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ORIGINAL ARTICLE

IGF2 is Deregulated During the Development of Uterine Cervical Carcinoma in Indian Patients

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Abstract

Uterine cervical carcinoma (CACX) is one of the leading causes of deaths in Indian women. Chromosomal alterations including 11p15.5 locus were reported in CACX. Consequently, we strived for the first time to understand the molecular status of the candidate gene Insulin-like growth factor 2, *IGF2* (11p15.5) in Indian CACX patients ($n = 128$). DNA copy number (CN) analysis using CGH-SNP analysis showed no genetic alteration and it was further validated by comparison with publicly available CN datasets. But promoter hypo-methylation during the progression of CACX was observed and also found to be concordant with publicly available DNA methylation datasets. Interestingly, we found diverse expression of *IGF2* transcript in both normal cervical epithelium (NCE) and CACX tumors. Similar heterogeneous expression pattern was seen in publicly available expression datasets as well. Finally, protein expression analysis in NCE showed concordance with transcript expression but tumors showed frequent low expression. Log-rank test showed a difference (p-value $= 0.057$) in overall survival between cases with and without alteration for *IGF2* in Indian CACX patients. Collectively, our study proposes that regulation of *IGF2* expression in NCE appeared to be multifaceted and deregulation during the development of CACX resulted in the differential expression.

Keywords Insulin-like growth factor 2 (IGF2) · Cervical cancer (CACX) · Normal cervical epithelium (NCE) · Gene regulation · Methylation-specific PCR (MSP)

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Introduction

Cervical cancer (CACX) ranks as the fourth most frequent carcinoma among females worldwide (Jemal et al. 2011). In developing nations like India, CACX is the second most frequent cancer among women with high rates of incidence (122,844) and mortality (67,477) (Kaarthigeyan 2012). It was identified that untreated persistent infection of high-risk human papillomavirus (hrHPV) is the primary cause of CACX although, several other etiological factors including use of oral contraceptives, precocious marriage, multiparity, and smoking were seen to influence the risk of CACX (Walboomers et al. 1999). Long latency period for the development of CACX in only a subset of hrHPV-infected women indicates involvement of additional genetic and epigenetic alterations (Lazo 1999). One such frequently altered chromosomal 11p15.5 locus bears the Insulin-like growth factor 2 (*IGF2*) gene (Narayan and Murty 2010).

IGF2 encodes a mitogenic peptide which controls cell survival, proliferation, and metabolism by binding with the Receptor Tyrosine Kinases (RTKs) like Insulin receptor isoform A (IR-A) and Insulin-like growth factor 1 receptor (IGF1R) with varying affinities. It is also reported that bioavailability of IGF2 is regulated through its binding with the Insulin-like growth factor 2 receptor (IGF2R) missing the tyrosine kinase domain (Brouwer-Visser and Huang 2015).

IGF2 consists of nine exons of which only exons 7, 8, and a part of exon 9 encode for the pre-proprotein (180 residues). During post-translational modifications, a signal peptide (24 residues) is cleaved off, finally leading to 156 residues of IGF2 proprotein. In an adult human, *IGF2* transcripts were shown to be regulated by four different promoters (P1–P4). Moreover, the expression of *IGF2* gene is known to be controlled through genomic imprinting as in most normal human tissues it is expressed only from the paternal allele while the maternal allele remained silent (Brouwer-Visser and Huang 2015). Moreover, it is well known that DNA methylation influences expression of genes and its anomaly leads to deregulation of genes in diseases including cancer (Baxter et al. 2014; Schlosberg et al. 2017). In several human tumors like ovarian cancer, breast cancer and colorectal cancer, expression of *IGF2* have been reported to be deregulated primarily due to aberrant DNA methylation (Brouwer-Visser and Huang 2015; Hidaka et al. 2018). In CACX, till now, studies investigated the molecular alterations of *IGF2* in comparatively smaller number of French and South Korean patients only. It was found that loss of imprinting (LOI) and differential promoter usage may contribute for the deregulation of expression of *IGF2* gene in CACX (Douc-Rasy et al. 1996; Kim et al. 2002; Vidal et al. 2014). However, no study involving large CACX patient population was performed so far, especially in Indian population. Moreover, correlation between IGF2 transcript and protein expression in both normal cervix and primary cervical tumors were not performed to understand the functional role of IGF2 protein in the development of the disease.

Here, for the first time in Indian population, we carried out a comprehensive analysis to explore the promoter methylation and its impact on the expression of

IGF2 transcript and protein expression both in normal and tumor tissues of the uterine cervix.

Materials and Methods

Patients, Tumor Tissues, and Cell Lines

A total of 128 CACX cancer patients were selected for the present study after power (85%) calculations. Primary cervical lesions and normal cervical epithelial (NCE) tissues were collected from the hospital section of Chittaranjan National Cancer Institute, Kolkata after appropriate approval of the Institutional Ethical Committee and informed written consent from the patients. Normal cervical epithelial (NCE) tissue was obtained from patients who underwent hysterectomy for benign reasons. Adenocarcinoma and previously treated CACX patients were excluded only. Cervical squamous cell carcinoma samples (CSCC) were all included. Chosen samples were then graded and staged according to FIGO classification (Fig. 1). Samples were frozen immediately after collection at -80ºC for genomic DNA isolation. For RNA isolation, part of the tissue was directly collected in TRIzol reagent (Invitrogen, San Diego, CA) and for immunohistochemical analyses, a portion of tissue was paraffin-embedded after formalin-fixation. The 128 primary lesions include 22 pre-malignant cervical intraepithelial neoplasia (CIN) lesions (9 low-grade CIN I and rest high-grade CIN II/III), 51 stage I/II CSCC tumors and 55 stage III/IV CSCC tumors (Table 1). The CACX cell lines (HeLa and SiHa) were procured from National Centre for Cell Sciences, Pune, India and were grown according to the supplier's recommendations.

Microdissection and DNA Extraction

Cryosections (5 µm) of the samples were microdissected under a dissecting microscope (Leica MZ 16, Germany) using surgical knives to remove contaminant cells. Microdissected samples which were then enriched with > 70% dysplastic epithelium/tumor cells were taken for DNA isolation by standard phenol/chloroform method (Mazumder Indra et al. 2011; Roychowdhury et al. 2017).

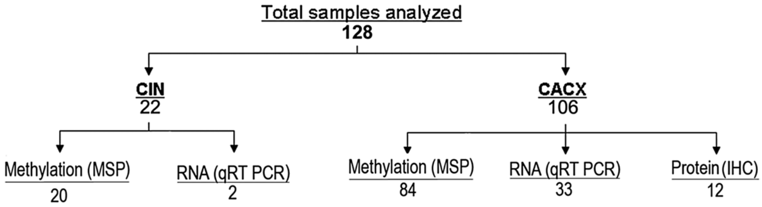


Fig. 1 Brief workflow and utilization of samples. MSP: Methylation-specific PCR; IHC: Immunohistochemistry

Table 1 Demography of CACX patients participated in the study

Clinical features	No. of patients	Mean age (years)
Tumor stage		
CIN	22	41
Stage I	31	47
Stage II	20	51
Stage III	48	51
Stage IV	7	47
Tumor differentiation		
Dysplasia	22	41
Well	13	51
Moderate	78	50
Poor	15	48
Lymph node		
Node +	37	53
Node−	91	50
Parity (live births only)		
Low (0–4)	77	45
High (≥ 5)	51	53
Age at sexual debut		
Early (12–19)	74	48
Late (> 19)	54	48
HPV positivity		
HPV +	118	49
HPV−	10	43

Promoter Methylation Analysis by Methylation-Specific PCR (MSP)

The methylation status of *IGF2* promoter (P3) was done by methylation-specific PCR (MSP) after bisulfite modification of the DNA (Herman et al. 1996). Briefly, appropriate amount (5µg) of genomic DNA was denatured with 0.2M NaOH for 15 min at 37 °C. Cytosines were sulfonated in the presence of 3 M sodium-bisulfite (Sigma Chemical Co., USA) and 10 mM hydroquinone (Sigma Chemical Co., USA) for 16 h at 55 °C. Subsequently, the DNA samples were desalted with Wizard DNA Clean-Up System (Promega, Madison, WI, USA) and desulfonated in 0.3M NaOH at 37 °C for 15 min. Finally, the treated DNA samples were ethanol precipitated and resuspended in Tris-EDTA buffer (Mazumder Indra et al. 2011). The PCR of the modified DNA was performed with methylated specific (M) primers MF: TTTAAGTTTGGTTTAGATTCGGTTC and MR: CGA AAAATCCTTACTAATTATTACGAC set (amplicon size: 221bp) and unmethylated specific (U) primer UF: TTAAGTTTGGTTTAGATTTGGTTTG and UR: CAAAAAATCCTTACTAATTATTACAAC set (amplicon size: 220bp). PCR products were then run on 2% agarose gels, visualized under UV illumination and photographed.

Total RNA Isolation and IGF2 mRNA Expression Analysis

Expression of the *IGF2* transcripts was analyzed in 17 NCE tissues, 35 cervical lesions, and 2 CACX cell lines (Roychowdhury et al. 2017). Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen, USA). Power SYBR-green assay (Applied Biosystems, USA) was performed for real-time quantification with primers FP: CCTTGGACTTTGAGTCAAATT and RP: GGTCGTGCCAATTACATTTCA (amplicon size: 293bp). β2-microglobulin (B2M) gene was used as endogenous control and FP: GTGCTCGCGCTACTC TCTCT and RP: TCAATGTCGGATGGATGAAA primers (amplicon size: 153bp) were used. The comparative threshold cycle (ddCt) method was employed to determine the relative level of gene expression (Livak and Schmittgen 2001). A log2^ (−ddCt) value of ± 0.1769 (Fold change $\geq \pm 1.5$) was considered as threshold for over/under-expression.

Protein Expression Analysis of IGF2 by Immunohistochemistry

Immunohistochemical (IHC) analysis of IGF2 protein was done in 7 NCE and 12 cervical lesions (Mazumder Indra et al. 2011). Briefly, formalin-fixed, paraffin-embedded (FFPE) tissue sections of 3–5 µm thickness were deparaffinized and then a 45-min antigen retrieval step in boiling 10mM sodium citrate buffer (pH 6.0) followed by blocking with Bovine serum albumin (BSA). It was then reacted overnight with primary antibody for IGF2 (ab9574; Abcam Inc., Cambridge, MA) at a dilution of 1:50 at 4 °C. Next day, after washing with 1X Phosphate buffered saline (PBS), goat anti-rabbit IgG horseradish peroxidase conjugate (G21234, Thermo Fisher Scientific) was added at 1:500 dilutions. The slides were developed using 3-3′ diaminobenzidine (DAB 0430, Amresco Inc.) as the chromogen and 0.5% H_2O_2 (1072090250, Merck) as substrate, followed by counter-staining with hematoxylin (517-28-2, Merck). The staining intensity (1 = weak, 2 = moderate and 3 = strong) and the percentage of positive cells ($<1=0$, 1–20 = 1, 20–50 = 2, 50–80 = 3 and $>80=4$) were detected and the final evaluation of expression was done (0–2 = low, 3–5 = intermediate and 6–7 = high) (Perrone et al. 2006).

Analysis of Publicly Available Datasets

NCBI GEO GSE76911 is a catalog of genomic alterations of Indian CSCC patients ($n=11$) using high-resolution CGH-SNP microarray done previously (Roychowdhury et al. 2017). Normalized log ratio greater than 0.0 (positive values) indicates amplification while deletion is depicted with negative values (<0.0). Further, copy number of *IGF2* gene was compared with two CNV datasets, "TCGA cervix" consists of 95 peripheral blood lymphocytes (PBL), 7 normal cervical epithelium (NCE), and 105 CSCC samples and "Scotto cervix" which consists of 7 NCE and 79

CSCC samples. In addition, two expression datasets were used namely, "Biewenga cervix" (5 NCE and 40 CSCC samples) and "Zhai cervix" (10 NCE and 21 CSCC samples). Status of *IGF2* was obtained from these CNV and expression datasets using Oncomine® Research Edition (https://www.oncomine.org), which is an online application that integrates and unifies high-throughput cancer profiling data across a large number of cancer types (Rhodes et al. 2004). To validate the promoter (P3) methylation status of *IGF2* in larger population, we have extracted the beta-values of the corresponding Illumina Human Methylation (HM) 450K array probes ($n=27$) located within 1kb upstream (+strand) of *IGF2* (NM_00612) transcription start site (TSS) from TCGA (3 NCE and 252 CSCC samples) and NCBI GEO GSE46306 (20 NCE and 6 CSCC samples) datasets (Cancer Genome Atlas Research et al. 2017; Farkas et al. 2013). The mean of the beta-values of both the normal and tumor samples for each 27 probes were plotted for comparison. Beta-value of 0.8–1.0 means methylation and beta-value of 0.0–0.2 depicts no methylation while beta-values in between (0.2 and 0.8) represent partial methylation of CpGs (Farkas et al. 2013). For comparison of protein expression of IGF2, we looked into The Human Protein Atlas (https://www.proteinatlas.org) which aims to map all the human proteins in cells, tissues, and organs (Ponten et al. 2008).

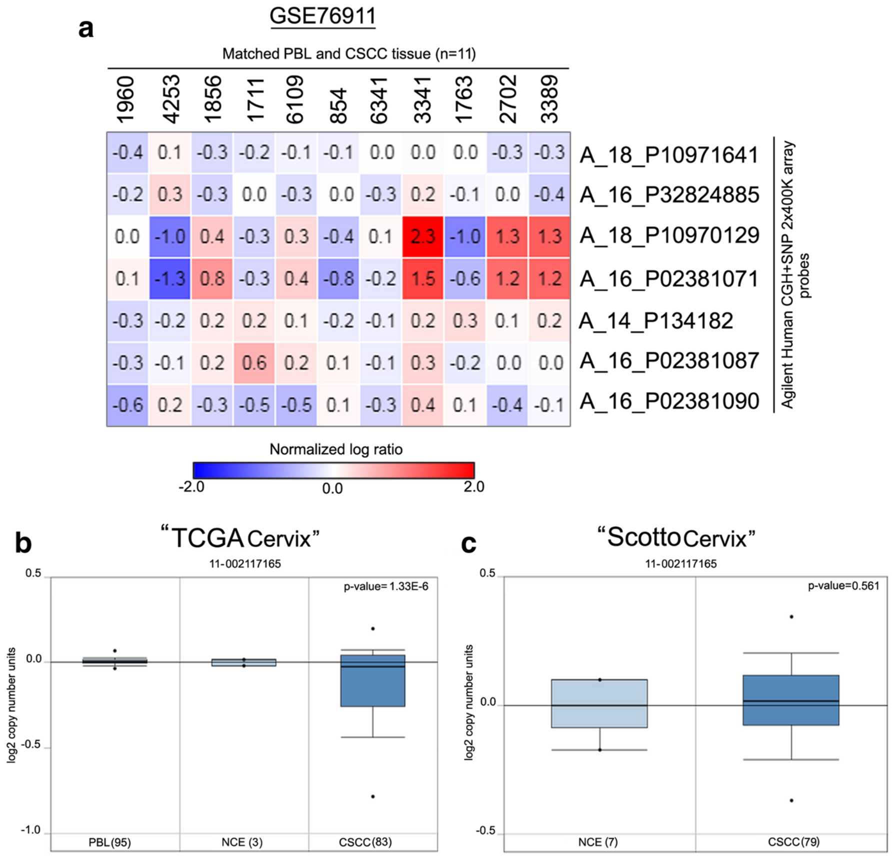
Statistical Analysis

Chi-square analysis was used to determine the association between tumors genetic profile and different clinicopathological features. Survival analysis was performed by Kaplan–Meier method in 82 CACX samples. Cox-proportional hazards regression model predicted patient's survival in relation to several prognostic factors like clinical stage, tumor grade, HPV infection, lymph node involvement, parity, and alterations of the candidate gene. Probability value (p-value) ≤ 0.05 was considered statistically significant. All the statistical analyses were performed using statistical programs Epi Info 6.04, SPSS 10.0 (SPSS, Chicago, IL). Histograms and Box plots were done using Microsoft Excel 2007 and GraphPad Prism 7.01 softwares (Mazumder Indra et al. 2011).

Results

Copy Number Variation (CNV) Analysis of IGF2 Locus

Analysis of copy number using high-resolution CGH-SNP dataset (GSE76911) of Indian patients revealed that there is no significant change (mean normalized log ratio of − 0.036) in copy number of *IGF2* locus (Fig. 2a). Moreover, the data were found concordant with two publicly available CNV datasets, "TCGA cervix" and "Scotto cervix" (Fig. 2b). So, we looked into the methylation status of *IGF2* promoter.



	1960	4253	1856	1711	6109	854	6341	3341	1763	2702	3389
A_18_P10971641	-0.4	0.1	-0.3	-0.2	-0.1	-0.1	0.0	0.0	0.0	-0.3	-0.3
A_16_P32824885	-0.2	0.3	-0.3	0.0	-0.3	0.0	-0.3	0.2	-0.1	0.0	-0.4
A_18_P10970129	0.0	-1.0	0.4	-0.3	0.3	-0.4	0.1	2.3	-1.0	1.3	1.3
A_16_P02381071	0.1	-1.3	0.8	-0.3	0.4	-0.8	-0.2	1.5	-0.6	1.2	1.2
A_14_P134182	-0.3	-0.2	0.2	0.2	0.1	-0.2	-0.1	0.2	0.3	0.1	0.2
A_16_P02381087	-0.3	-0.1	0.2	0.6	0.2	0.1	-0.1	0.3	-0.2	0.0	0.0
A_16_P02381090	-0.6	0.2	-0.3	-0.5	-0.5	0.1	-0.3	0.4	0.1	-0.4	-0.1

Fig. 2 DNA copy number analysis of *IGF2* locus. **a** High-resolution CGH-SNP analysis in Indian patients (GSE76911) showed no differential change in copy number which is evident from the heatmap of the normalized log ratio of the seven probes spanning *IGF2*. The copy number of IGF2 was further validated in two publicly available datasets **b** "TCGA cervix" and **c** "Scotto cervix"

Methylation Profile of IGF2 Promoter in Cervical Tissues

Promoter P3 is an important promoter and was commonly reported to be deregulated in several diseases including cancer. Promoter methylation status was analyzed in normal tissues, primary CACX samples, and cell lines. All normal tissues were found to be unmethylated. The methylation frequency of P3 promoter decreased from 40% (8/20) in CIN to 30.8% (12/39) in stage I/II to 22.2% (10/45) in stage III/IV tumors (Fig. 3). So, overall it was observed that there was frequent hypo-methylation across both normal and tumor uterine cervical tissues. It was further confirmed by the analysis of the 27 Illumina HM450K probes of the two datasets. The two datasets showed similar pattern and mean beta-values of both normal and tumor tissues were below 1 (hypo-methylation). On the other hand,

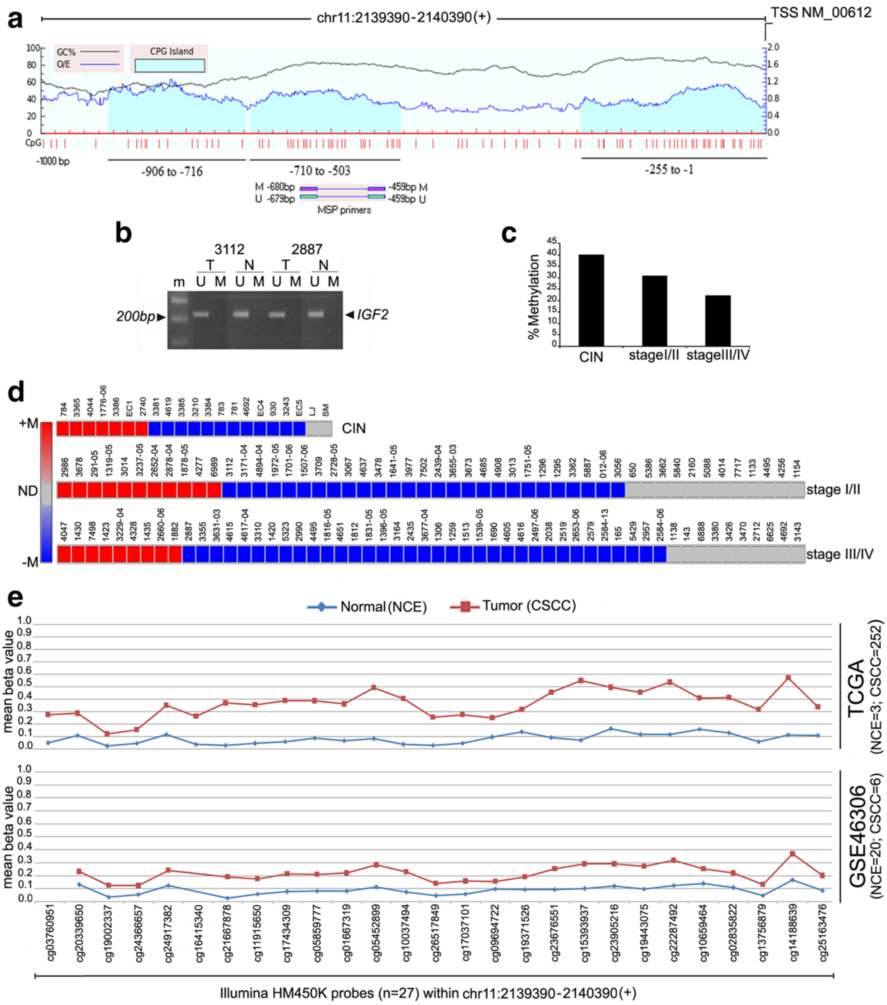


Fig. 3 Methylation-specific PCR (MSP) analysis of *IGF2* P3 promoter. **a** Illustration of CpG islands across −1000 bp upstream (chr11: 2,139,390–2,140,390) of *IGF2* transcript encompassing P3 promoter. O/E denotes CpG observed/expected ratio. Both the methylated (M) and unmethylated (U) MSP primer sets were also marked here. **b** Representative agarose gel showing both the tumor and the adjacent normal tissue of the samples 3112 and 2887 to be unmethylated. **c** Gradual decrease in promoter methylation was observed from CIN to stage I/II and stage III/IV CACX patients as depicted by the histogram. **d** Methylation profiles of individual samples across different stages of the CACX. **e** Validation of methylation status of *IGF2* P3 in two publicly available Illumina HM450K methylation datasets TCGA and NCBI GEO GSE46306. There were 27 probes within −1000 bp upstream (chr11: 2,139,390–2,140,390) of *IGF2* transcript. NCBI GEO GSE46306 dataset does not contain data for the probes cg03760951 and cg16415340. Both the datasets showed similar beta-value (mean) distribution which is concordant with the observed hypo-methylation of the P3 promoter

the CACX cell lines, HeLa (HPV18 positive) and SiHa (HPV16 positive) were found to be methylated for the gene.

Expression (mRNA) Profile of IGF2 in Normal Cervical Epithelium and Tumor Lesions

To ascertain the effect of alteration on the expression of *IGF2*, the analysis was done in NCE, primary CACX samples, and cell lines. Strikingly, *IGF2* was heterogeneously expressed in NCE and 41.17% (7/17) samples showed no expression. Similarly, the tumor samples had also showed heterogeneous mRNA expression across different tumor samples. When compared with the normal samples having *IGF2* expression, there was no significant fold change between normal and tumor samples with median log2^(−ddCt) of -0.06 in CIN ($n=2$), median log2^(−ddCt) of 0.07 in stage I/II ($n=17$), and median log2^(−ddCt) of -0.01 in stage III/IV ($n=17$). Similarly, heterogeneous expression was observed in two datasets where “Biewenga Cervix” showed significant over-expression for both the probes, while “Zhai Cervix” showed significant under-expression for both the probes (Supplementary Fig. 1 and Fig. 4). Interestingly, the frequent promoter hypo-methylation of *IGF2* was concordant with its expression in NCE tissues but CACX samples showed no such correlation. IGF2 was found to be highly under-expressed (median log2^(−ddCt) = − 1.56) in both the CACX cell lines and were concordant with their promoter hypermethylation.

Expression Analysis of IGF2 Protein in Normal Cervical Epithelium and Tumor Lesions

Protein expression in NCE was heterogeneous because 57.14% (4/7) showed high expression while rest 42.85% (3/7) showed low/no expression of IGF2 protein. It was found to be concordant with the mRNA expression of normal tissues. All the epithelial cell layers including basal, parabasal, and spinous cell layers showed similar intensity of nuclear expression in IGF2 expressed NCE samples. Remarkably, all the tumor tissues ($n=12$) showed low/no expression. Among these samples, 33.34% (4/12) showed both low expressions in both mRNA and protein but the rest 66.67% (8/12) showed high mRNA expression which was discordant with their protein expression.

Clinicopathological Correlation

The clinical outcome of the patients ($n=82$) was investigated for a period up to 5 years. Log-rank test uncovered a difference (p-value = 0.057) in overall survival between cases with and without alteration for the gene (Fig. 5c). Multivariate Cox regression model (data not shown) found no significant relative risks of *IGF2* alteration with other prognostic factors (clinical stage, tumor grade, lymph node involvement, parity, age at sexual debut, and HPV infection) on overall survival of the patients.

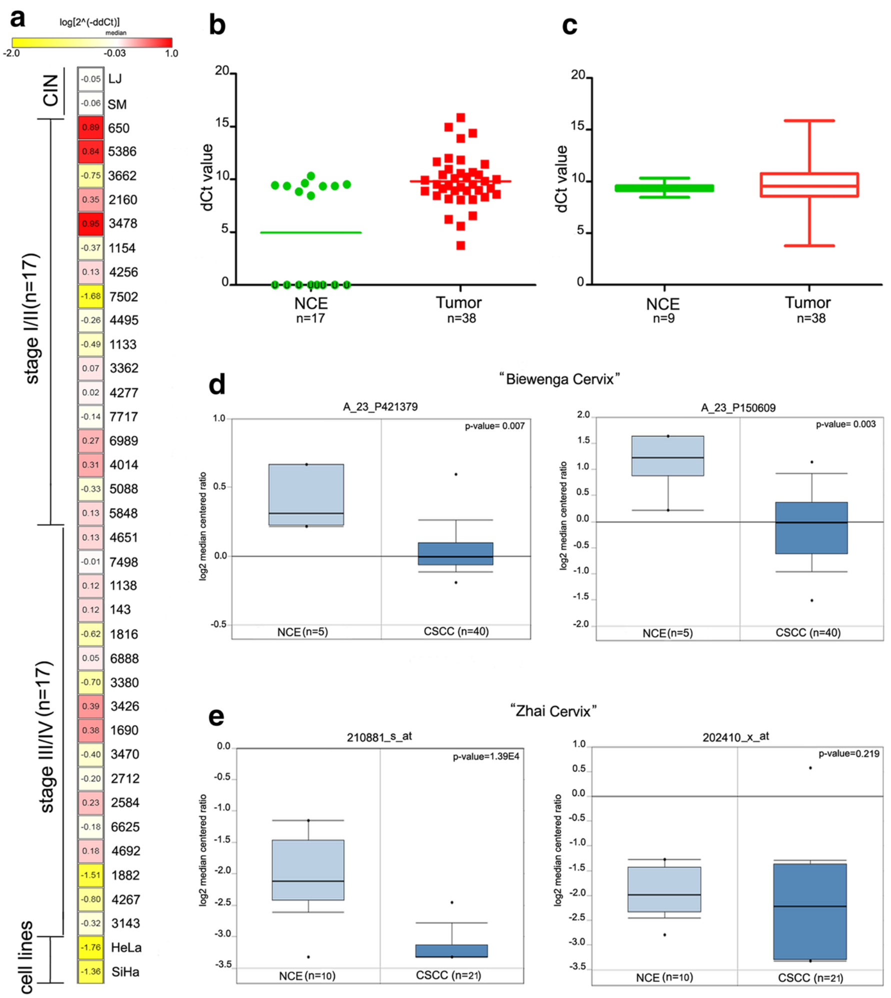


Fig. 4 Expression analysis of *IGF2* transcript. **a** Heatmap representation of log [2^(−ddCt)] depicting heterogeneous differential expression of *IGF2* transcript. **b** Boxplot showing dCt values both in NCE and tumor tissues. In eight NCE samples, no expression (marked U for undetermined) of *IGF2* transcript was observed. **c** Whisker plots showing no difference in median dCt values of the NCE (showing expression) and tumor samples but the diverse expression pattern was evident. **d** In publicly available dataset "Biewenga cervix," *IGF2* showed down-regulation in tumors with respect to NCE for both the probes. **e** On the other hand in another dataset "Zhai cervix" both the NCE and tumor tissues were poorly expressed as evident from their negative expressions. These contrasting results in the two datasets were concordant with the diverse expression of *IGF2* transcript in Indian patients

Discussion

The primary aim of the investigation was to unravel the molecular status of *IGF2* during the progression from normal cervical epithelium (NCE) to cervical squamous cell carcinoma (CSCC).

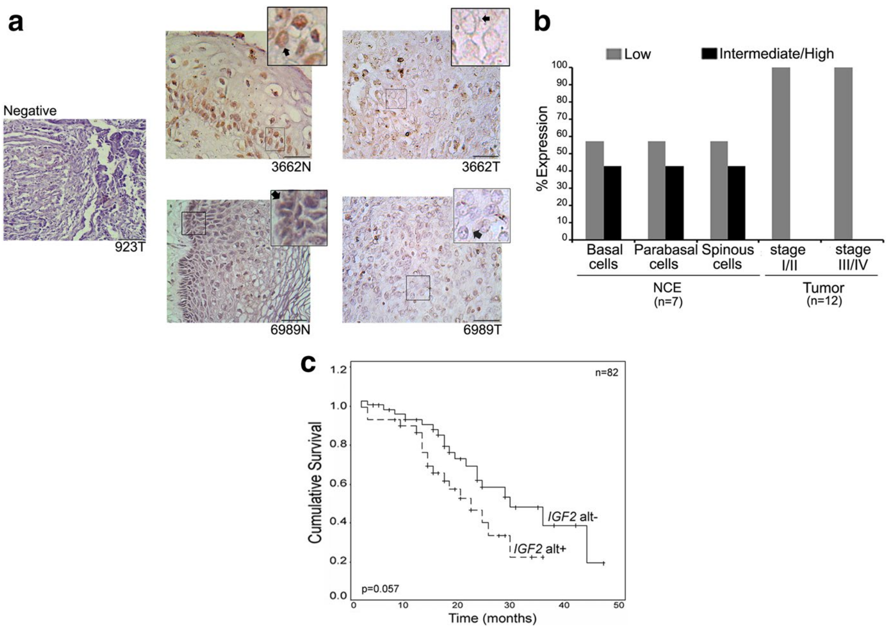


Fig. 5 Expression analysis of IGF2 protein. **a** Heterogeneous expression of IGF2 protein in NCE was observed as sample 3662 showed nuclear expression while sample 6989 does not show any expression. In the tumor counterpart, frequent low/no expression was seen. **b** Histogram showed the expression pattern of IGF2 across different cellular layers of NCE and different stages of the tumor. **c** Kaplan–Meier survival analysis of alteration of *IGF2* in Indian CACX patients

IGF2 is located at chromosomal 11p15.5 locus which is reported to be frequently altered during CACX development (Narayan and Murty 2010). Though, multifaceted deregulations of a candidate gene are observed during cancer development, copy number variation (CNV) and promoter DNA methylation status predominantly influence the mRNA and protein expressions (Moarii et al. 2015). So, in the beginning, analysis of CNV of *IGF2* locus revealed that there is no significant change (mean normalized log ratio of − 0.036) in DNA copy number. The data were further validated using datasets containing larger samples. It was found concordant with two publicly available CNV datasets, “TCGA cervix” and “Scotto cervix.” To the best of our knowledge, we report for the first time that there was no change in DNA copy number of *IGF2* locus in Indian CACX patients. Similar to CACX, CNV in *IGF2* locus is also infrequent in other cancers but The Cancer Genome Atlas (TCGA) study of colorectal cancer (CRC) samples found a focal amplification of the *IGF2* gene in 7% of the studied tumors leading to its over-expression in CRC (Brouwer-Visser and Huang 2015).

Then we probed into the methylation status of *IGF2* promoter. Now, *IGF2* is expressed from the reverse (−) strand and has several promoters P0, P1, P2, P3, and P4 (Brouwer-Visser and Huang 2015). Promoter P1 is located quite upstream almost near the insulin gene and it is active only in the adult human liver. Promoters P0 and P2–P4 are utilized during fetal development and in several malignancies the activation of these promoters was detected, especially, promoter P3 which is also often the most active promoter in immortal cell lines (Brouwer-Visser and Huang 2015). Hence, we focused on the methylation status of P3 promoter located within 1kb (chr11: 2,139,390–2,140,390) upstream of *IGF2* (RefSeq NM_000612). All normal tissues were unmethylated for P3 promoter. Similar frequent hypo-methylation was observed across CIN (60%) to stage I/II to 22.2% (69.2%) and stage III/IV (77.8%) CACX patients. To further confirm the frequent hypo-methylation of *IGF2* P3 promoter, we analyzed all the 27 Illumina HM450K probes located within the 1kb upstream region in two datasets. Strikingly, TCGA and NCBI GEO GSE46306 datasets results showed similar methylation status. For all the probes, mean beta-values for normal samples in both the datasets were close to 0 depicting no methylation and in tumor tissues also they were below 1 but higher than the normal tissues. Here also, the methylation status of P3 promoter of *IGF2* was revealed for the first time in Indian CACX patients. In other gynecological cancers like in breast cancer patients, increased hypo-methylation of the P3 promoter is reported (Brouwer-Visser and Huang 2015). Similar reports were also found in ovarian cancers as well (Qian et al. 2011). Both HeLa (HPV18 positive) and SiHa (HPV16 positive) cell lines were found methylated for the gene showing no relationship with high-risk HPV subtype.

To ascertain the effect of promoter methylation on the expression of *IGF2*, the mRNA expression analysis was done in NCE, primary CACX samples, and cell lines. Remarkably, *IGF2* was heterogeneously expressed in NCE where almost half (41.17%) of the individuals showed no expression. The protein expression analysis also revealed concordant observations where some NCE showed nuclear expression in the basal, parabasal, and spinous cell layers while others showed no expression. Similar expression across epithelial cell layers indicated no association of IGF2 protein expression with that of cellular differentiation. Moreover, IGF2 is predicted to be a secreted protein so, we expected its localization as cytoplasmic/membranous but instead it was observed to be nuclear in NCE tissues. To understand such observation, we looked into the Human Protein Atlas, where it was frequently found to be localized to the nucleoplasm of the analyzed cell lines (https://www.proteinatlas.org/ENSG00000167244-IGF2/cell#human) as well (Thul et al. 2017).

On the other hand, differential expression analysis with the normal samples (having *IGF2* expression) showed no significant fold change between normal and tumor samples. Though, heterogeneity was observed in tumor samples, such heterogeneous expression was observed in two publicly available datasets as well, where “Biewenga Cervix” showed significant over-expression and “Zhai Cervix” showed significant under-expression for *IGF2* transcript. Interestingly, hypermethylation of *IGF2* promoter was found to be concordant with its under-expressed (median log2^(−ddCt) = − 1.56) in both the CACX cell lines. Here, an important observation was made that unlike NCE, the frequent promoter hypo-methylation of *IGF2* was not concordant with its expression in CACX samples.

It was evident from the results that in NCE, our candidate gene *IGF2* showed no change in DNA copy number but hypo-methylation in P3 promoter was found with 59% of samples showing mRNA expression and 57% showing nuclear localization of its protein across cell layers. However, in CACX, we observed no differential change in DNA copy number and frequent hypo-methylation was observed during its different clinical stages. The differential mRNA expression also showed no significant change [median log2^(−ddCt) = − 0.03]. Similarly, low protein expression of IGF2 protein in CACX indicated that there is no change in differential protein expression when compared with 43% of NCE samples (with no expression of IGF2 protein) but it may be depicted as down-regulation when compared with the NCE samples having IGF2 expression. These observations clearly indicate that there may be other gene regulatory mechanisms other than promoter methylation which are in play in normal epithelial squamous tissues of the cervix and which get further deregulated during carcinogenesis (Brouwer-Visser and Huang 2015).

Previous studies in CACX only focused on the transcript expression with relationship to loss of imprinting and promoter methylation. To the best of our knowledge, this is for the first time the present study has shown an association between transcript and protein expression of IGF2 in same set of normal and CACX samples. Moreover, promoter methylation during tumor progression was also considered here as well. Hence, it has emerged from the present study that regulation of *IGF2* expression is multifaceted and needs to be explored further.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to declare.

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