A 7 gene expression score predicts for radiation response in cancer cervix

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Abstract

Background: Cervical cancer is the most common cancer among Indian women. The current recommendations are to treat the stage IIB, IIIA, IIB and IVA with radical radiotherapy and weekly cisplatin based chemotherapy. However, Radiotherapy alone can help cure more than 60% of stage IIB and up to 40% of stage IIB patients.

Methods: Archival RNA samples from 15 patients who had achieved complete remission and stayed disease free for more than 36 months (No Evidence of Disease or NED group) and 10 patients who had failed radical radiotherapy (Failed group) were included in the study. The RNA were amplified, labelled and hybridized to Stanford microarray chips and analyzed using BRB Array Tools software and Significance Analysis of Microarray (SAM) analysis. 20 genes were selected for further validation using Relative Quantitation (RQ) Taqman assay in a Taqman Low-Density Array (TLDA) format. The RQ value was calculated, using each of the NED sample once as a calibrator. A scoring system was developed based on the RQ value for the genes.

Results: Using a seven gene based scoring system, it was possible to distinguish between the tumours which were likely to respond to the radiotherapy and those likely to fail. The mean score ± 2 SE (standard error of mean) was used and at a cut-off score of greater than 5.60, the sensitivity, specificity, Positive predictive value (PPV) and Negative predictive value (NPV) were 0.64, 1.0, 1.0, 0.67, respectively, for the low risk group.

Conclusion: We have identified a 7 gene signature which could help identify patients with cervical cancer who can be treated with radiotherapy alone. However, this needs to be validated in a larger patient population.
Background
Cervical cancer is a preventable but the most common cancer among Indian women and second most common cancer among women worldwide [1,2]. More than 500,000 women are expected to develop the disease every year worldwide. The tragedy is worsened by most of the women presenting in locally advanced disease as well. Human Papilloma virus has been identified as an important factor in the development of cervical cancer, but it alone is not sufficient and requires additional events [3]. Cervical cancer progresses through initial dysplastic or pre-malignant stages before becoming invasive. The evolution to an invasive cancer may take up to 15 years or more [4]. PAP smear testing has been found to aid in early detection of pre-malignant lesions, thereby preventing the morbidity and mortality of cervical cancer. However, organized screening is primarily available in the Western countries and most of the developing countries including India do not have an organized screening program.

The standard treatment currently recommended for stage IIB, IIIA, IIIB and IVA has been concurrent chemo-radiotherapy, usually with weekly cisplatin based chemotherapy [5]. This is based on the data from 5 randomized trials which had shown a 30 -50% benefit in reducing the risk of death compared to the radiotherapy only arm. While the addition of chemotherapy is clearly beneficial, not all patients may need it. Currently, there are no reliable ways of identifying the individuals who may respond to the radical radiotherapy from those who will not respond.

The use of molecular approaches, such as gene expression studies, has helped tailor treatment based on the molecular characteristics. The Oncotype Dx testing for breast cancer is an example of using the newer techniques to help identify patients who may not need chemotherapy or advancing disease. This study aims to develop a test which can be used routinely to predict radiation response. More recently a few studies have used microarray based technology to identify gene signatures predictive of radiation response [12-14] however, none of them had validated their genes using further techniques such as Real time Polymerase Chain Reaction (PCR).

Avoiding morbidity and the cost of chemo-radiation in cervical cancer would be relevant particularly in a developing country. This study aims to develop a test which can help identify patients who may not need chemotherapy and who can be cured only with radical radiotherapy.

Methods
Archival total RNA extracted from punch biopsy samples collected in RNA later (Ambion, Austin, Tx; Cat no: AM7021) and stored in the tumour bank after an informed consent were used, after obtaining the Institutional Ethical committee's approval for the study. The RNA had been extracted from the biopsy samples using the RNeasy RNA extraction kit (Qiagen, Gmbh, Hilden; Cat no: 74106) as per the manufacturer’s instructions.

Twenty five patients' samples were included in the study. The criteria for inclusion in the study were as follows: 1. good quality RNA as assessed by Bio-analyser (RIN 6 or above); 2. paired paraffin block having at least 70% tumor cells; 3. sufficient quantity of RNA be available; 4. patient should have completed prescribed radiotherapy and follow-up information till death/last disease free status be available.

All the patients had received radical radiotherapy, with 6 MV X-ray from Linear accelerator and brachytherapy with HDR-Ir192. The total dose given was between 64 - 66 Gy, over 56 - 60 days. The median and mean follow-up period among the NED group was 43 months and 42 months, respectively, with a minimum and maximum period of follow up of 38 and 45 months, respectively. All the Failed patients had been followed up till progression/relapse/death. Complete remission refers to complete resolution of measurable disease by clinical examination, hematologic, biochemical or radiological examination; Partial remission refers to 50% or more disease reduction lasting longer than one month; Progression refers to worsening or advancing disease.

HPV testing
The quality of the DNA was assessed by amplifying for β globin prior to HPV testing which was done using GP5+ and GP6+ primers [15]. HPV16 and 18 typing was done using Nested Multiplex Polymerase Chain Reaction (NMPCR) technique [16]. SiHa DNA for HPV16, HeLa DNA for HPV18 (positive controls) and C33A DNA (negative control) were included in all runs.

Microarray experiment
1 μg of total RNA from the tumour sample and universal RNA (Stratagene; Cat no: 740000-41) were reverse transcribed using Array script at 42°C for 2 hrs to obtain cDNA using the Amino Allyl MessageAmp II aRNA amplification kit (Ambion, Austin, Tx; Cat no: AM1797). The cDNA was amplified by in-vitro transcription in the presence of T7 RNA polymerase; aRNA thus obtained was purified and quantitated in NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). 20 μg of tumour aRNA was labelled using NHS ester of Cy5 dye and control universal aRNA was labelled using NHS ester of Cy3 dye. The Cy3 and Cy5 labelled aRNA was used for hybridization onto the microarray chips from Stanford Functional Genomics Facility (SFGF, Stanford, CA) containing 44,544 spots, for 16 hrs in Lucidea SlidePro hybridization
chamber (GE Health Care, Uppsala, Sweden) at 42°C. After hybridization, slides were washed in 0.1× SSC, 1× SSC followed by 0.1× SSC and dried.

The slides were scanned in ProScanArray (PerkinElmer, Shelton, CT, USA). Griding was done using Scan array Express software package (version -4). The integrated or mean intensity of signal within the spot was calculated. The files were saved as GPR files.

All the raw data files have been submitted to GEO with an assigned GEO accession number - GSE14404.

Microarray data analysis
The Foreground Median intensity for Cy3 and Cy5, Background Median intensity for Cy3 and Cy5, spot size data were imported into BRB-ArrayTools software [17] using the Import wizard function. Background correction was not done. Global normalization was used to median centre the log-ratios on each array in order to adjust for differences in labelling intensities of the Cy3 and Cy5 dyes. The data was analysed using the Class comparison module in the BRB-Array Tools software. The normalized Log ratios were also imported into Significance Analysis of Microarray (SAM) [18] software and analysed.

Class Comparison in BRB-Array Tools
We identified genes that were differentially expressed among the two classes (NED VS Failed) using a random-variance t-test. The random-variance t-test is an improvement over the standard separate t-test as it permits sharing information among genes about within-class variation without assuming that all genes have the same variance [19]. Genes were considered statistically significant if their p value was < 0.0015. In addition a two fold difference was required between the two classes.

SAM Analysis
The normalized log ratios of all the samples were imported into SAM software and analysed. First a two class unpaired analysis with 100 permutations was done. A delta value of 0.96 and a fold difference of 2 was used to identify the genes differentially expressed. In addition, Survival analysis for significant genes was done using 100 permutations and with a delta of 0.85.

Quantitative Real time PCR
High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA; Cat no: 4368814) was used to reverse transcribe 2 μg of total RNA from the 23 samples in a 20 μl reaction volume. In two samples, due to the limiting amount of RNA, 0.75 μg was used for the cDNA synthesis.

These cDNA samples were used for real time PCR amplification assays using TaqMan® arrays formerly TaqMan® Low density arrays (TLDA) (Applied Biosystems, Foster City, CA; Cat no: 4342261). The fluorogenic, FAM labelled probes and the sequence specific primers for the list of genes with endogenous control 18S rRNA were obtained as inventoried assays and incorporated into the TaqMan® array format. Quadruplicate (n = 23) and duplicate (n = 2; with limiting amount of RNA for cDNA synthesis) cDNA template samples were amplified and analysed on the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA).

The reaction set up, briefly, consisted of 1.44 μg of cDNA template made up to 400 μl with deionised water and equal amounts of TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA; Cat no: 4304437). 100 μl was loaded into each of the 8 ports of the array (2 ports comprise of one sample replicate on the array). Thus, the samples run as duplicates were only loaded into 4 ports of the array. Thermal cycling conditions included a 50°C step for 2 minutes, denaturation for 10 min at 94°C followed by 40 cycles consisting of 2 steps: 97°C for 30 seconds and 59.7°C for 1 minute for annealing and extension.

The raw data from the Prism 7900HT sequence detection system was imported into the Real-Time StatMiner™ software for statistical analysis of the data. Among the endogenous reference genes included on the array (18S ribosomal gene; UBC, β2 microglobulin), UBC was chosen after visualizing the global Ct value distribution, for normalizing the data (see Additional file 1). The TLDA assays were run at LabIndia Instruments Pvt Ltd laborataries at Gurgaon, New Delhi.

Development of the scoring method
Relative quantification is based on the relative expression of a target gene versus a reference gene. The UBC normalized Delta Ct values were imported into the Excel spread sheet for obtaining the RQ values for the 24 samples (one sample which had not worked in RQ-RT-PCR was excluded), using each NED sample once as a calibrator. The genes were provided a score of 2, if the RQ value was >2. This analysis was done initially on all the selected genes taken up for validation and then on genes which had greater than two fold difference between the two classes, with an attempt to obtain a group of genes which can help distinguish between the two classes. The Mean score, the standard error (SE), the mean score ± 1SE and mean score ± 2SE were calculated and the Mean ± 2SE was used for categorizing as either High risk or Low risk. The cut-off was set at 40% (5.6) of the potential total score possible.

Sensitivity is the probability for a NED sample to be correctly predicted as Low risk; Specificity is the probability for a Failed sample to be correctly predicted as High risk;
PPV is the probability that a sample predicted as Low risk actually belongs to class NED; NPV is the probability that a sample predicted as High risk actually does not belong to NED [17].

Statistical Analysis
Kaplan Meier survival analysis was used to study the disease free survival (DFS) in the two classes, using the Stata 10 software. This was done initially based on the stage of the disease and then based on the gene signature risk stratification (Score ≤ 5.60 and score >5.60). Log rank test was used to assess the survival difference.

Results
The patients’ clinico-pathological status and their response to treatment are given in Table 1. Twenty four of the tumours were Squamous cell carcinomas (16 Large cell non-keratinizing, 4 large cell keratinizing and 4 unspecified) and one was a poorly differentiated carcinoma. Fourteen were HPV16 positive, 7 were HPV18 positive and 4 were HPV16 and 18 subtype negative (but HPV positive).

Using different methods, as described above, genes that were found to be differentially expressed between the two classes (NED and Failed) were obtained. The List of genes significant by different methods of analysis are given as a Additional file 2.

Twenty genes were selected for further validation by RQ-PCR using the Taqman Low Density Array card (TLDA) format (Table 2). These 20 genes formed part of the 95 genes selected for analysis using the TLDA format. Most of the additional genes were those which had been found to be differentially expressed between cervical cancer and normal cervix; cervical cancer and CIN1; CIN3 and CIN1; CIN3 and Normal cervix. Apart from the mandatory endogenous 18S rRNA included in the TLDA cards, based on the microarray data, UBC and β2 microglobulin, were included as additional endogenous reference genes.

One of the sample CXL19-hov160 which had worked in microarray did not amplify in the RQ-TLDA assay and had to be removed from further analysis. In addition, OPTC and C11orf9 genes did not amplify in any of the samples.

Table 1: Clinico-pathological and disease free status of the patients

| ID    | Stage | Grade | HPE  | Subtype | HPV Subtype | Treatment given | Response | Outcome (NED/Failed) | Site of failure | DFS (months) | OS (months) |
|-------|-------|-------|------|---------|-------------|-----------------|----------|----------------------|----------------|--------------|-------------|
| hOAe145 | IIB   | III   | SCC  | LCNK   | 16          | RT CR          | NED      | 44                   |                | 44           | 44          |
| hOAe146 | IIB   | III   | SCC  | LCNK   | Negative    | RT CR          | NED      | 38                   |                | 38           | 38          |
| hOAe152 | IIB   | III   | SCC  | LCNK   | 18          | RT CR          | NED      | 45                   |                | 45           | 45          |
| hOAe153 | IIB   | III   | SCC  | LCK    | 16          | RT CR          | NED      | 45                   |                | 45           | 45          |
| hOV156  | IIB   | III   | SCC  | LCNK   | 18          | RT CR          | NED      | 43                   |                | 43           | 43          |
| hOV160  | IIB   | III   | SCC  | LCNK   | 16          | RT CR          | NED      | 44                   |                | 44           | 44          |
| hOV161  | IIB   | III   | SCC  | LCNK   | 18          | RT CR          | NED      | 41                   |                | 41           | 41          |
| hOV164  | IIB   | III   | SCC  | LCNK   | Negative    | RT CR          | NED      | 38                   |                | 38           | 38          |
| hOV165  | IIB   | III   | SCC  | LCNK   | 16          | RT CR          | NED      | 43                   |                | 43           | 43          |
| hOV182  | IIA   | III   | SCC  | LCNK   | 16          | RT CR          | NED      | 39                   |                | 39           | 39          |
| hOV183  | IIB   | III   | SCC  | LCNK   | 18          | RT CR          | NED      | 45                   |                | 45           | 45          |
| hOV189  | IIB   | III   | SCC  | LCNK   | 16          | RT CR          | NED      | 40                   |                | 40           | 40          |
| hOV190  | IIB   | III   | SCC  | LCNK   | 16          | RT CR          | NED      | 42                   |                | 42           | 42          |
| hOV196* | IIB   | II-III| SCC  | LCNK   | 16          | RT+CT PR      | Failed   | Local                | 0              | 6            | 6           |
| hOV198  | IIB   | III   | SCC  | LCNK   | 16          | RT PR          | Failed   | Local                | 0              | 13           | 13          |
| hOV199  | IIB   | III   | SCC  | LCNK   | 18          | RT CR          | Failed   | Distant              | 10             | 34           | 34          |
| hOV200  | IIB   | II-III| SCC  | LCK    | 16          | RT PR          | Failed   | Local                | 0              | 24           | 24          |
| hOV217  | IIB   | III   | SCC  | LCNK   | 18          | RT PR          | Failed   | Local                | 0              | 7            | 7           |
| hOV218  | IIB   | III   | SCC  | LCNK   | 18          | RT CR          | Failed   | Distant              | 18             | 23           | 23          |
| hOV220  | IIB   | III   | SCC  | LCNK   | 16          | RT PR          | Failed   | Local                | 0              | 11           | 11          |
| hOV232  | IIA   | III   | SCC  | LCNK   | 16          | RT CR          | NED      | 46                   |                | 46           | 46          |
| hOV240  | IIB   | III   | SCC  | LCNK   | 16          | RT CR          | NED      | 38                   |                | 38           | 38          |
| hOV241  | IIB   | III   | PD   | Not specified | Negative    | RT PR          | Failed   | Local                | 0              | 12           | 12          |
| HOV242  | IIB   | II    | SCC  | LCK    | 16          | RT PR          | Failed   | Local                | 0              | 15           | 15          |
| hOV247  | IIB   | III   | SCC  | LCK    | 16          | RT PR          | Failed   | Local                | 0              | 7            | 7           |

* - One patient had received concurrent chemo-radiotherapy and had failed, was also included in the study
NED = No evidence of disease; SCC = Squamous cell carcinoma; PD = Poorly differentiated; LCNK = Large cell non-keratinizing;
LCK = Large cell keratinizing; CR = Complete remission; PR = Partial remission; RT = Radiotherapy; CT = Chemotherapy
The RQ values after calibrating with the NED samples (Mean) for all the 95 genes showed two additional genes, UBE2C and IGF2BP2, to be overexpressed in the Failed samples compared to the NED samples. These two genes had initially been chosen for validation of the differentially expressed genes between cancer and normal cervix. After excluding the genes which did not amplify, we now had 20 genes for further analysis (see Additional file 3).

There were 10 genes (including UBE2C and IGF2BP2) with more than 2 fold difference between the two classes. In addition, 4 genes had a fold change between 1 and 2. There was only 44% (8/18) concordance between the microarray data and the Relative quantitation RT-PCR data, when a 2 fold difference was used as the criteria. The best concordant rates were with the SAM survival analysis, wherein of the 11 genes taken up for validation, 8 were concordant (73%).

We then tried to analyse the RQ data using each of the NED sample once as a calibrator. The focus was on genes which had an over-expression of at least two fold between the two classes. A scoring system was then developed as described in the Methods section. Using this we found that the seven gene score (UBE2C, MMP3, DCUN1D5, SDCCAG8, IGF2BP2, CCL18 and FST) can help distinguish between the Failed and the NED samples. Table 3 gives the mean score of the 24 experiments, the standard deviation, the standard error (SE), and score ± 2SE (see Additional file 4 for the details). Using a cut-off value of > 5.60, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) are 0.64, 1.0, 1.0, 0.67, respectively, for the Mean ± 2SE. At this cut-off point, 9 of the 14 (64) NED samples were classified as low risk, while all the failed samples were classified as high risk.

The Kaplan Meier survival curves are given in Figures 1 and 2, the former based on the stage of the disease and the latter based on the gene expression signature.

**Discussion**

Cervical cancer although a preventable disease, still kills a significant number of women. Most of the tertiary centres in developing countries still see patients in advanced stages. Radiotherapy has been the main stay of treatment and the addition of concurrent cisplatin based chemotherapy has been found to improve cure rates. However, not all patients will need concurrent chemotherapy, which has its own cost implications and morbidity, particularly in a developing country. With the emphasis shifting to tailoring therapy to individuals based on the patient and tumour characteristics, it is essential to develop newer tests which can help identify individuals who can be cured only with radiotherapy. This paper provides one such approach, wherein a seven gene score was found to help distinguish those who are likely to be cured with radiotherapy.
therapy alone from those who may require additional forms of treatment.

The concordance between the microarray and the RQ-PCR data on genes differentially expressed with more than 2 fold was 44% (8/18). SAM Survival analysis had identified 13 genes (of the 14 genes, one was a duplicate -YAP1) as being differentially expressed and 11 of these genes were included in our validation. One gene did not have a suitable assay for the TLDA format. Of the 11 genes tested, 8 were found to be concordant in the RQ-RT-PCR validation (73% concordance rate). The SAM Survival analysis had correctly identified 5 of the 7 genes which we had used for the prediction.

One group had addressed the issue of concordance, when they compared Affymetrix data with Real time quantitative data for 48 genes. Their concordance rate was around 69% and they have reported that this may be usually due to differences in the target transcripts being identified by the two platforms (microarray versus Real time PCR) [20].

Klopp et al [14] had used microarray analysis on samples obtained prior to treatment and 48 hours after start of chemoradiation in 12 patients and found that a 58 gene signature can help predict recurrence. Others have developed either a Lymph node prediction model using 156 gene signatures, which had a prediction accuracy of 77% in predicting lymph node metastasis [21] or thermo-radio-response prediction model using a 35 gene signature [22]. Wong et al [13] used supervised clustering analysis to classify radiosensitive and radioresistant tumours in 13 patients.

Six of the seven genes, identified in our study, are known to be involved in cancers, with some of them having a role in inducing therapeutic resistance.

**Insulin like growth factor 2 mRNA binding protein 2/IGF2BP2/IMP2**

This oncofetal protein functions by binding to the 5’UTR region of IGF2 and thereby regulating its translation. Yisraeli (2005) [23] had suggested that the VICKZ family, to which IGF2BP2/IMP2 belongs, have a role to play in cell polarity and migration, cell proliferation and cancer. Autoantibodies to IGF2BP2 has been detected in different cancers including hepatocellular carcinoma [24,25].

**Ubiquitin-conjugating enzyme E2C/UBE2C/UBCH10**

The gene is a member of the E2 ubiquitin-conjugating enzyme family, playing key roles in regulation of cell cycle. The gene was found to be overexpressed in cervical

| Table 3: Scores based on RQ-RT-PCR values |
|-------------------------------------------|
| MEAN SCORE   | STDEV  | SE   | SCORE+2SE | SCORE-2SE |
|---------------|--------|------|-----------|-----------|
| Dct.CXL103-HOV220 | 9.57   | 1.95 | 0.52      | 10.61     | 8.53      |
| Dct.CXL108-HOV199  | 7.86   | 1.99 | 0.53      | 8.92      | 6.79      |
| Dct.CXL120-HOV241  | 12.86  | 1.51 | 0.4       | 13.67     | 12.05     |
| Dct.CXL121-HOV247  | 10     | 1.36 | 0.36      | 10.73     | 9.27      |
| Dct.CXL17-HOV200   | 7.86   | 2.66 | 0.71      | 9.28      | 6.44      |
| Dct.CXL46-HOV198   | 7.43   | 2.98 | 0.8       | 9.02      | 5.84      |
| Dct.CXL86-HOV218   | 8.68   | 2.32 | 0.62      | 8.92      | 5.62      |
| Dct.CXL90-HOV217   | 7.14   | 2.57 | 0.69      | 8.52      | 5.77      |
| Dct.CXL95-HOV242   | 13.14  | 1.29 | 0.35      | 13.83     | 12.45     |
| Dct.CXMO18-HOV196  | 8.86   | 1.7  | 0.46      | 9.77      | 7.95      |
| Dct.CXL11-HOV240   | 1.14   | 1.29 | 0.35      | 1.83      | 0.45      |
| Dct.CXL3-HOV183    | 3.86   | 2.14 | 0.57      | 5         | 2.71      |
| Dct.CXL4-HOV164    | 1.86   | 1.83 | 0.49      | 2.84      | 0.88      |
| Dct.CXL6-HOV232    | 4.43   | 2.38 | 0.64      | 5.7       | 3.16      |
| Dct.CXL20-HOV182   | 4.29   | 2.46 | 0.66      | 5.6       | 2.97      |
| Dct.CXL22-HOV161   | 3.71   | 2.7  | 0.72      | 5.16      | 2.27      |
| Dct.CXL3-HOV165    | 5.57   | 2.85 | 0.76      | 7.09      | 4.05      |
| Dct.CXL35-HOAIE145 | 1.29   | 1.86 | 0.5       | 2.28      | 0.29      |
| Dct.CXL52-HOAIE152 | 4.29   | 1.9  | 0.51      | 5.3       | 3.27      |
| Dct.CXL62-HOAIE153 | 1.43   | 1.65 | 0.44      | 2.31      | 0.55      |
| Dct.CXL73-HOAIE146 | 3.43   | 2.98 | 0.8       | 5.02      | 1.84      |
| Dct.CXL82-HOV189   | 4.86   | 2.32 | 0.62      | 6.09      | 3.62      |
| Dct.CXL9-HOV156    | 4.57   | 3.18 | 0.85      | 6.27      | 2.87      |
| Dct.CXL19-HOV190   | 2.57   | 1.99 | 0.53      | 3.63      | 1.51      |
cancers [27] and was reported to contribute to chemotherapy resistance in breast cancer [28]. Donata et al [29] have reported over-expression in low grade astrocytomas and in glioblastomas, wherein they could contribute to therapeutic resistance.

**Follistatin/FST**
This protein inhibits follicle stimulating hormone release. FST has been shown to be associated with Basal cell carcinomas [30]. In addition, integrin alpha6beta4, which is involved in apoptosis resistance, invasion, metastasis, can induce the expression of several genes, including FST, which could help mediate these processes [31].

**Matrix metallopeptidase 3 (stromelysin1; progelatinase)/MMP3**
MMP3 has been reported to be expressed in lymph node metastasis and in recurrent cervical cancers [32]. MMP3 has been suggested to have a role in modulating chemotherapeutic response in head and neck cancers [33] and has been shown to play a role in breast tumour carcinogenesis [34].

**Chemokine (C-C motif) ligand 18/CCL18/MIP4/AMAC/DCCK1/PARC**
CCL18/PARC has been found to be overexpressed in the serum of acute lymphoblastic leukaemia patients [35] and in the ascitic fluid of patients with ovarian carcinoma [36].

Serologically defined colon cancer antigen 8/SDCCAG8/Centrosomal colon cancer autoantigen protein/CCCAP
Autologous antibody responses to SDCCAG8 has been reported in colon and ovarian cancers [37,38]. It is a protein integral to the centrosomes [39].

**Defective in cullin neddylation 1, domain containing 5/DCUN1DS**
Little is known about the function of this gene, particularly in cancer.

**Conclusion**
Locally advanced cancer of the cervix is now being treated with chemo-radiotherapy. However, nearly 60% of stage
IIB and 40% of stage IIIB can be cured with radiotherapy only. Currently there are no reliable ways of identifying those patients who can be cured with radiotherapy alone.

Using microarray technique followed by Relative Quantitation Real Time PCR (RQ RT-PCR), we have identified a 7 gene signature with differential expression between cervical cancers with and without recurrence/progression after radiation treatment. This finding needs to be validated in an independent larger patient population.

**Conflict of interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
TR conceived the study; acquired, analysed & interpreted the data and drafted and revised the article. NV standardized and performed the microarray experiments and was involved in the drafting of the article. KS was involved in the acquisition and analysis of the microarray data and in the drafting of the article. SS carried out all the pathological studies and assessment of samples for the microarray studies. GS was involved in the clinical management and data analysis and follow-up of the patients. MB performed the microarray experiments. LN was involved in sample collection and processing and analysis. All the authors read and approved the final version of the manuscript.

**Additional material**

- **Additional file 1**
  - Ct values for reference genes. Provides the Ct values of the reference genes.
  - [Click here for file](http://www.biomedcentral.com/content/supplementary/1471-2407-9-365-S1.JPEG)

- **Additional file 2**
  - Genes significant by different methods of analysis of the microarray data. The data shows the list of genes identified to be significant by different methods of the microarray analysis.
  - [Click here for file](http://www.biomedcentral.com/content/supplementary/1471-2407-9-365-S2.XLS)
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Identification and validation of genes involved in cervical tumourigenesis

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Abstract

Background: Cervical cancer is the most common cancer among Indian women. This cancer has well defined pre-cancerous stages and evolves over 10-15 years or more. This study was undertaken to identify differentially expressed genes between normal, dysplastic and invasive cervical cancer.

Materials and methods: A total of 28 invasive cervical cancers, 4 CIN3/CIS, 4 CIN1/CIN2 and 5 Normal cervix samples were studied. We have used microarray technique followed by validation of the significant genes by relative quantitation using Taqman Low Density Array Real Time PCR. Immunohistochemistry was used to study the protein expression of MMP3, UBE2C and p16 in normal, dysplasia and cancers of the cervix. The effect of a dominant negative UBE2C on the growth of the SiHa cells was assessed using a MTT assay.

Results: Our study, for the first time, has identified 20 genes to be up-regulated and 14 down-regulated in cervical cancers and 5 up-regulated in CIN3. In addition, 26 genes identified by other studies, as to playing a role in cervical cancer, were also confirmed in our study. UBE2C, CCNB1, CCNB2, PLOD2, NUP210, MELK, CDC20 genes were overexpressed in tumours and in CIN3/CIS relative to both Normal and CIN1/CIN2, suggesting that they could have a role to play in the early phase of tumorigenesis. IL8, INDO, ISG15, ISG20, AGRN, DTXL, MMP1, MMP3, CCL18, TOP2A AND STAT1 were found to be upregulated in tumours. Using Immunohistochemistry, we showed over-expression of MMP3, UBE2C and p16 in cancers compared to normal cervical epithelium and varying grades of dysplasia. A dominant negative UBE2C was found to produce growth inhibition in SiHa cells, which over-expresses UBE2C 4 fold more than HEK293 cells.

Conclusions: Several novel genes were found to be differentially expressed in cervical cancer. MMP3, UBE2C and p16 protein overexpression in cervical cancers was confirmed by immunohistochemistry. These will need to be validated further in a larger series of samples. UBE2C could be evaluated further to assess its potential as a therapeutic target in cervical cancer.
The advent of microarray based technology has helped study the expression patterns of more than 40,000 genes at a time [4]. Several groups have used microarray based technology to look for differentially expressed genes in the different stages of cervical tumorigenesis [5,6]. Few studies have followed up and validated the microarray data in a large number of genes [7,8]. The objective of our study was to identify genes differentially expressed between normal cervix, CIN1/CIN2, CIN3/CIS and invasive cervical cancer, using oligo-microarray technique, validate the genes so identified using Relative quantitation Real Time Polymerase Chain Reaction (RQ-RT-PCR) and detect potential biomarkers for early diagnosis and therapeutic targets.

Methods
Archival total RNA extracted from punch biopsy samples from patients with cervical cancer, collected in RNA later (Ambion, Austin, USA; Cat no: AM7021) and stored in the tumour bank after an informed consent were used, after obtaining the Institutional Ethical committee’s approval for the study. The RNA had been extracted from the biopsy samples using the RNeasy RNA extraction kit (Qiagen, Gmbh, Hilden; Cat no: 74106) as per the manufacturer’s instructions.

Twenty eight cervical cancer patients’ samples were included in the study. The criteria for inclusion in the study were as follows: 1. good quality RNA as assessed by Bio-analyser (RIN 6 or above); 2. paired paraffin block having at least 70% tumour cells; 3. sufficient quantity of RNA be available; 4. patient should have completed prescribed radiotherapy and follow-up information till death/last disease free status be available.

In addition, 5 normal cervix tissues from women who underwent hysterectomy for non-malignant conditions or for non-cervical cancer were included. Four CIN1/CIN2 and 4 CIN3/CIS (one CIN3/CIS was included for RQ-RT-PCR analysis directly) were also included after informed consent. The Normal and CIN samples underwent frozen section to confirm their histopathologic status and the samples were immediately snap frozen in liquid nitrogen. RNA was extracted from the samples using the RNeasy RNA extraction kit, as described above.

HPV Testing
The quality of the DNA was assessed by amplifying for β globin and only then HPV testing was done using GP5+ and GP6+ primers [9]. HPV16 and 18 typing was done using Nested Multiplex Polymerase Chain Reaction (NMPCR) technique [10]. SiHa DNA for HPV16 and HeLa DNA for HPV18 (positive controls) and C33A DNA (negative control) were included in all runs.

Microarray experiment
1 μg of total RNA from the tumour/CIN/Normal sample and universal RNA (Stratagene; Cat no: 740000-41) were reverse transcribed using Arrayscript at 42°C for 2 hrs to obtain cDNA using the Amino Allyl MessageAmp II aRNA amplification kit (Ambion, Austin, USA; Cat no: AM1797). The cDNA was amplified by in-vitro transcription in the presence of T7 RNA polymerase; aRNA thus obtained was purified and quantitated in NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). 20 μg of tumour/CIN/Normal aRNA was labelled using NHS ester of Cy5 dye and the control universal aRNA was labelled using NHS ester of Cy3 dye. The Cy3 and Cy5 labelled aRNA was used for hybridization onto the microarray chips from Stanford Functional Genomics Facility (SFGF, Stanford, CA) containing 44,544 spots, for 16 hrs in Lucidea SlidePro hybridization chamber (GE Health Care, Uppsala, Sweden) at 42°C. After hybridization, slides were washed in 0.1× SSC, 1× SSC followed by 0.1× SSC and dried.

The slides were scanned in ProScanArray (PerkinElmer, Shelton, CT, USA). Griding was done using Scan array Express software package (version -4). The integrated or mean intensity of signal within the spot was calculated. The files were saved as GPR files.

All the raw data files have been submitted to GEO with an assigned GEO accession number - GSE14404.

Microarray data analysis
The Foreground Median intensity for Cy3 and Cy5, Background Median intensity for Cy3 and Cy5, spot size data were imported into BRB-ArrayTools software [11] using the Import wizard function. Background correction was not done. Global normalization was used to median centre the log-ratios on each array in order to adjust for differences in labelling intensities of the Cy3 and Cy5 dyes. The data was analysed using the Class comparison and Class prediction modules in the BRB-Array Tools software. In addition, Lowess normalization was also done separately and the data analysed using the modules mentioned above. The normalized Log ratios were also imported into Significance Analysis of Microarray (SAM) [12] software and analysed.

Class Comparison in BRB-Array Tools
We identified genes that were differentially expressed among the four classes (Normal, CIN1/2, CIN3/CIS, Cancer) using a random-variance t-test. The random-variance t-test is an improvement over the standard separate t-test as it permits sharing information among genes about within-class variation without assuming that all genes have the same variance [13]. Genes were considered statistically significant if their p value was < 0.01. In addition a two fold difference was required...
between the Cancer and Normal, CIN3/CIS and Normal, CIN1/2 and Normal. The same was repeated with the Lowess normalized data using the same criteria.

**Class prediction in BRB-Array Tools**

We developed models for utilizing gene expression profile to predict the class of future samples based on the Diagonal Linear Discriminant Analysis and Nearest Neighbour Classification [11]. The models incorporated genes that were differentially expressed among genes at the 0.01 significance level as assessed by the random variance t-test [13]. We estimated the prediction error of each model using leave-one-out cross-validation (LOOCV) as described [14]. Leave-one-out cross-validation method was used to compute mis-classification rate. From the list, genes were sorted further based on 2 fold difference between Cancer versus CIN1/2 & Normal, CIN3/CIS versus CIN1/2 & Normal, and CIN1/2 versus Normal. The same was repeated with the Lowess normalized data using a significance value of 0.01.

**SAM Analysis**

The normalized log ratios of all the samples were imported into SAM software and analysed. A Multiclass analysis with 100 permutations was done. A delta value of 0.96 and a fold difference of 2 was used to identify the genes differentially expressed.

**Quantitative Real time PCR**

High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA; Cat no: 4368814) was used to reverse transcribe 2 μg of total RNA from the 38 samples in a 20 μl reaction volume. In 3 samples, due to the limiting amount of RNA, 0.75 μg was used for the cDNA synthesis.

These cDNA samples were used for real time PCR amplification assays using TaqMan® arrays formerly TaqMan® Low density arrays (TLDA) (Applied Biosystems, Foster City, CA; Cat no: 4342261). The fluorescent, FAM labelled probes and the sequence specific primers for the list of genes with endogenous control 18S rRNA were obtained as inventoried assays and incorporated into the TaqMan® array format. Quadruplicate (n = 38) and duplicate (n = 3; with limiting amount of RNA for cDNA synthesis) cDNA template samples were amplified and analysed on the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The reaction set up, briefly, consisted of 1.44 μg of cDNA template made up to 400 μl with deionised water and equal amounts of TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA; Cat no: 4304437). 100 μl was loaded into each of the 8 ports of the array (2 ports comprise one sample replicate on the array). Thus, the samples run as duplicates were only loaded into 4 ports of the array. Thermal cycling conditions included a 50°C step for 2 minutes, denaturation for 10 min at 94°C followed by 40 cycles consisting of 2 steps: 97°C for 30 seconds and 59.7°C for 1 minute for annealing and extension.

The raw data from the Prism 7900HT sequence detection system was imported into the Real-Time Statminer™ software for statistical analysis of the data. Among the endogenous reference genes included on the array (18S ribosomal gene; UBC, β2 microglobulin), UBC and β2 microglobulin were chosen after visualizing the global Ct value distribution, for normalizing the data (Supplementary figure 1). The TLDA assays were run at LabIndia Instruments Pvt Ltd laboratories at Gurgaon, New Delhi.

**Immunohistochemistry (IHC)**

IHC was done for MMP3 protein expression in 5 Normal cervical tissue, 30 dysplasias of varying grade (CIN1 - 11; CIN2 - 8; CIN3/CIS - 11) and 27 invasive cervical cancers. A 3 layered ABC technique was used as described previously [15]. MMP3, monoclonal antibody (Sigma Aldrich, India; cat no: M6552) was used at a dilution of 1:75 and with wet antigen retrieval method. Positive control (section from a pancreatic cancer) and negative control (omission of primary antibody) were included in each run. The slides were scored by SS and TR independently and where discordant, jointly. The scoring was based on percentage of tumour cells immunoreactive (negative - 0; <25% = 1; 25-50% = 2; 51-75% = 3; >75% = 4), intensity of immunoreactivity (negative - 0; + - 1; ++ - 2; +++ - 3) and the compartment stained (cytoplasmic, nuclear or stromal). The scores obtained were added and the threshold was set at above the scores seen in the Normal cervical tissue (maximum score seen in Normal cervical tissue was 8). Hence tissues with a score of 9 or above were considered to overexpress MMP3.

p16 IHC was done as described previously [16] on 5 normal cervical tissue, 31 dysplasias of varying grades (CIN1 - 12; CIN2 - 8; CIN3/CIS - 11) and 29 tumours. Slides were scored as reported previously [16].

UBE2C IHC was done as above using wet autoclaving with a hold time of 5 minutes. Rabbit UBE2C polyclonal antibody (Millipore, USA - catalogue no: AB3861) was used at 1 in 100 dilution. The scoring was done similar to the scoring of MMP3 staining, with the maximum score seen in normal cervical tissue being 6. Hence a score of 7 or above was considered to be overexpression.

**UBE2C in cervical cancer cell lines**

Taqman Real time PCR was done for UBE2C levels in SiHa, C33A, HeLa, ME180, BU25K and HEK293 (Human embryonic kidney cells) cell lines. GAPDH was used to normalize the data.
Dominantly negative UBE2C, in which Cysteine 114 is replaced by Serine, leading to loss of catalytic activity [17] was introduced into SiHa cells, using Fugene 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions using a 3:2 Fugene/DNA ratio. The effect on growth was assessed using the MTS assay (Promega) in the SiHa wild type (WT), in SiHa with pcDNA vector alone (SiHa pcDNA) and in SiHa with dominant negative UBE2C (SiHa DN-UBE2C).

Statistical analysis
Comparison between group means was assessed using a one-way ANOVA and multiple-comparison correction by Holm-Sidak method using Sigmaplot version 11.0. Fisher’s exact test (2 tailed) was used to assess significance of IHC immuno-reactivity between cancer and dysplasias.

Results
The stage distribution of the invasive cancer cases was as follows: IB - 2, IIA - 4, IIB - 18 and IIIB - 4. Twenty seven of the tumours were Squamous cell carcinomas (18 Large cell non-keratinizing, 5 large cell keratinizing and 4 unspecified) and one was a poorly differentiated carcinoma. Eighteen were HPV16 positive, 6 were HPV18 positive and 4 were HPV16 and 18 subtype negative (but HPV positive). All the Normals were HPV negative while one CIN1/2 and all the CIN3/CIS were HPV16 positive.

Using different methods, as described above, genes that were found to be differentially expressed between the four classes (Normal, CIN1/2, CIN3/CIS and Cancer) were identified. We did not use a Training set and a Test set for the Class Prediction model but used LOOCV for cross-validation and obtain the misclassification error. The list of genes significant by different methods of microarray analysis is given in the Additional File 1 (AF1).

Sixty nine genes were selected for further validation by RQ-PCR using the Taqman Low Density Array card (TLDA) format (Additional File 2). These 69 genes formed part of the 95 genes selected for analysis using the TLDA format. The additional genes were those which had been found to be differentially expressed between the responders and non-responders to radiotherapy only treatment. Apart from the mandatory endogenous 18S rRNA included in the TLDA cards, based on the microarray data, UBC and β2 microglobulin, were included as additional endogenous reference genes.

Two of the samples CXL19-hov160 and CXM024-hov210 which had worked in microarray did not amplify satisfactorily in the RQ-TLDA assay and had to be removed from further analysis. In addition, RPS3A gene did not amplify in any of the samples.

The RQ values after calibrating with the Normal samples (Mean) for all the 94 genes showed 8 additional genes to be overexpressed; 4 (ASB16, CCL18, FST, THOC6) in Cancers, 1 (KLK9) in CIN3/CIS and 3 (RASSF6, TMEM123 and GLB1L3) in CIN1/2 samples. These 8 genes had initially been chosen for validation of the differentially expressed genes between responders and non-responders to radiotherapy. After excluding the genes which did not amplify, we now had 76 genes for further analysis.

Of the 31 genes which had been selected based on a greater than 2 fold difference between cancer versus CIN1/2 & Normal, 28 were concordant between the microarray data and the RQ-RT-PCR (Concordant rate of 90%). Three of four genes selected based on higher level of expression in Normals compared with all other classes showed concordance between the different methods of analysis. In the case of CIN1/2, concordance was seen in 6/7 genes (86%). However, with CIN3, this dropped to 41% (11/27). In four additional genes, there was a two fold greater difference between CIN3/CIS and Normal but not with CIN1/2. The overall concordance rate between the microarray data and the RQ-RT-PCR was 70% (48/69).

The list of genes validated and found to have a greater than 2 fold difference compared to the Normal, in the 3 different classes (Cancer, CIN3/CIS and CIN1/2) is given in Table 1. Figure 1 provides the fold change relative to Normal for these genes.

The genes were grouped on the basis of whether or not they were known to be involved in cervical tumorigenesis (Tables 2 and 3). Gene Ontology mapping was done using Babelomics software [18], which showed an over-representation of genes involved in cell cycle, cell division, catabolic process and multi-cellular organismal metabolic process. The genes identified to be differentially expressed were then analysed for specific pathways of relevance by manual curetting of data from published literature and online databases. The genes were grouped under the following categories: 1. Cell cycle regulatory genes (n = 13); 2. Interferon induced genes (n = 5); 3. Ubiquitin pathway (n = 5); 4. Myc Pathway [19] (n = 12); 5. HPV-E6/E7 related genes [20] (n = 14); 6. RNA targeting genes (n = 3) (details are given in Additional File 3). In addition, 40 genes in our list were found to be potentially regulated by p53 family of genes [21] (Additional File 4).
Table 1 Rq Values For The Genes Relative To Normal (Continued)

| GENES WITH LESS THAN TWO FOLD DIFFERENCE IN THE 3 CLASSES |
|----------------------------------------------------------|
| CIN3 | CALML5 | 0.51194 | 10.2307 | 5.18399 | 0.00709 | 0.005123751 |
| CKNB1 | 10.6825 | 10.44 | 2.79343 | 0.0175 | 0.009329125 |
| CKNB2 | 6.74715 | 7.52496 | 2.54768 | 0.00064 | 0.008585426 |
| EBP | 2.05888 | 5.0445 | 3.49571 | 0.00027 | 0.008585426 |
| FLI14635 | 1.40964 | 6.98147 | 3.89307 | 0.22913 | 0.365061765 |
| KLK9 | 11.7606 | 30.8271 | 26.324 | 0.01049 | 0.065711253 |
| NUSAP1 | 1.92037 | 6.57218 | 1.75075 | 0.00533 | 0.049729841 |
| PCNA | 1.76308 | 26.4579 | 0.92838 | 0.01586 | 0.009329125 |

CIN1

| B4GALT1 | 1.29547 | 0.63469 | 2.21031 | 0.05915 | 0.166490075 |
| CAPNS2 | 0.32306 | 1.98671 | 2.3676 | 0.01723 | 0.09528905 |
| CD36 | 0.71359 | 10.6158 | 14.14 | 0.00889 | 0.052209988 |
| CRNN | 0.00653 | 0.40855 | 12.1177 | 0.01104 | 0.027439506 |
| CST8 | 0.17911 | 0.90884 | 4.5624 | 0.02063 | 0.029296967 |
| CXCL14 | 0.00072 | 1.49396 | 11.7379 | 0.07384 | 0.18626486 |
| DAPL1 | 0.02534 | 1.3546 | 6.09254 | 0.00067 | 0.027439506 |
| DIB | 0.40666 | 2.15767 | 3.96854 | 0.00449 | 0.031848688 |
| DYNL1 | 5.20441 | 4.20119 | 6.97185 | 0.07896 | 0.185551839 |
| FBLN1 | 0.15796 | 0.34282 | 2.85624 | 0.05894 | 0.166490075 |
| GJA1 | 0.16263 | 0.60199 | 3.58862 | 0.0017 | 0.029296967 |
| GLB1L3 | 0.11823 | 0.47805 | 5.23721 | 0.04676 | 0.16283811 |
| HEPBP2 | 0.82928 | 1.50603 | 2.15449 | 0.03358 | 0.144693437 |
| HOPX | 0.04259 | 0.57251 | 2.37465 | 0.07856 | 0.185551839 |
| KRT10 | 0.50079 | 7.42996 | 10.2777 | 0.03009 | 0.029296967 |
| KRTDAP | 0.00637 | 0.61777 | 10.8535 | 0.00441 | 0.031848688 |
| MAFA | 1.18718 | 2.08598 | 4.31406 | 0.0029 | 0.029296967 |
| RASS6F | 2.55599 | 3.073 | 8.63678 | 0.00189 | 0.029296967 |
| SLURP1 | 0.00845 | 0.56373 | 7.04202 | 0.00117 | 0.027439506 |
| TMEM123 | 2.77691 | 1.00492 | 19.4803 | 0.03995 | 0.150195313 |

Gene symbols in bold italics indicate those which were not concordant between microarray and RQ-RT-PCR analysis.

Using IHC, we studied the protein expression for MMP3 in 5 normal cervical tissues, 30 dysplasias of varying grades and 27 invasive cancers. Using a semi-quantitative scoring system and a cut-off threshold set based on the normal cervical tissue staining, 6/30 dysplasias and 11/27 invasive cancers were found to overexpress MMP3 protein (Figure 3A). Among the patients...
Table 2 Genes Identified as Up or Down-Regulated In Cervical Cancers For The First Time

| No. | GENE SYMBOL | FUNCTION | CANCERS WHEREIN UP-REGULATION REPORTED |
|-----|-------------|----------|----------------------------------------|
| 1   | AGRN        | Basement membrane component | Hepatocellular carcinoma (HCC) [27], Synovial sarcoma [28] |
| 2   | CCL18       | Attracts lymphocytes towards dendritic cell | Gastric cancer [29] |
| 3   | CKS1B       | Cell cycle | Breast cancer [30] etc. |
| 4   | COL7A1      | Epithelial basement membrane organization | Oesophageal cancer [31] |
| 5   | DNYLL1/DLC1 | Inhibits neuronal nitric oxide synthase | Breast cancer [32] |
| 6   | FERMT1      | Cell adhesion; TGF-β signaling | Lung and colon cancer [33] |
| 7   | FST         | Activin antagonist; inhibits synthesis and secretion of FSH | Wilm’s tumour, Basal cell carcinoma [34] |
| 8   | IGF2BP2     | Regulate translation of target mRNA | Testicular cancer [35], HCC [36] |
| 9   | IL8         | Inflammatory cytokine | Several cancers, HR-HPV+VIN [37] |
| 10  | KLK9        | Serine protease | Bladder cancer [38], breast cancer [39] |
| 11  | MELK        | Leucine zipper kinase | Colo-rectal cancer, lung cancer [40], brain cancer [41] |
| 12  | PLCD2       | Lysyl hydroxylase | Glioblastoma [42] |
| 13  | RASSF6      | Tumour suppressor | Low levels detected in HeLa cell line [43] |
| 14  | SLC16A1     | Monocarboxylate transporter | Neuroblastoma [44] |
### Table 2 Genes Identified as Up or Down-Regulated In Cervical Cancers For The First Time (Continued)

| S NO. | GENE SYMBOL | FUNCTION | REFERENCES |
|-------|-------------|----------|------------|
| 15    | SMC4        | Chromosome condensation; DNA repair | Breast cancer [45] |
| 16    | APOBEC3B    | RNA editing enzyme | None to date |
| 17    | ASB16       | Ubiquitin pathway | None to date |
| 18    | NUP210      | Nuclear pore complex | None to date |
| 19    | THOC6       | Spliceosome associated protein | None to date |
| 20    | TMEM123     | Induces Pro-onclosis type cell death | None to date |

**CIN3**

| S NO. | GENE SYMBOL | FUNCTION | REFERENCES |
|-------|-------------|----------|------------|
| 1     | CCNB2       | Cell cycle | Lung [46], pituitary tumours [47] |
| 2     | EBP         | ER Protein | ALK+ Anaplastic large cell lymphoma [48] |
| 3     | NUSAP1      | Microtubule associated protein | Melanoma [49] |
| 4     | CALML5      | Calcium binding protein | Psoriasis [50] |
| 5     | FLJ44635    | TPT1-like protein | None to date |

### CANCERS WHEREIN DOWN-REGULATION REPORTED

| S NO. | GENE SYMBOL | FUNCTION | REFERENCES |
|-------|-------------|----------|------------|
| 1     | C20orf114   | Innate immunity | Nasopharyngeal cancer [51] |
| 2     | CRNN        | Tumour suppressor | Tongue cancer [52] |
| 3     | CSTB        | Thiol proteinase inhibitor, anti-metastatic | Laryngeal cancer [53] |
| 4     | DBI         | Intracellular carrier for Acyl-COA esters | Overexpressed in Brain tumours [54] |
| 5     | FCGBP       | Maintenance of mucosal surface | Prostate [55] |
| 6     | HOPX        | Tumour suppressor | Choriocarcinoma [56] and lung cancer [57] |
| 7     | SLURP1      | Anti-tumour, anti-angiogenic | Hypopharyngeal cancer; Can induce apoptosis in Kaposi’s sarcoma [58] |
| 8     | SPINK5      | Serine protease inhibitor | Tongue cancer [59] |
| 9     | TFF3        | Protect mucosa | Down-regulated in highly invasive colon cancers [60] and in thyroid cancers [61] |
| 10    | CAPNS2      | Thiol protease | None to date |
| 11    | DAPL1       | Epithelial differentiation | None to date |
| 12    | GLB1L3      | Galactosidase beta1 like | None to date |
| 13    | HEBP2       | Heme binding protein | None to date |
| 14    | KRTDAP      | Keratinocyte differentiation | None to date |

### Table 3 Genes Known To Be Up or Down-Regulated In Cervical Cancers Found Also In Our Study

#### UP-REGULATED

| S NO. | GENE SYMBOL | FUNCTION | REFERENCES |
|-------|-------------|----------|------------|
| 1     | CCNB1       | Cell cycle | Overexpressed in cervical cancers [62] |
| 2     | CDC20       | Cell cycle | HR-HPV E2 interaction [63] |
| 3     | CDC25B      | Cell cycle | FOXM1 increases Cyclin B1, CDC25B, Cyclin D1 in cervical cancers [64] |
| 4     | CDH3        | Cell adhesion | P-Cadherin predominant cadherin in high grade cervical dysplasia [65] |
| 5     | CDKN2A      | Cell cycle | Overexpressed in high grade dysplasia and invasive cancers [66] |
| 6     | CKS2        | Cell cycle | Overexpressed in cervical cancer [67] |
| 7     | DTX3L       | Ubiquitin pathway | Overexpressed in cervical cancer [68] |
| 8     | INDO/IDO1   | Immuno-suppression | Role in inducing immunosuppression in the tumour milieu [69] |
| 9     | ISG15/S1P2  | Ubiquitin like protein | Over-expressed in invasive cancer [70] |
| 10    | ISG20       | Exonuclease with higher affinity for RNA | Up-regulated by HPV E6 [71] |
| 11    | KRT17       | Intermediate filament, marker for epithelial "stem cells" | Overexpressed in cervical cancer [72] |
| 12    | LAMB3       | Basement membrane protein | HR-HPV-E6 inhibits miR-218 which regulates LAMB3 [73] |
| 13    | MCM4        | Cell cycle | Widely expressed in cervical cancers [74] |
| 14    | MCM6        | Cell cycle | Over-expressed in cancer [75] |
Table 3 Genes Known To Be Up or Down-Regulated In Cervical Cancers Found Also In Our Study (Continued)

|    | Description                        | Relation to Cervical Cancers |
|----|------------------------------------|-----------------------------|
| 15 | MMP1                               | Breakdown of extracellular matrix Overexpressed in cervical cancers [76] |
| 16 | MMP3                               | Breakdown of extracellular matrix Metastatic lymphnodes in cervical cancer harbour MMP3 positive tumour cells [77]; Increased in stroma in cancers [78] |
| 17 | SLC2A1                             | Glucose transporter Expressed in CIN and invasive cervical cancers [79] |
| 18 | STAT1                              | Transcription activator Overexpressed in cervical cancers [80] |
| 19 | TK1                                | Thymidine kinase Up-regulated in invasive cancers [81] |
| 20 | TOP2A                              | Toposomerase Overexpressed in cervical cancers [82] |
| 21 | UBE2C                              | Ubiquitin pathway Overexpressed in cervical cancer [76] |

CIN3

1. PCNA  Cell cycle Major up-regulation of PCNA upon progression to CIN3 [83]

DOWN-REGULATED

1. FBLN1  Cell adhesion; tumour suppressor E6 binds to Fibulin1 and modulates its activity [84]
2. CD36   Cell adhesion CD36 down-regulated in high grade dysplasias and cancer [85]
3. CXCL14 Immunoregulatory cytokine Down-regulated in cervical and head and neck cancers [86]
4. GJA1   Gap junction Down-regulated in CIN3 and invasive cancers [87]
5. KRT10  Intermediate filament Down-regulated in invasive cancer [72]

Figure 2  Inter-relationship of our validated genes with known Transcription factors and E6 & E7 protein. Bold arrows indicate stimulatory effect; dotted arrows indicate inhibitory effect. Dot-Dash arrow refers to unknown effect.
whose tumours had been treated only with radical radiotherapy and had been followed up for a minimum period of 3 years, over-expression was seen in a greater number of tumours that failed treatment (6/9) compared to those free of disease at 3 years (2/12) (p = 0.03). p16 was found to be overexpressed in 19 of 31 dysplasias of varying grade and in 27/29 cancers (p = 0.005) (Figure 3B).

Using IHC, we found UBE2C to be overexpressed in 28/32 cancers, 2/11 CIN3/CIS and none of the CIN1 or 2 (Fisher’s exact test p = 2.2 e^{-11}) (Figure 3C). Using RQ RT-PCR, UBE2C was found to be overexpressed by more than 2 fold in SiHa, HeLa, C33A and ME180 relative to the HEK293 cells (Figure 4A). The growth of SiHa cells transfected with dominant negative UBE2C was significantly reduced at 48 and 72 hours compared to SiHa WT and SiHa transfected with pcDNA vector alone (p < 0.001) (Figure 4B).

**Discussion**

There was good overall concordance between the microarray and the RQ-RT-PCR data. The lower concordance rate seen with the CIN3/CIS may be due to the additional CIN3 sample processed directly using RQ-RT-PCR. The relative quantitation values with and without the additional sample is given as Additional File 5. The concordance rate between microarray and semi-quantitative RT-PCR in the study by Gius et al [8] was less than 50%, using the standard microarray data analysis package.

There were several instances, wherein, a small difference in Microarray (above the 2 fold mandatory criteria) sometimes translated to large differences with RQ-RT-PCR (e.g. p16, MMP1, MMP3) and vice versa (e.g. CD36). This reinforces the point about the limitation of the microarray technique and it does emphasize the need for further validation, using assays like RQ-RT-PCR.

HPV16 was the predominant subtype seen in the invasive cancers and CIN3/CIS. However, we did not look for all the high risk subtypes and hence cannot exclude multiple subtype infection. Four of the cancers were HPV positive but HPV16 and 18 negative, suggesting that other high risk subtypes could be involved. None of the normal cervical tissues were HPV positive.

The genes that were for the first time, found to be over-expressed in cervical cancers compared to Normal cervix, is given along with information in which other cancers they have been reported to be overexpressed (Table 2A). Our study, for the first time, has identified 20 genes to be up-regulated in cervical cancers and 5 in CIN3; 14 genes were found to be down-regulated. In addition, 26 genes identified by other studies, as to playing a role in cervical cancer, were also confirmed in our study. UBE2C, CCNB1, CCNB2, PLOD2, NUP210, MELK, CDC20 were overexpressed in tumours and in CIN3/CIS relative to both Normal and CIN1/CIN2, suggesting that they could have an important role to play in the early phase of tumorigenesis. Among the genes which were up-regulated in cancers compared to that of
Normal, CIN1/2 or CIN3/CIS, IL8, INDO, ISG15, ISG20, AGRN, DTXL, MMP1, MMP3, CCL18, TOP2A AND STAT1 are likely to play an important role in the progression of the disease.

STAT1 gene has a bi-phasic level, a rise in CIN1/2, drop in CIN3/CIS and a significant rise in invasive cancers. STAT1 has been considered generally to be a tumour suppressor, while STAT3 and STAT5 are known to be proto-oncogenes. However, recent studies have shown STAT3 to have both oncogenic and tumour suppressor function [22]. It could be that in cervical cancer, STAT1 may be protective in the early phase of growth.
HPV infection but could function as a proto-oncogene in the invasive stages of the disease. Highly invasive melanoma cell lines had high levels of STAT1 and c-myc [23].

The study by Lessnick et al., [24] showed that introduction of the potentially oncogenic EWS-FLI transcript into the fibroblasts, resulted in growth arrest rather than transformation. Knocking out p53 using HPV E6 helped overcome the growth arrest but was not sufficient to induce malignant transformation. The study used microarray to identify genes differentially expressed between the EWS-FLI transfected and the mock transfected cell line and found several genes related to growth promotion down-regulated. Our study had several genes [19] overlapping with theirs. Thirteen genes from our study were found to be HPV E6/E7 related genes[20] and 40 of the genes in our list were found to be potential p53 Family Target genes[21] (Additional File 3). In addition, there were 12 myc regulated genes, (MYC Cancer database at http://www.myc-cancer-gene.org/) of which CSTB which has been reported to be down-regulated by myc, was down-regulated in CIN3/CIS and in Cancer [19].

p16 gene, a tumour suppressor has been reported to be over-expressed in dysplasias and invasive cancer of the cervix. Several studies have tried to use this as a marker in the PAP smears for more reliable interpretation of the smear. von Knebel's group from Germany [25], had developed an ELISA to detect p16 in the cervical cell lysates, and reported a 96% sensitivity to pick up high grade dysplasias. Subsequently, the p16 ELISA assay was compared with Hybrid Capture 2 and was found to have comparable sensitivity and a slightly better specificity (46.9% versus 35.4%) [26]. Our RQ-RT-PCR data shows a gross over-expression of p16 in the CIN3 and invasive cancers (>250 fold). In our series of dysplasias and cancers, p16 protein was found to be over-expressed in invasive cancers compared to the dysplasias.

UBE2C will need to be studied further to assess its potential as a target for the treatment of cervical cancer.

Additional material

Additional file 1: List of genes differentially expressed identified by microarray analysis
Additional file 2: List of genes taken up for validation
Additional file 3: Identified genes linked to specific pathways
Additional file 4: p53 family regulated genes
Additional file 5: Relative Quantitation with and without CXM180

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Authors’ contributions

TR conceived the study; acquired, analysed & interpreted the data and published the data. GS was involved in the clinical management and data analysis and follow-up of the patients. All the authors read and approved the final version of the manuscript.

Conlicts of interests

The authors declare that they have no competing interests.

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Dominant negative Ubiquitin-conjugating enzyme E2C sensitizes cervical cancer cells to radiation

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Abstract

Purpose: To find the radiation sensitivity of human cervical carcinoma cell lines and to investigate the effect of the dominant negative-Ubiquitin-conjugating enzyme E2C (DN-UBe2C) on cell proliferation and radiation response.

Materials and methods: Radiation sensitivities of human cervical cell lines (SiHa, HeLa, BU25TK, ME 180, and C33A) were analyzed by assessing their cell survival after irradiation by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Soft agar cloning assay, growth curve and radiation response of DN-Ube2C stably transfected SiHa and HeLa cell lines were assessed by MTS assay and Clonogenic assay.

Results: Difference in sensitivity to radiation was observed among the cervical cancer cell lines studied. SiHa was found to be the most resistant cell line whereas C33A cells were the most sensitive. The growth rate of SiHa and HeLa transfected with DN-Ube2C was significantly reduced compared to vector control. Furthermore, DN-Ube2C-mediated radiosensitivity was correlated with a significant decrease in resistance to radiation by SiHa and HeLa cells after transfection with the DN-Ube2C when compared to control cultures.

Conclusion: These results suggested that the Ubiquitin-conjugating enzyme E2C (Ube2C) gene is a potential therapeutic target for cervical cancer treatment.

Keywords: Ube2C, dominant negative-Ube2C, cervical cancer, radiation sensitivity, therapeutic target

Introduction

Cervical cancer is the second most common cancer in women, worldwide. About 80% of cervical cancer cases occur in developing countries where, in many regions, it is the most common cancer among women (Ferlay et al. 2010). Asia accounts for more than half of the world’s cervical cancer cases and deaths because of its immense population. The Crude Incidence Ratio (CIR) of cervical cancer as per Madras Metropolitan Tumor Registry (MMTR) is 17.2 per 100,000. Lack of resources for organized screening of cervical cancer has mainly contributed to burden of the disease in developing countries (Ihun et al. 2010). The majority of the cases (about 70%) presenting at the Institute are in the advanced stages (Stage III and IV) and despite adequate treatment some patients fail therapy. Hence there is a need to identify novel therapeutic targets which can modify radiation response for a favorable therapeutic outcome. Large scale gene expression studies, functional characterization and validation of individual genes are an important means to understand the molecular mechanisms behind disease progression and treatment response. Studies on specific genes involved in malignant transformation and therapeutic resistance will help identify therapeutic targets, which when coupled with conventional therapy will further improve the treatment outcome.

Ubiquitin-dependent proteolysis by the 26s proteosome is involved in regulating various cellular processes including cell cycle progression, signal transduction and differentiation. Ubiquitin/proteosome system involves activation of three distinct enzymes – Ubiquitin-activating enzyme E1, Ubiquitin-carrier or conjugating enzyme E2s, and Ubiquitin-protein ligase E3. The process of proteolysis occurs through sequential activation of these enzymes that link chains of the polypeptide co-factor, Ubiquitin, onto proteins that are to be degraded by proteosome (Glickman and Ciechanover 2002).

Cell cycle is controlled by a complex series of signaling pathways, including the Ubiquitin proteosome pathway which intricately modulates the cell cycle progression. The Ubiquitin-conjugating enzyme E2C (Ube2C/UbcH10) is a part of this process; by interacting with Ubiquitin-ligating enzyme (E3), it is involved in ubiquitination of mitotic cyclins A and B, as well as securin (Bastians et al. 1999), Townsley et al. (1997), reported a dominant negative-Ubiquitin-conjugating enzyme E2C (DN-Ube2C) variant of the wild-type Ube2C by changing the catalytic cysteine to serine, at residue 114, which resulted in blockage of cyclin ubiquitination and destruction in vitro. Overexpression of Ube2C has been demonstrated in a variety of malignancies such as colorectal, thyroid, lung, breast, ovary and lymphomas corresponding to their normal tissues (Warrenfeltz et al.)

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2004, Kadara et al. 2009, Loussouarn et al. 2009, Troncone et al. 2009, Chen et al. 2010, Guerriero et al. 2010).

We have recently shown that, UBE2C is over expressed in cervical cancer (Rajkumar et al. 2011) and it is one among the seven-gene signature whose expression pattern could help identify patients to be treated with radiotherapy alone (Rajkumar et al. 2009). Our study had shown the SiHa cells expressing the DN-UBE2C to have growth inhibition, relative to the parental and the vector-only transfected SiHa cells (Rajkumar et al. 2011). In the present study, we have explored the role of DN-UBE2C in radiation response.

Materials and methods

Cell culture

The human cervical carcinoma cell lines used in this study were SiHa, HeLa, Bu2STK, ME180 and C33A were grown in Dulbecco’s Modified Eagle Medium (DMEM), (Hi media Laboratories, Mumbai, India) containing 10% foetal bovine serum (FBS), (Gibco Laboratories, Carlsbad, CA, USA), and Penicillin/streptomycin (Hi media Laboratories). Cells were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Cell irradiation

Cells were irradiated using 6MeV linear accelerator – Clinac 2300 C/D (Varian Medical systems, Inc. Palo Alto, CA, USA) with a source to bolus distance of 1 cm. A field size of 20 × 20 cm was used for 25 cm² flask. The cells were seeded into 25 cm² flask and irradiated using 6 MeV X-rays. A single dose of 2, 4, 8 and 10 Gy was delivered at a dose rate of 300 MU min⁻¹. The cells from the five cell lines were irradiated in duplicates and independently.

Radiation sensitivity studies on cervical cancer cell lines

Radiation sensitivities of cervical cell lines such as SiHa, HeLa, Bu2STK, ME180 and C33A were analyzed by assessing their cell survival. The cells were seeded into 25 cm² flasks and exposed to single doses of 2, 4, 8 and 10 Gy of 6 MeV X-ray from the linear accelerator. Similarly, SiHa and HeLa cells stably expressing plasmid encoding for Flag epitope alone (pcDNA/FLAG) or plasmid encoding for Flag epitope-tagged DN-UBE2C (pcDNA/FLAG/DN-UBE2C), maintained in DMEM (Hi media Laboratories) supplemented with 10% FBS (Gibco Laboratories) and 20 µg/ml of Hygromycin (Invitrogen, Carlsbad, CA, USA) were also irradiated. After irradiation, cell count was performed and 1 × 10³ cells were seeded in quadruplicates into 96-well tissue culture plates. Cells were allowed to grow for 7 days and cell viability was then assessed using the MTS assay (Promega Pte Ltd, Singapore).

Sub cloning and purification of dominant negative-UBE2C

Addgene plasmid 8505-UbcH10 DN (Townsley et al. 1997) was obtained from Addgene, Inc. The sequence encoding the DN-UBE2C were amplified using UBE2C open reading frame primers (FP: 5’-GGATCCATGGCTTCCAAAAC 3’; RP: 5’-CTCGAGTCAGGGTCTCTGGCT 3’) and sub cloned into pcDNA/FLAG 3.1 mammalian expression vector at BamHI and XhoI restriction sites using standard techniques. Plasmid DNA purification was performed using the Zyppy plasmid miniprep kit (Zymo Research Corporation, Irvine, CA, USA). Sub cloned DN-UBE2C, with mutation at its active site was confirmed by DNA sequencing using UBE2C open reading frame primers. Sequencing was performed using Big dye terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed in ABI Prism 310 Genetic Analyzer (Applied Biosystems). After confirming the identity of the sequence a midiprep of pcDNA/FLAG/DN-UBE2C construct was prepared using a QIA filter Midiprep Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

Transfection of SiHa and HeLa cells with dominant negative-UBE2C

SiHa and HeLa cultures at 70% confluence were trypsinized, and cells were transferred to six-well plates. Each well contained 2 × 10⁶ cells and was incubated for 24 h in DMEM (Hi media Laboratories) with 10% FBS (Gibco Laboratories) and 1% penicillin/streptomycin (Hi media Laboratories). Transfection with DN-UBE2C plasmids was performed using Fugene HD Transfection Reagent (Roche Diagnostics GmbH, Germany) according to the manufacturer’s instructions. Ratio of Fugene to plasmid DNA was maintained at 3:1. The cells were maintained in OptiMEM™ medium (Invitrogen) for 48 h and then cells stably expressing empty pcDNA/FLAG or pcDNA/FLAG/DN-UBE2C were maintained in DMEM supplemented with 10% FBS and 20 µg/ml of Hygromycin (Invitrogen).

Immunoblotting

SiHa and HeLa cells stably expressing empty pcDNA-FLAG or pcDNA-FLAG/DN-UBE2C were washed once in cold phosphate buffered saline (PBS) and lysed in a RIPA lysis buffer containing (50 mM Tris HCl (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)). The lysates were clarified by centrifugation at 14,000 rpm at 4°C for 10 min. Protein concentrations were estimated by Pierce® BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) and boiled in gel loading buffer for 5 min before electrophoresis. Proteins were subjected to SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) followed by transfer to polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Buckinghamshire, UK); completeness of transfer was assessed using SEE-BLUE prestained protein standards (Invitrogen). After blocking with Tris-Buffered Saline and Tween-20 (TBST)-MILK (25 mM Tris, pH 7.4, 200 mM NaCl, 5% non-fat milk powder), the membrane was incubated with the primary ANTI-FLAG® antibody (Sigma, St Louis, MO, USA) overnight, at room temperature. To ascertain that equal amounts of protein were loaded, the Western blots were incubated with antibodies against the β-actin protein (Sigma). After washing, membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:5000) for 60 min (at room temperature) and the reaction was detected with a western blotting detection system ECL plus (Amersham Biosciences).
Cell proliferation assay
Cell proliferation was assessed using CellTiter 96 AQ<sub>ueous</sub> One Solution Cell Proliferation Assay (Promega), as per the manufacturer’s instructions. All experiments for SiHa and HeLa transfected DN-UBE2C were performed along with wild-type and pcDNA/FLAG alone transfected SiHa and HeLa as controls, in triplicates at cell density of 1 × 10⁴ cells/well in a 96-well format. Cells were allowed to grow in DMEM (Hi media Laboratories) medium containing 10% FBS (Gibco Laboratories) and 20 µg/ml of Hygromycin (Invitrogen). Cell proliferation was then assessed at different time points (0, 24, 48, 72 and 96 h), using the MTS assay.

Soft agar assay
Soft agar colony forming assay was performed by suspending 3 × 10⁴ cells in 0.2% low-melting point agarose (Becton Dickinson and Company, MD, USA) dissolved in DMEM (Hi media Laboratories) containing 10% FBS (Gibco Laboratories) with 20 µg/ml of Hygromycin (Invitrogen) and plated in six-well plates consisting of DMEM containing 10% FBS (Gibco Laboratories) in a solidified 0.5% agarose medium. All experiments for SiHa and HeLa transfected DN-UBE2C were performed along with wild-type and pcDNA/FLAG alone transfected SiHa and HeLa as controls, in duplicates. The plates were incubated for 4–5 weeks after which a colony count was performed.

Clonogenic assay
SiHa and HeLa cultures at 70% confluence were trypsinized, and cells were seeded into 25 cm² flasks and exposed to single doses of 2 and 4 Gy of 6 MV x-ray from the linear accelerator. Similarly, SiHa and HeLa cells stably expressing empty pcDNA/FLAG or pcDNA/FLAG/DN-UBE2C, maintained in DMEM (Hi media Laboratories) supplemented with 10% FBS (Gibco Laboratories) and 20 µg/ml of Hygromycin (Invitrogen) were also irradiated. After irradiation, cell count was performed and 1 × 10³ cells were seeded in duplicates into six-well tissue culture plates. The plates were incubated for two weeks after which staining using Clonogenic assay reagent (containing 50% Ethanol and 0.25% 1,9-dimethyl-methylene blue) and colony count was performed.

Statistical analysis
Comparison between group means was assessed using a one-way ANOVA and multiple-comparison correction by Holm-Sidak method using Sigmaplot version 11.0.

Results
SiHa cells are resistant to radiation
Differences in response to radiation were observed in all five cell lines studied. Figure 1 shows the results of radiation response of different cell lines to single doses of 2, 4, 8 and 10 Gy of 6 MV x-ray from the linear accelerator. These data are expressed as mean percent survival of individual cell line at different dose points. At 2 Gy, sensitivity was similar in SiHa, HeLa, followed by Bu25TK, C33A and ME180. SiHa was the most resistant cell line showing 30% survival at 10 Gy, whereas C33A cells was the most sensitive cell line showing 30% survival even at 4 Gy. ME180 and Bu25TK showed moderate sensitivity at 10 Gy with 15 and 17% survival, respectively.

Dominant negative-UBE2C represses cell proliferation
DN-UBE2C with cysteine-to-serine mutation at residue 114 of its active site was cloned into pcDNA 3.1 mammalian expression vector and confirmed by sequencing and double enzymatic digest before transfection into cervical cancer cell lines. SiHa and HeLa cells stably expressing pcDNA/FLAG/DN-UBE2C were confirmed by Western blotting (Figure 2a). SiHa and HeLa cells which overexpressed wildtype UBE2C were transfected with DN-UBE2C to study its effect on anchorage-independent growth on soft agar and cell proliferation. Anchorage-independent cell growth on soft agar showed significantly reduced numbers of colonies in both DN-UBE2C transfected SiHa and HeLa cells when compared with vector alone transfected cells as controls ($P < 0.05$; Figure 2b).

Significant decrease in cell proliferation was observed in DN-UBE2C transfected cell lines when compared with wild type ($P < 0.05$), across the time point. The growth rate DN-UBE2C transfected SiHa cells which originally showed a fourfold increase in UBE2C levels, showed a significant decrease in cell proliferation at 48 and 96 h ($P < 0.001$), when compared

![Image](image-url)

Figure 1. Radiation sensitivity of cervical cancer cells (SiHa, HeLa, Bu25TK, ME180 and C33A) to different doses. MTS assay was used to generate the percent survival compared to untreated controls, with assays run in quadruplicate twice. Error bar represents standard deviation ($n = 4$), independent experiments.
with pcDNA alone transfected SiHa cells (Figure 2c). Similarly, HeLa cells transfected with DN-UBE2C showed significant decrease in cell proliferation at 24, 72 and 96 h, respectively, when compared to vector-alone transfected HeLa cells. *P*-value of < 0.05 was considered significant (Figure 2d).

**Dominant negative-UBE2C transfected cells are sensitive to radiation**

Radiation sensitivity of the SiHa and HeLa cells transfected with DN-UBE2C was assessed by MTS assay. Significant decrease in resistance to radiation at doses of 2, 4, and 8 Gy by DN-UBE2C transfected SiHa cells was observed when compared to pcDNA vector alone transfected SiHa cells (*p* = 0.006, < 0.001 and < 0.001, respectively) (Figure 3a). Similarly, HeLa cells transfected with DN-UBE2C showed significant decrease in their resistance to radiation at doses of 2 and 4 Gy (*p* = < 0.001) when compared to pcDNA vector alone transfected HeLa cells. Overall significant level of < 0.05 was observed between all groups (Figure 3b). Similarly the radiation sensitivity was also assessed by Clonogenic assay. SiHa (Figure 3c) and HeLa (Figure 3d) cells transfected with DN-UBE2C showed significant decrease in resistance to radiation at 4 Gy (*p* = < 0.05 and 0.006, respectively), when compared to pcDNA vector alone transfected cells.

**Discussion**

In developing countries, cancer of uterine cervix remains as a leading cause of death from gynecological malignancies. Although both surgery and radiotherapy are used as the primary therapies for early cervical cancer, surgery alone is rarely used for patients with advanced stage disease (Chung et al. 2005). Currently, concomitant chemotherapy and radiotherapy is the preferred modality for patients with locally advanced cervical cancer (Green et al. 2001). In spite of different modalities of treatment, cervical cancer is responsible for 275,000 deaths in 2008, about 88% of which occur in developing countries (Perlay et al. 2010) indicating a greater need for targeted therapies with the understanding of molecular mechanisms behind disease progression and treatment response.

We have previously showed that UBE2C was overexpressed in cervical tumors and might be contributing to therapeutic resistance. We have also reported earlier expression of UBE2C in cervical cancer cell lines by quantitative real-time PCR (Rajkumar et al. 2011). Among the cell lines analyzed, UBE2C mRNA was found to be highest in SiHa (four-fold) followed by about two-fold upregulation in C33A, ME180 and HeLa, whereas, Bu2STK showed least expression. UBE2C expression levels in cervical cancer cells are consistent with a previous report which studied expression of 17 E2 genes. UbcH10 (UBE2C) was expressed at very low to undetectable levels in normal tissues, whereas numerous cancerous cell lines expressed it at high levels (Okamoto et al. 2003).

Our current study on the radiation response of the cervical cancer cell lines indicated that SiHa cells as the most resistant and C33A as most sensitive. The observed radiation
sensitivities were similar to the previously reported data (Saxena et al. 2005). When we compared the radiation sensitivities of cervical cancer cell lines along with the UBE2C expression status, we found no correlation since Bu2STK, which showed moderate resistance to radiation, had in fact the lowest levels of expression whereas C33A, which was the most sensitive, had higher expression levels of UBE2C than Bu2STK. However SiHa cells, which showed the highest levels of expression of UBE2C, were also the most radioresistant. These results indicate that other factors independent of UBE2C pathway might also contribute to radiation sensitivity.

Studies on a variety of tumors have shown light on UBE2C as a potential oncofugic factor. Okamoto et al. (2003) reported that stable transfectants of NIH3T3 cells overexpressing UBE2C caused an efficient incorporation of BrdUrd, an accelerated growth rate, an increase in saturation density, and a promotion of anchorage-independent growth. In the light of our previously published data on UBE2C expression levels in cervical cancer tissues, we proceeded to investigate the effects of repression of activity of UBE2C in cervical cancer cells. To this end we employed a mutant version of UBE2C whose dominant negative effect was previously demonstrated. Townsley et al. (1997) reported that mutation of UBE2C active site cysteine to serine results in a dominant negative phenotype, with the mutant UBE2C protein competitively inhibiting cyclin B ubiquitination and degradation and inhibits destruction of both cyclin A and B. Comparison of anchorage-independent growth on soft agar and cell proliferation between vector alone transfected and stably transfected DN-UBE2C showed significant decrease in number of colonies formed and cell proliferation in DN-UBE2C transfected cells when compared to the vector control in both SiHa and HeLa cells. A similar finding was reported by Lin et al. (2006) when Seg-1, an esophageal adenocarcinoma-derived cell line expressing high levels of UBE2C, transfected with a dominant negative form of UBE2C resulted in a significant decrease in cell proliferation.

Furthermore, to explore the role of DN-UBE2C in radiation response, we have studied the sensitivity of SiHa and HeLa transfected DN-UBE2C to radiation. Significant decrease in resistance to radiation by SiHa and HeLa cells transfected with DN-UBE2C was observed when compared to vector-alone transfected controls. To further validate our finding, we did a conventional two-week Clonogenic assay which also revealed that DN-UBE2C transfected SiHa and HeLa cells showed increased sensitivity to radiation compared to vector controls. Shekhar et al. (2002) found that overexpression of Ubc2/Rad6 (Ubiquitin-conjugating enzyme E2A [RAD6 homolog]) induced anchorage-independent growth of recipient cells, indicating that deregulated expression of Ubc2/Rad6 is involved in malignant transformation. The RAD6 gene of Saccharomyces cerevisiae encodes a ubiquitin-conjugating (E2) enzyme and is required for the repair of damaged DNA, mutagenesis, and sporulation. RAD6 mRNA levels were found to be elevated in cells exposed to UV light, and at all UV doses the increase in mRNA levels was rapid and occurred within 30 min after exposure to UV (Madura et al. 1990).

We have shown that SiHa and HeLa cells which overexpressed UBE2C and showed a relatively higher resistance level, became radiosensitized following transfection with dominant negative form of UBE2C (compared to vector-alone transfected cells); this effect might possibly be due
to competitive inhibition of functional wild-type UBE2C. However, the precise molecular mechanisms by which DN-UBE2C sensitizes cells to radiation are unclear and needs further investigation. UBE2C is an integral part of the Ubiquitin proteasome pathway and is involved in cell cycle control and progression. Tumor cells are more susceptible to proteosome inhibition due to their rapid division and disordered regulatory pathway. Targeting the proteosome pathway especially, UBE2C may result in favorable therapeutic outcome. In summary, we have confirmed that UBE2C is overexpressed in cervical cancer cell lines, repression of UBE2C by DN-UBE2C decreases cell proliferation. To the best of our knowledge, this is the first report to demonstrate that inhibition of UBE2C sensitizes cervical cancer cells to radiation. These results imply that UBE2C can be a potential therapeutic target.

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Declaration of interest

The authors report no declaration of interest. The authors alone are responsible for the content and writing of the paper.

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