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RESEARCH ARTICLE

Molecular and Circulatory Expression of Insulin Growth Factors in Indian Females with Advanced Cervical Cancer

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Abstract

Background: Recent studies have demonstrated an association between insulin growth factor (IGF) and insulin growth factor binding protein-3 (IGFBP-III) serum levels and increased risk for various cancers. However, little information is available on clinical implications of the IGF system in Indian patients with cervical cancer. This study explored associations by analyzing their expression profiles in cervical cancer cases. Materials and Methods: Totals of 50 patients with advanced cervical cancer and 40 healthy controls were enrolled. Human papillomavirus (HPV) and cervical biopsy sample were obtained from all participating women. Circulatory levels were estimated by ELISA and the tissue expression was assessed using RT-PCR and Western blotting. Results: Levels of IGF-I and II showed significant increase whereas IGFBP-III showed significant decline in all patients as compared to controls. Spearman correlation analysis between IGFs and HPV status showed significant correlations. Conclusions: We demonstrated elevated circulating levels and tissue expression of IGF-I and IGF-II in advancer cancer cervix patients, as compared with controls, with a converse trend being apparent for IGFBP-III. In future, associations of the IGF system and clinical outcome of cervical cancer patients in post treatment samples might point to significance in disease mapping as a prognostic marker after validation with a larger patient series.

Keywords: Insulin growth factors - insulin growth factor binding protein - cervical cancer - human papilloma virus

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Introduction

Cervical cancer is the second most common cancer among women worldwide. Virtually 100% of cervical cancers contain the HPV DNA sequences from a high-risk oncogenic genital HPV (Zur et al., 2002). The most important players are HPV-16, found in 50-70% of cases, and HPV-18, found in 7-20% of cases (Munoz et al., 2006; Koutsky et al., 2009). However, only a small fraction of those infected by HPV develop cancer, indicating that other factors contribute to the progression to cervical cancer. Despite intensive investigation, the tumor biology of this disease is still largely unknown (Huang et al., 2008). Recent studies by our group have investigated the role of mediators of inflammation like cytokines and oxidative stress and therapeutic response in patients with advanced cervical cancer (Sharma et al., 2007; 2009; 2010; Goswami et al., 2008).

The insulin-like growth factors (IGFs) are mitogens that play a key role in regulating cell proliferation, differentiation, apoptosis, and transformation. The insulin-like growth factor 1 (IGF-I) system is comprised of ligand (IGF-I), receptor (IGF-IR) and a family of binding proteins (IGF-BPs). Insulin-like growth factor binding protein 3 (IGFBP-III) is the major serum carrier of IGF to IGF-IR. Most actions of IGF-I are mediated through IGF-IR signalling, resulting in mitogenic and metabolic functions in vivo as well as in vitro (Yu et al., 2000). IGFBPs play a role in controlling the interaction between IGFs and IGF-IR by binding to IGFs and thereby blocking their binding to IGF-IR (Yu et al., 2000). In some situations, the binding of IGFBPs to IGFs can protect the IGFs from degradation, thereby increasing IGF activity (Chen et al., 1994; De Mellow et al., 1998). IGF-I, IGF-II and IGFBPs have also been investigated as possible predictors of cancer risk in several cancers (Hankinson et al., 1998; Wolk et al., 1998; Kaaks et al., 2000; Wu et al., 2000; Renehan et al., 2004). Of the IGFBPs, IGFBP-III has been the most intensively studied because it is the main IGFBP found in plasma.

Prospective and retrospective studies have demonstrated an association between IGF-I, IGF-II and IGFBP-III serum levels and increased risk for various cancers (Hankinson et al., 1998; Wolk et al., 1998; Kaaks et al., 2000; Wu et al., 2000; Renehan et al., 2004). It has been shown in animal models that circulating IGF-I levels may play a significant role in carcinogenesis (Shen et al., 2006). Some

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studies have suggested that serum levels of IGF-I, IGF-II or IGFBP-III may be useful biomarkers for assessing risk of SIL or cervical cancer development (Mathur et al., 2003; Wu et al., 2003; Schaffer et al., 2007; Harris et al., 2008; Lee et al., 2010). In this study, we have explored the expression of the IGF system in cervical cancer patients and evaluated its potential association with HPV status by analysing its circulatory and cellular expression.

Materials and Methods

Fifty patients with advanced cervical cancer (International Federation of Gynecology and Obstetrics [FIGO] stage IIIa-IVa), 40 healthy controls were enrolled in this study. These patients had attended the Gynaecology Cancer Clinic, Lok Nayak Hospital, New Delhi, India, as a result of either their own gynecological conditions or through the mass cancer campaign conducted by one of the researchers under the auspices of the Directorate of Health, Government of Delhi. Human papillomavirus (HPV) samples and a cervical biopsy sample were obtained from all participating women.

A total of 40 normal cervical-tissue samples were obtained from surgeries performed for reasons other than a diagnosis of cervical cancer (such as total hysterectomies for benign indication such as dysfunctional uterine bleeding, fibroid and prolapse) and showed normal cytology and no dysplasia. These cases were treated as normal controls. The exclusion criteria were pregnancy at the time of sampling, chronic diseases such as diabetes mellitus or autoimmune diseases, acute infections at the time of sampling, and an immune-compromised state. The study protocol was approved by the hospital ethics committee and all patients voluntarily gave informed consent.

Tissue samples were collected by routine punch biopsy from the uterine cervix of patients of having advanced cervical cancer. Furthermore, clinical staging was done according to the FIGO method during visual inspection and assessment. The same tissue samples were used for histopathologic studies. An experienced pathologist reviewed all histologic samples. On histopathologic examination in cancer cases, the tissue samples were divided on the basis of cell differentiation status from well to poorly differentiated SCCs and adenosquamous carcinoma. The biopsy samples were collected in ice-cold phosphate buffered saline (pH 7.4) and immediately transported to the laboratory on ice for further processing.

Human papillomavirus (HPV) sampling and determination

DNA was prepared from the pellets obtained after extraction of cervical scrapings or section in TRAP lysis buffer using the guanidium isothiocyanate (GTC)-diatom-procedure (Boom et al., 1990). DNA was dissolved in 100 mL of 100 mmol/L Tris HCl, 1 mmol/L EDTA (pH 7.0), and stored at -20 degree Celsius until use. PCR and HPV typing were performed as described earlier (Sharma et al., 2007).

Blood collection

A total of 4 mL of venous blood was taken from study subjects into tubes free of endotoxins. Serum was separated from the blood by centrifuging it for 10 min at 3,000 rpm and stored at -80^{0}C for further use.

ELISA for IGF-I, IGF-II, and IGFBP-III

Commercially available ELISA kits were used to determine the serum levels of IGF-I, IGF-II, and IGFBP-III. The mean intra- and inter-assay variation coefficients for the controls were, respectively, 4.1% and 8.6% for IGF-I, 9.8% and 13.8% for IGF-II and 4.2% and 11.9% for IGFBP-III. ELISA was performed according to the manufacturer's instructions. Standards, controls, and samples were tested in duplicate. The IGF-I, IGF-II, and IGFBP-III serum concentrations were determined from the standard curve by matching the absorbance readings with the corresponding IGF-I, IGF-II, and IGFBP-III concentrations.

Tissue processing

The cervical tissues were collected and washed in ice-cold wash buffer containing 10 mmol/L HEPES-KOH (pH 7.5), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, and 1 mmol/L dithiothreitol. After washing, the pellet was homogenized in cold lysis buffer containing 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L $MgCl_2$, 1 mmol/L ethylene glycol tetra-acetic acid (EGTA), 0.1 mmol/L phenyl methyl sulfonyl fluoride (PMSF), 5 mmol/L ß-mercaptoethanol, 0.5% 3-cholamidopropyl-dimethyl-ammonio-1-propane sulphonate (CHAPS), and 10% glycerol. The suspension was incubated on ice for 30 minutes with intermittent shaking. Following centrifugation at 3,000 rpm for 10 minutes, the clear supernatant was separated carefully and quickly stored at -80°C. The protein concentrations of the cell lysates were determined by spectrophotometry performed according to the Bradford method.

Western blot analysis of IGF-I, IGF-II, and IGFBP-III

Thirty micrograms of whole-cell protein lysate {in 1× Tris-glycine-sodium dodecyl sulfate (SDS) sample buffer with 100 mM dithiothreitol (DTT)} from each patient and control was separated by SDS-PAGE in 15% (for IGF-I and IGF-II) and 10% (for IGFBP-III) separation gels. The gels were transferred to nitrocellulose membranes for Western blot analysis. The membranes were blocked in 5% BSA for 2 hours at 4°C and then, were incubated with primary antibodies (Rabbit anti-IGF-I (Abcam, UK) at a dilution of 1:6000, Rabbit Anti-IGF-II (Abcam, UK) at a dilution of 1:10000 and Rabbit Anti-IGFBP-III (Abcam, UK) at a dilution of 1:1000) in TBS-T at 4°C overnight. Anti-beta Actin antibody (Abcam, UK) was used as a loading control. The membranes were re-blocked for 1 hour at room temperature (RT) before incubation with secondary antibodies for 1 hour at room temperature. After they were washed with TBS-T, the membranes were incubated with peroxidase-conjugated streptavidin at a dilution of 1:40,000 in TBS-T for 1 hour at RT. The membranes were incubated with a chemiluminescent Western blotting detection system.

RT-PCR

The total RNA was extracted, reverse transcribed, and

subjected to real time RT-PCR using primers for IGF-I (forward,5'-TTTCAACAAGCCCACAGGGT-3';reverse, 3'-GGAGTCTGTCCGTAGCACC-5'),IGF-II (Forward 5'-CTGGAGACGTACTGTGCTACCCCC-3' and reverse 5'-GTGTCATATTGGA AGAACTTGCCC-3'), IGFBP-III (forward 5'-CTCTGCGTCAACGCTAGTGC-3'; reverse 5'-CGGTCTTCCTCCGACTCACT-3') and for beta-actin as a housekeeping gene control (forward 5'-CTGGGACGACATGGAGAAAA-3'; reverse 5'-AAGGAAGGCTGGAAGAGTGC-3'). The RT-PCR amplification products were gel electrophoresed, stained with ethidium bromide, and imaged with a gel documentation system.

Results

Patients mean age was 50.6 (SD±8.04) while the controls mean age was 49.2 (SD±9.6). Out of 50 patients, 23 (46%) were of educational background of 10+2 or lower whereas in controls 17 (34%) were of educational background of 10+2 or lower. 24 patients (48%) were earner in the range of 3000-8000 whereas 16 (40%) controls earned 8000 and above. 16% of patients were smoker as compared to controls (15%). 2% of patients were drinker as compared to 2.5% (controls). Table 1 demonstrates the HPV status in all the subjects. 27 patients are positive for HPV-16 and 29 patients are positive for HPV-18. 6 patients show positivity for both HPV-16 and HPV-18.

Table 2 illustrates that the levels of Insulin Growth Factors (IGF-I & II) showed significant increase (p<0.01 and 0.005) whereas Insulin Growth Factors binding protein-3 (IGFBP-III) showed significant decline (p<0.002) in all cancer patients, in comparison with controls. The mRNA expression and protein expression of IGF-I & 2 was invariably higher in the patients, whereas it was found low for IGFBP-III in all cases of cancer cervix on RT-PCR and Western blot analysis (Figures 1 and 2). This expression was relatively higher in the samples which were positive for HPV 16 and 18 both as compared to HPV 16 or HPV 18 positive subjects only. On Spearman correlation analysis between growth factors and HPV status, we have found a statistical significant correlation (p<0.05) in advanced cancer cervix patients.

Table 1. HPV Status

Group		HPV Status	
		HPV-16	HPV-18
Healthy controls (N=40)		-	-
Patients Groups (N=50):	Group I (n=21±6)	+	-
	Group II (n=23±6)	-	+
	Group III (n=6)	+	+

Table 2. ELISA Values of Insulin Growth Factors and Their Binding Protein

	Patients	Controls	p
IGF-I (ng/ml)	151.2±18.4	102.1±12.4	0.01
Mean±S.D. (Range)	(70.6-182.3)	(34.5-127.6)	
IGF-II (ng/ml)	903.3±238.9	482.2±156.8	0.005
Mean±S.D. (Range)	(404.2-1328.7)	(98.2-604.3)	
IGFBP-III (ng/ml)	1902.4±580.4	3382.7±620.6	0.002
Mean±S.D. (Range)	(452.3-2201.8)	(2053.9-5289.2)	

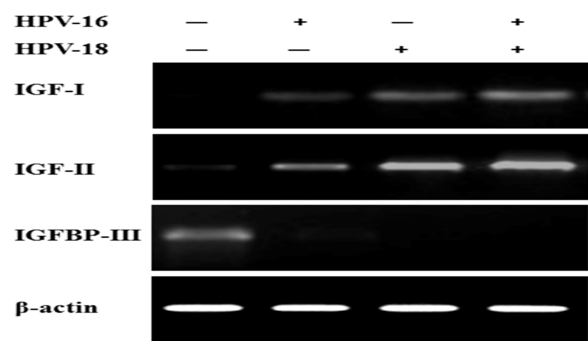

Figure 1. RT-PCR Results of IGF-I, IGF-II, IGFBP-III and β-actin for Healthy Control {HPV-16 (-ve) and 18 (-ve)} and Three Different Group of Patients {HPV-16 (+ve) and 18 (-ve), HPV-16 (-ve) and 18 (+ve) and HPV-16 (+ve) and 18 (+ve)}

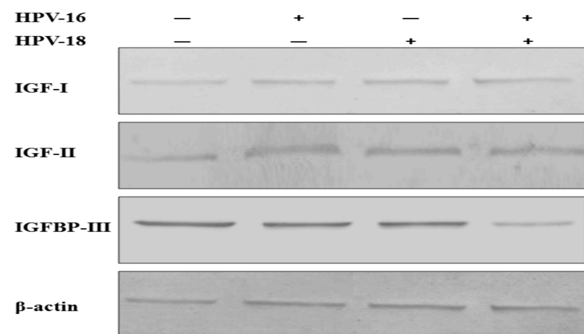

Figure 2. Western Blot Analysis of IGF-I, IGF-II, IGFBP-III and β-actin for Healthy Control {HPV-16 (-ve) and HPV-18 (-ve)} and Three Different Groups of Patients {HPV-16 (+ve) and 18 (-ve), HPV-16 (-ve) and 18 (+ve) and HPV-16 (+ve) and 18 (+ve)}

Discussion

Recent advancement in cancer research endow with the incidental evidence that the insulin growth factor (IGF) influences malignancy (Zumkeller et al., 2001). IGFs are peptide hormones that play a vital role in the control of cell proliferation, differentiation, and apoptosis in many cell types (Grimberg et al., 2000; 2003; LeRoith et al., 2003). IGF-I, by binding to its receptor, influences cell transit from the G1 to S phase by stimulating the mitogen-activated protein (MAP) kinase signal transduction pathway, which increases the production of cyclin D1. IGFs also contribute to suppression of apoptosis and increased cell growth by enhancing the production of Bcl proteins and inhibiting the production of Bax proteins (Collett-Solberg et al., 2000; Ali et al., 2003; Baserga et al., 2003; Dupont et al., 2003; Lelbach et al., 2005).

High levels of IGF-I in circulation are reported to be positively associated with risk of several common tumors (Hankinson et al., 1998; Wolk et al., 1998; Kaaks et al., 2000; Wu et al., 2000; Renehan et al., 2004) and recent data suggest a possible additional role of IGF-I in cervical tumorigenesis (Grimberg et al., 2000). Another study (Wu et al., 2000) showed that the mean plasma levels of IGF-I and the molar ratio of IGF-I/IGFBP-III were

higher in patients with advanced or poorly differentiated disease than in patients with early or well-differentiated lung cancer. There is a report (Wolk et al., 1998) which concludes that elevated serum IGF-I levels may be an important predictor of risk for prostate cancer. Another report (Kaaks et al., 2000) also associated the elevated levels of IGF-I with increased colorectal cancer risk. Another study (Lee et al., 2010) reported lower levels of IFG-I in cervical cancer patients as compared to controls as well as CIN groups. Another group (Jozefiak et al., 2008) also demonstrated lower levels of IGF-I in squamous cell carcinoma patients (HPV+) as compared to controls (HPV+ or HPV-). Lower levels of IFG-I and IGF-I: IGFBP-III molar ratio in cervical cancer patients were reported in another study (Serrano et al., 2006). In another study they (Serrano et al., 2007) have observed very low IGF-I mRNA levels and also found no significant difference between control and other groups (LSIL and HSIL). In this study we have observed elevated levels of IGF-I at mRNA as well as protein level in advanced cervical cancer patients which are in concordance with the results of studies in other cancers but are in contradiction with the results of the studies on cervical cancer and CIN.

IGF-II mRNA levels (Serrano et al., 2007) were significantly lower in LSIL than in control group but its expression in HSIL and cervical cancer was similar to the one observed in controls. Another study (Mathur et al., 2000; 2003) reported significantly increased serum IGF-II levels in pretherapy cervical cancer patients and posttherapy persistent/recurrent cervical cancer as compared to controls. After therapy, the IGF-II levels returned to normal in both CIN and cervical cancer patients under remission. There is contradiction among studies reported so far. Some reports showed elevated levels of serum IGF-II while others found expression as similar to controls. Contradiction in results of several studies on IGF-II incited us to analyze and validate its expression by using RT-PCR, ELISA and Western Blotting. In this study we have observed higher levels of IGF-II at mRNA as well as at protein level.

IGFBPs play a role in controlling the interaction between IGFs and IGF-IR by binding to IGFs and thereby blocking their binding to IGF-IR. In some situations, the binding of IGFBPs to IGFs can protect the IGFs from degradation, thereby increasing IGF activity (Collett-Solberg et al., 2000; Grimberg et al., 2000; Lelbach et al., 2005). Because of their role in the regulation of IGFs, IGFBPs have also been investigated as possible predictors of cancer risk in several cancers (Chen et al., 1994; Hankinson et al., 1998; Wolk et al., 1998; Kaaks et al., 2000; Wu et al., 2000). In cancer patients, the IGFBP-III levels may be controlled by the increased levels of IGFBP-III proteases, leading to an unopposed increase in IGF-I and -II levels. Besides binding to IGFs, IGF-BP3 is known to have proapoptotic properties both dependent on and independent of p53 and IGFs. IGF-BP3 can stimulate apoptosis and inhibit cell proliferation directly and indirectly. Hence, IGF-BP3 may have an IGF-independent anti-proliferative and anti-tumorigenic action on cancer cells (Collett-Solberg et al., 2000; Grimberg et al., 2000; Ali et al., 2003; Lelbach et al., 2005). IGFBP-III mRNA levels were significantly lower in cervical cancer than in controls (Serrano et al., 2007). In a study (Lee et al., 2010) lower levels of IFGBP-3 were reported as compared to controls as well as CIN groups. Another study (Jozefiak et al., 2008) also demonstrated lower levels of IGFBP-III in squamous cell carcinoma patients (HPV+) as compared to controls (HPV+ or HPV-). In this study we have also found lower levels of IGFBP-III at mRNA as well as protein level which is in concordance with the previous findings.

Recent studies reported additional evidence of an association between the IGF axis and cervical neoplasia (Mathur et al., 2003; Wu et al., 2003; Schaffer et al., 2007; Harris et al., 2008; Lee et al., 2010). A study investigated the association of IGF-I system and clinical outcome of cervical cancer, by analysing the serum levels of IGF-I, 2 and IGFBP-III, and tissue expression in the patient with different HPV status (Wu et al., 2003). The results suggest the important function of IGF-I system which correlated with HPV status in predicting the clinical outcome of advanced cervical cancer patients. Similar positive associations were reported (Mathur et al., 2003) with high levels of IGF-II (a related growth factor) and low levels of IGFBP-III. Another study demonstrated that the plasma levels of IGF-I and the IGF-I/IGFBP-III molar ratio might be useful for the early detection of cervical lesions (Lee et al., 2010). Another group prospectively assessed the influence of circulating IGF-I levels on the natural history of oncogenic HPV (Harris et al., 2008). Levels of IGF-I in young women may be inversely associated with high-grade cervical intraepithelial neoplasia, a precursor to cervical cancer (Schaffer et al., 2007). Our results showed strong association of IGFs with the HPV status in Indian patients with advanced cancer cervix and are in concordance with the findings of the previous studies (Mathur et al., 2003; Wu et al., 2003; Schaffer et al., 2007; Harris et al., 2008; Lee et al., 2010).

The strength of the present work lies in the fact that the circulating levels and tissue expression (as studied by mRNA expression by RT-PCR and protein expression by Western blot analysis) of insulin growth factors and IGFBP-III in Indian females with cancer cervix and their correlation with HPV status were analyzed in a single study. Such a comprehensive analysis of IGF/IGFBP-III axis in Indian females with cancer cervix, as detailed above has not been attempted before, to the best of our knowledge.

In recent years, evidence has been mounting that the IGF axis may be involved in human cancer progression (Baserga et al., 2003; Dupont et al., 2003; Renehan et al., 2004; Pollak et al., 2008) and can be targeted for therapeutic intervention. Our findings in patients with advanced cervical cancer add to the evidence in this context. In future, the association of IGF-I, IGF-II system and clinical outcome of cervical cancer patients in post treatment samples might add its significance in disease mapping as prognostic marker. It also indicates their possible usefulness towards developing newer therapeutic drugs, their targets and assessment of the clinical outcomes of disease in future.

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