median=3.26 *versus* 2.12; p=0.003; Figure 4a).

Correlations between gene expression profiles, hemoglobin level, and age. Higher levels of expression of GAPDH were observed in patients co-expressing IGF2 and IGF1R and who had hemoglobin levels ≤11g/dl (Figure 4b), with a significant correlation (Fisher's test, p=0.05). IGF2 and IGF1R expression had a non-significant trend to increase with the age of the patients (Fischer's test, p=0.255 for IGF2, p=0.555 for IGF1R).

Analysis of gene expression profiles and radiotherapy outcome. In bi-variate analysis, IGF1R gene expression was significantly higher in the non-responders *versus* the responders (55.6% *versus* 25%, respectively; Fisher's test, p=0.05). We did not identify any other marker predictive of radiotherapy response within this analysis (Table III).

In multivatiate analysis IGF1R expression statistically inversely correlated to radiotherapy response (p=0.018, Pearson's chi-square test). Accordingly, patients with expression of IGF1R had a 28.6-fold greater risk of treatment failure. No association was observed between clinical characteristics or hemoglobin levels and response to treatment (Table IV).

Discussion

In this study, we found an increased risk of radiotherapy failure in patients expressing IGF1R in tumor samples obtained from HPV-positive invasive squamous cell

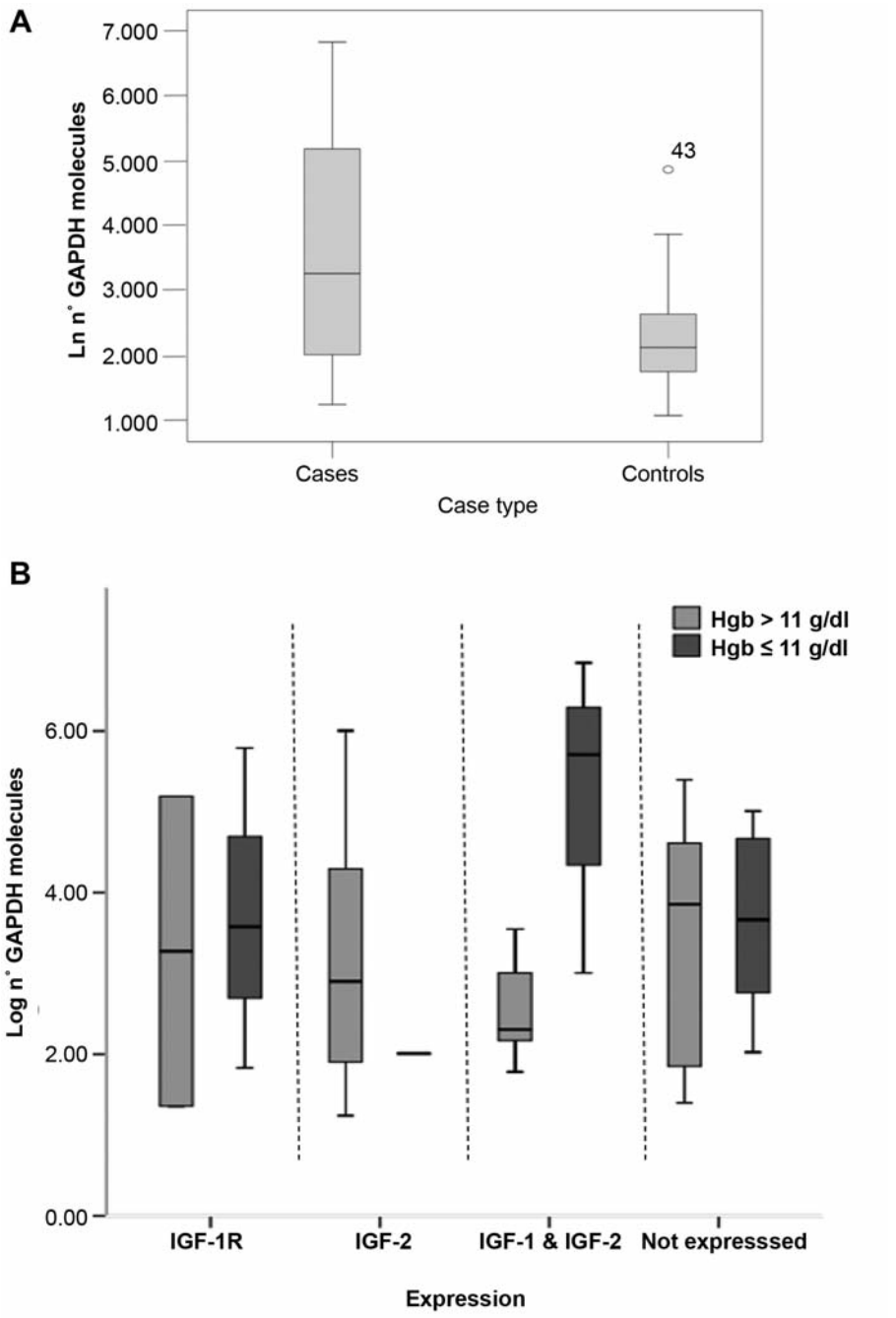

Figure 4. *Expression profiles of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A: Cancer biopsies cases and Control (normal cervical tissue from non-oncological patients undergoing hysterectomy). B: Relationship between the expression levels of GAPDH and hemoglobin (Hgb) levels in cases that expressed and did not express Insulin-like growth factor-1 receptor (IGF1R) and IGF2 and that co-express IGF1R and IGF2.*

carcinomas of the uterine cervix. To our knowledge, this is the first report to support a possible clinical use of *IGF1R* transcription levels as a predictive biomarker for response to radiation therapy.

In fact, IGF1R has already been described as a prognostic factor for survival. Huang *et al*. showed that recurrence-free and overall survival rates were significantly lower among patients with high-grade expression of tissue IGF1R, in comparison with those with low-grade expression. On the other hand, the IGF1 serum antigen failed to predict cancer death and recurrence (25). Similarly, other authors described that the association of IGF1R with two other oncoproteins

Table III. *Bi-variate analysis of response to radiotherapy according to clinicopathological characteristics.*

	Complete response (n=20)	No response (n=18)	*p*-Value
Median tumor size (cm) (SD)	6.7 (1.7)	7 (1.9)	0.5+
Median hemoglobin (g/dl) (SD)	12 (2.3)	10.9 (2.3)	0.3+
FIGO stage (%)			
IIB	4 (20)	3 (16.6)	0.6*
IIIB-IVA	16 (80)	15 (83.4)	
Histological differentiation (%)			
Well	2 (7.5)	5 (7.7)	0.3#
Moderate	11 (81.1)	10 (76.9)	
Poor	3 (11.3)	1 (15.4)	
Treatment RT (%)			
Complete	19 (75)	15 (83.3)	0.3*
Incomplete	1 (5)	3 (16.4)	
Expression			
IGF1R			
Negative	15 (75)	8 (44.4)	0.05*
Positive	5 (25)	10 (55.6)	
IGF2R			
Negative	11 (55)	9 (50)	0.5*
Positive	9 (45)	9 (50)	
GADPH	20 (22.6)	18 (38.46)	0.5#

+Mann-Whitney test; * Fisher's exact test; #Pearson's chi-square test. FIGO: International Federation of Gynecology and Obstetrics; RT: radiotherapy; IGF1R: insulin-like growth factor receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Table IV. *Multivariate analysis of response to radiotherapy according to clinicopathological characteristics.*

Variable	RR	*p*-value*	95% CI
Tumor size	1.33	0.23	0.82-2.13
FIGO stage			
IIB	1.0		
IIIB-IVA	0.72	0.76	0.08-6.07
Treatment			
Complete	1.0		
Incomplete	9.2	0.17	0.63-13.31
Expression			
IGF1R	26.8	0.018	1.74-41.15
IGF2	3.62	0.21	0.46-28.04
IGF2 & IGF1R	3.68	0.19	0.51-26.10

* Pearson's chi-square test. RR: Relative risk; CI: confidence interval; IGF1R: insulin-like growth factor receptor.

[B-cell lymphoma 2 (BCL-2) and major vault protein] were prognostic factors for poor locoregional disease-free survival, distant disease-free survival, disease-free survival, and cause-specific survival, in 50 patients treated for cervical cancer (26). Generally, while a prognostic biomarker provides information about the patients' overall cancer outcome, regardless of therapy, a predictive biomarker gives information about the effect of a therapeutic intervention. However, if our results were validated, it is likely that IGF1R could be considered as both a prognostic and a predictive biomarker.

It has been reported that IGF1R and IGF2 are ubiquitously expressed in all human tissues (27, 28). Nonetheless, in cervical cancer, the production of IGF2 and IGF1R may be increased to convey signals that up-regulate gene transcription, in order for cells to thrive and increase resistant tumoral phenotypes. Tumor microenvironment, growth factors, cytokines and steroid hormones may also induce IGF1R overexpression (20, 28-30). Signaling by means of the IGF1R pathway up-regulate downstream oncoproteins involved in cell survival, anti-apoptotic pathways and DNA repair mechanisms. Among them, PI3K, MAPK and 14-3-3 protein may increase radiation resistance (28, 29). In addition, our results are consistent with previous reports regarding the influence of IGF1R on radioresistance of fibroblasts, prostate, and breast cancer cell lines (10, 11, 29). Yu *et al*., reported that the contribution of IGF1R expression to radiotherapy resistance may be modest (10). Conversely, even low radioresistance, as described in mutation of *RAS* or in high expression of epidermal growth factor receptor (*EGFR*), may be crucial for clinical outcome if it is propagated or amplified in each fraction of radiotherapy (5, 10).

Herein, GAPDH expression levels correlated with the expression of IGF1R and the co-expression of IGF1R and IGF2. GAPDH and IGF1R pathways may be both implicated in the tumoral cell glycolytic metabolism (8, 18). Interestingly, higher levels of expression of GAPDH were observed in patients co-expressing IGF2 and IGF1R and who had hemoglobin levels ≤11 g/dl. This could indicate that IGF1R pathway activation may inhibit the transport of glucose across the plasma membranes through the down-regulating effects of PI3K on Glucose transporter 1 (GLUT1), -3, and -4 systems. Consequently, this could lead to an activation of the cellular glycolytic GADPH pathway (31-33).

Conclusion

Our findings suggest an association between IGF1R expression and response to radiotherapy. In our opinion, this study contributes to highlight the remarkable role of IGF1R in cervical cancer, which has already been described as a significant prognostic factor in cervical cancer. Future larger studies are warranted to validate IGF1R as a predictive biomarker and evaluate its usefulness as a potential target for therapy.

Conflicts of Interest

All Authors declare no conflicts of interest.

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