CDC27 protein is involved in radiation response in squamous cell cervix carcinoma

T Rajkumar¹*, G Gopal¹, G Selvaluxmi² and K R Rajalekshmy³
¹Dept. of Molecular Oncology; ²Dept. of Radiation Oncology; ³Dept. of Immunology/Hematology, Cancer Institute (WIA), Adyar, Chennai 600 020, India

Received 15 March 2005; revised 3 September 2005

In the present study, an attempt was made to identify genes involved in radiation response in cervix carcinoma. Differential display technique was used to study the expression profiles of tumour biopsy samples obtained from patients, responding and not responding to treatment. The samples were obtained prior to radiotherapy and subsequent to treatment with Tele-radiation at 10 Gray (Gy). One of the differentially expressed cDNAs, when sequenced was identified to be CDC27. Immunohistochemical analysis of pre- and post-treated tumour samples from fifteen patients showed the down-regulation of expression of CDC27 protein in seven patients. Down-regulation was associated with poorer response to radiotherapy. Cervical cancer cell lines SiHa and C33A were irradiated and their nuclei were stained for expression of CDC27 and analyzed using fluorescent-activated cell sorting (FACS). Down-regulation of CDC27 protein in the irradiated SiHa cell line was associated with greater survival fraction, compared to the irradiated C33A cell line, which had only slight fall in the level of CDC27 protein. This is the first study to suggest a role for CDC27 in radiation response. However, a larger cohort is needed to further confirm the value of CDC27 protein as a predictive marker, for radiation response in cervix cancer.

Keywords: cdc27, cervix cancer, predictive markers, radiation response, differential display, prognosis

IPC Code: G01N33/574

Cervix cancer is the most common cancer among Indian women with a crude incidence rate of 26/100,000 (Madras metropolitan tumour registry (MMTR), 1998). Most of the patients (80%) are in stages II B and III B and more than 80% are high-grade tumours. Squamous cell carcinomas account for nearly 90% of these cancers, while adeno-squamous carcinoma and adenocarcinoma make up the remaining 10%. Although standard radiotherapy treatment is available, nearly 40-60% cases fail the treatment. Thus, there is a need for better predictive markers that could identify high-risk women, who are likely to fail the treatment with standard therapy, so that additional chemotherapy/other measures could be instituted to improve the cure rate. In our earlier study of cell cycle regulatory molecules in cervical cancer, c-myc over-expression was found to be an indicator of poor prognosis in stages II B and III B. Besides, Bcl2, EGFR, c-erbB2, Ki67 and mitotic index, MMP2 and TIMP2 were also reported to be the indicators of poor prognosis in cervical cancer.

Gene expression analysis can help study radiation-induced changes in cells, which could help identify molecules that could act as surrogate markers for radiation response and also help predict response to a particular modality of treatment. Based on changes in gene expression levels and protein function in response to radiation, genes having roles in various cellular process, such as signal transduction, cell death, DNA repair and cell cycle have been identified as potential modifiers/markers of radiation response.

Differential display (DD) has been extensively used to study gene expression to identify differentially expressed novel and known genes. A major advantage of this technique is that both tumour suppressors (which are likely to be down-regulated)
and oncogenes (which are likely to be up-regulated) can be identified. Also, the use of relatively small quantities of the sample\(^\text{13}\) makes it an ideal technique to study gene expression changes in tissue biopsy samples. However, one of the drawbacks of this procedure is a high rate of false positive candidate sequences identified. In our recent study on SiHa cell line, using DD, we identified kDec03 MRPS28 as being expressed in response to radiation\(^\text{19}\).

In the present study, we used DD to identify differentially expressed genes in tumour biopsy samples, obtained from patients who had responded or not responded to the radiotherapy. The tumour biopsies were obtained prior to and after 10 Gy radiation treatment. One of the genes \(\text{cdc27}\) was found differentially expressed and this was confirmed by immuno-histochemical analysis for the CDC27 protein. Down-regulation of the CDC27 protein was associated with poorer response to radiotherapy.

**Materials and Methods**

**Cell culture**

Two human cervical cancer cell lines SiHa and C33A were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 100 \(\text{U/ml}\) penicillin, and 100 \(\mu\text{g/ml}\) streptomycin, all obtained from Sigma Chemical Co., USA. The cultures were maintained at 37°C in air plus 5% carbon dioxide humidified incubator.

**Patients**

Fifteen patients, who were diagnosed to have cervical cancer, were included in the study, after an informed consent. Initial punch biopsy samples were obtained prior to and after completion of 10 Gray (Gy) dose of tele-radiation treatment. The patients were treated with external beam radiotherapy using 6 MeV linear accelerator (200 cGy/fraction/day/5days a week/for 5 weeks) and low dose rate brachytherapy (dose rate 180-190 cGy/hr). The total dose delivered to point A was 70-72 Gy. Brachytherapy was done on completion of 50 Gy dose of radiation. In two of the stage III B patients, who had completed 50 Gy treatment, the tumour regression was minimal and intra-cavitary radiotherapy could not be done, as the canal was not defined, hence the whole pelvic dose was taken up to 66 Gy. All the patients were followed up until unequivocal evidence of failure or death. All the failures were found to be due to partial remission, followed by progression of disease, which was usually observed within 3-6 months after completion of treatment.

The two tumour biopsy samples obtained from each patient at each time point, were processed as follows — one of the sample was formalin-fixed, paraffin-embedded and processed for histopathological and immuno-histochemical evaluation, while the other was snap frozen in liquid nitrogen, immediately after collection.

**RNA isolation and RT-PCR**

Total RNA was isolated from biopsy samples using Trizol\textsuperscript{TM} (Life Technologies, Inc., Rockville, MD), according to manufacturers protocol, and was treated with DNase (Amersham Biosciences, UK). RT-PCR for Abl gene c-DNA was performed to check for DNA contamination. Briefly, 0.2 \(\mu\text{g}\) of total RNA was reverse transcribed using M-MuLV reverse transcriptase (Amersham Biosciences, UK) in 20 \(\mu\text{M}\) reaction volume containing 200 \(\mu\text{M}\) dNTP mix, 1 \(\mu\text{g}\) random-hexamers (Amersham Biosciences, UK), 2 \(\mu\text{l}\) 5xRT buffer and 1 \(\mu\text{M}\) of M-MuLV. The 2 \(\mu\text{l}\) from the reaction was PCR amplified using Abl primers (forward primer-5'-GGCCGATGACATCGACACCGT-3'; and reverse primer-5'-ATGGTACCAAGGAGCTTTCTCC-3') in a 20 \(\mu\text{M}\) reaction volume containing 10 pmol each of the primers, 200 \(\mu\text{M}\) of dNTP mix, 0.2 \(\mu\text{M}\) of Taq (Amersham Biosciences, UK) and 2 \(\mu\text{l}\) 10xPCR buffer to check for genomic DNA contamination.

**Differential display (DD)**

DD was performed using total RNA isolated from pre- and post-treated (10 Gy) samples of a patient who responded and a patient who did not respond to the treatment using DD kits purchased from GenHunter Corp (Nashville, TN), according to manufacturer’s protocol. The anchor and arbitrary primers that led to the detection of CDC27 were 5'-AAGCTTTTTTTTTTT-3' and 5'-AAGCTTTTGCCTG-3'. Band isolation and re-amplification was performed as described\(^\text{13}\). The re-amplified fragment was cloned into a TA cloning vector (Promega Corp, US) and sequenced using M13-21 universal sequencing primer, in an ABI 310 genetic analyzer. The sequence was submitted to the GenBank database using the Blast-N search function for homology search.

5' Rapid amplification of cDNA end (5' RACE)

To further confirm the identity of the sequence, a 5'RACE was performed using SMART RACE kit (BD Clontech, Palo Alto, CA, US). The Oligo\textsuperscript{TM} software was used to design primers of the desired length. The DD was performed using total RNA isolated from pre- and post-treated (10 Gy) samples of a patient who responded and a patient who did not respond to the treatment using DD kits purchased from GenHunter Corp (Nashville, TN), according to manufacturer’s protocol. The anchor and arbitrary primers that led to the detection of CDC27 were 5'-AAGCTTTTTTTTTTTA-3' and 5'-AAGCTTTTGCCTG-3'. Band isolation and re-amplification was performed as described\(^\text{13}\). The re-amplified fragment was cloned into a TA cloning vector (Promega Corp, US) and sequenced using M13-21 universal sequencing primer, in an ABI 310 genetic analyzer. The sequence was submitted to the GenBank database using the Blast-N search function for homology search.
software (Medprobe, Norway) was used to design the downstream 3' primer based on the sequence of the DD fragment (3' primer- 5'GAG TCC CAA TAT (GCC CAT TAC 3'). The reaction was performed as per manufacturer's protocol. The 5' RACE reaction product was cloned into TA cloning vector (Promega Corp) and sequenced using universal sequencing primers. The sequence was compared with the GenBank database using the Blast-N search function.

**Immuo-histochemistry (IHC)**

IHC evaluation was used to study the expression pattern of CDC27 in pre-treated and post-treated (10 Gy) formalin-fixed, paraffin-embedded sections (15 cases) using avidin-biotin peroxidase complex method. Antigen retrieval was performed by autoclaving the sections in 10 mM citrate buffer (pH 6.0). The autoclaved sections were allowed to cool to room temperature for over 20 min and were incubated with anti-CDC27 mouse monoclonal antibody (clone AF3.1, Santa Cruz Biotechnology Inc., USA) at a dilution of 1:20 from 200 µg/ml stock. A cervix carcinoma tumour tissue section that was found to express CDC27 was used as the positive control, and for negative control the primary antibody was omitted. T Rajkumar (TR) and G Gopal (GG) scored the slides independently and where the scoring was not concordant, a joint review was done. Tumours were scored without the knowledge of the treatment outcome for the percentage of immuno-reactive tumour cells and the intensity of nuclear immuno-reactivity.

**Cell irradiation**

Cells were irradiated using the 6 MeV linear accelerator (Varian, Palo Alto, CA) with a source to bolus distance of 100 cm. A field size of 15 cm×25 cm was used for 175 cm² flask. The cells were seeded into the flask and irradiated using 6 MeV X-rays as described. A total dose of 10 Gy was delivered in five consecutive daily fractions of 2 Gy at a dose rate of 200 cGy min⁻¹. The interval between two doses was around 24 hr. The cells from the two cell lines were irradiated in duplicates, and independently.

**Fluorescent activated cell sorting (FACS)**

The irradiated cells were harvested by trypsinization along with the un-irradiated cells after approx. 18-20 hr after the final fraction. The nuclei from the cells were isolated in nucleus isolation buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM EDTA and 0.5% Triton X-100) after incubation for 15 min on ice with occasional gentle vortexing, pelleted at 1000 rpm, and re-suspended in ice-cold phosphate-buffered saline (PBS). The nuclei isolated from the un-irradiated and irradiated cells were washed once with 1% bovine serum albumin (BSA) in PBS and aliquoted into four tubes each. Two aliquots were incubated with 20 µl (200 µg/ml) of the anti CDC27 antibody (clone AF3.1, Santa Cruz Biotechnology Inc., USA) and the remaining two aliquots with equal volume of 1% BSA in PBS at 4°C for 40 min (negative control). After washing in 1% BSA-PBS, all the four aliquotes were treated with FITC-labeled anti-mouse antibody (DAKO) at a dilution of 1:20 and incubated for 30 min at 4°C. After washing in 1% BSA-PBS, FACS (Becton Dickinson FACSCalibur) scan was used to analyze the stained nuclei using the Becton Dickinson cell quest FACStation software. The M1 parameter was set for the negative control of each cell line at the specified dose (0 or 10 Gy) and the same was then applied to the corresponding antibody-treated sample. The experiment was repeated twice.

**Cell survival**

Cell survival after exposure to single doses was assessed by MTT assay as described. Briefly, 10⁶ cells seeded into 25 cm² flasks were exposed to single dose of 2, 4, 8, 10 Gy, respectively using 6 MeV X-rays delivered at 200 cGy min⁻¹. Experiments were conducted to assess the number of cells needed to be seeded into the wells of a 96 well plate culture plate so that it does not reach confluence after seven days of culture. For both SiHa and C33A, a seeding density of 1000 cells/well was found to be optimal and this was used in all further experiments. Irradiated and un-irradiated cells (1000 cells) were plated in a 96 well plate; a minimum of three replicates per dose were plated. The cells were incubated for 7 days and assayed using the cell titre MTT assay (Promega Corp, USA). Un-irradiated cells were used as controls to calculate the percent survival. The mean values for survival and the standard deviation were obtained from the raw data. The experiment was repeated twice.

**Statistical analysis**

Chi squared test with Mantel-Haenszel correction was used to assess the significance and p value.
Results

The clinical details of the fifteen patients included in the study and the scoring for CDC27 protein expression are given in Table 1. All the patients were treated with standard radical radiotherapy protocol and had provided tumour biopsy samples, prior to treatment and on completion of 10 Gy of radiation dose.

In the differential display, more than 30 fragments differentially expressed between the responder and the non-responder were identified (Fig. 1). The sequence of one of the differentially expressed band was found to be identical to the mRNA sequence of the human homologue of nuc2 (which is the CDC27 homologue in *Schizosaccharomyces pombe*), with a score = 452 bits (228), expect = e-124; identities = 231/232 (99%) in the Blast-N search. The sequence was further extended by RACE and was also found to be identical with the human homologue of nuc2 and hCDC27 (expect value = 0).

Since an antibody to CDC27 that would work on formalin-fixed, paraffin-embedded sections was available (clone AF3.1), immuno-histochemical evaluation was done on the paraffin-embedded sections from the pre- and the post-treated samples of the fifteen patients. The CDC27 expression was predominantly found in the nucleus and all the cases failed to show the positive staining in the cell nuclei, although the flow-cytometric analysis showed that the DNA content of the cells was not changed after treatment with either 10 Gy external beam radiotherapy or 2 Gy of ion beam radiation (10 Gy and 2 Gy). The cell lines and cell nuclei of the treated and untreated cells were also stained with DAB to show the expression of the protein CDC27 (Fig. 2). The expression of CDC27 was higher in the non-responders than in the responders.

Table 1—Tumour characteristics, disease status and CDC27 expression

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Stage</th>
<th>Grade</th>
<th>Disease status</th>
<th>CDC27 level in post-treated sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>III B</td>
<td>II</td>
<td>Progression - Failed</td>
<td>Down-regulated</td>
</tr>
<tr>
<td>2</td>
<td>III B</td>
<td>III</td>
<td>Disease-free</td>
<td>No change</td>
</tr>
<tr>
<td>3</td>
<td>III B</td>
<td>III</td>
<td>Progression - Failed</td>
<td>No change</td>
</tr>
<tr>
<td>4</td>
<td>II A</td>
<td>III</td>
<td>Progression - Failed</td>
<td>Down-regulated</td>
</tr>
<tr>
<td>5</td>
<td>III B</td>
<td>III</td>
<td>Progression - Failed</td>
<td>Down-regulated</td>
</tr>
<tr>
<td>6</td>
<td>III B</td>
<td>III</td>
<td>Progression - Failed</td>
<td>Down-regulated</td>
</tr>
<tr>
<td>7</td>
<td>III B</td>
<td>III</td>
<td>Progression - Failed</td>
<td>Down-regulated</td>
</tr>
<tr>
<td>8</td>
<td>III B</td>
<td>III</td>
<td>Progression - Failed</td>
<td>Down-regulated</td>
</tr>
<tr>
<td>9</td>
<td>III B</td>
<td>II</td>
<td>Disease-free</td>
<td>Up-regulated</td>
</tr>
<tr>
<td>10</td>
<td>III B</td>
<td>III</td>
<td>Progression - Failed</td>
<td>Down-regulated</td>
</tr>
<tr>
<td>11</td>
<td>III B</td>
<td>II</td>
<td>Disease-free</td>
<td>Up-regulated</td>
</tr>
<tr>
<td>12</td>
<td>III B</td>
<td>III</td>
<td>Progression - Failed</td>
<td>No change</td>
</tr>
<tr>
<td>13</td>
<td>III B</td>
<td>III</td>
<td>Disease-free</td>
<td>No change</td>
</tr>
<tr>
<td>14</td>
<td>III B</td>
<td>III</td>
<td>Progression - Failed</td>
<td>No change</td>
</tr>
<tr>
<td>15</td>
<td>III B</td>
<td>III</td>
<td>Disease-free</td>
<td>Up-regulated</td>
</tr>
</tbody>
</table>
showed nuclear immuno-reactivity for CDC27, although with varying percentages of tumour cell positivity and intensity. Most of the cases also demonstrated weak to moderate cytoplasmic reactivity. Of the fifteen patients' paired samples, seven showed down-regulation of CDC27 in the post-treated (10 Gy) tumour sections and all the seven patients failed treatment, five did not show alteration in the expression of CDC27 by way of percentage of tumour cell nuclei that were positive or the intensity of nuclear immuno-reactivity, and three showed up-regulation of CDC27 expression in the post-treated section. Among the eight cases, who showed no change or an up-regulation of CDC27 expression, five were disease-free and three had failed (p = 0.013). Fig. 2 shows the representative examples of CDC27 expression in pre- and post-irradiated samples from a responder (A & B) and a non-responder (C & D).

In order to corroborate the in vivo findings, cervical cancer cell lines SiHa and C33A which were shown to exhibit differential radio-sensitivity (C33A being more sensitive than SiHa), were studied for CDC27 expression. Our results also demonstrated a similar effect (Fig. 3). At 10 Gy dose, nearly 25% of the SiHa cells survived, compared to less than 5% of C33A.

Discussion

The standard prognostic indicators such as stage, grade, histologic sub-type will not be useful in our cervix cancer patients setting, wherein nearly 80%
The CDC27 protein (APC3), a 823-aa protein, is a member of the tetratrico peptide repeat (TPR) gene family24, the gene for which has been localized to long arm of chromosome 17q12-q2125. It is one of the eight subunits of the anaphase-promoting complex (APC)26 that is involved in separation of sister chromatids during the transition from metaphase to anaphase of mitosis, probably through ubiquitination of a centromere protein that regulates the sister chromatid separation process27. Thus, the APC plays an important role in mitosis. The other members of the APC family include BimE (APC1), CDC16 (APC6), CDC23 (APC8), APC2, APC4, APC5 and APC728.

The APC is involved in degradation of cyclin B during mitosis, which is a pre-requisite for the exit of the cell from mitosis28. Injection of anti-CDC27/Hs antibodies into logarithmically growing HeLa cells results in cell cycle arrest in metaphase with a normal spindle structure29. The APC requires Cdc20 for its activation in mitosis and a Cdc20-related protein CDH1 in G1 phase. The expression of Cdc20 is restricted to proliferating cells whereas, the APC and CDH1 are expressed in both proliferating and non-proliferating tissues30. The APC (with all its core subunits) is expressed in several differentiated cells including the terminally differentiated neurons and is tightly associated with CDH131. The APC-CDH1 complex was shown to have a high cyclin B ubiquitination activity in mitosis and as yet other roles in the G1 phase of cell cycle31.

The human homologue of fission yeast CDC27 has also been shown to be a functionally important subunit of DNA polymerase delta32,33 and in the interaction of DNA polymerase delta with the processivity factor proliferating cell nuclear antigen (PCNA), which is essential for effective replication of the genome. Thus, it appears that CDC27 protein may play a role in cell cycle regulation through several mechanisms.

Fas-induced activation of cdk's in Jurkat cells is shown to be due to caspase-3-mediated destruction of Wee1 (an inhibitory kinase of cdc2 and cdk2) and CDC27, resulting in a loss of APC function, leading to inhibition of cyclin degradation34. In spite of the increased cdk activity during Fas-induced apoptosis in Jurkat cells, mitosis did not occur. In endothelial cells, caspase-mediated destruction of p21CIP1 and p27KIP1 results in apoptosis35. However, in Jurkat cells it is not seen34, indicating that there may be different mechanisms.

In the current study using cell lines SiHa and C33A also corroborated the in vivo data, in as much as the less radio-sensitive cell line SiHa showing a marked down-regulation of CDC27, after exposure to 10 Gy radiation, compared to the radio-sensitive C33A (Figs 3 and 4).

References

1. Shanta V, Shanth 

Fig. 4—FACS analysis of expression of CDC27 protein by human cervical carcinoma cells [A & B]: Negative controls of C33A cell line in non-irradiated (A) and after 10 Gy radiation (B); (C & D): profiles of expression of CDC27 in C33A cell line stained with monoclonal antibody against CDC27 in non-irradiated (C) and after 10 Gy radiation (D); (E and F): negative controls of SiHa cell line in non-irradiated (E) and after 10 Gy radiation (F); (G and H): profiles of expression of CDC27 in SiHa cell line stained with monoclonal antibody against CDC27 in non-irradiated (G) and after 10 Gy radiation (H). A to H are representative profiles of one of the two measurements in one experiment]
mechanisms triggering apoptosis in different systems. In the cervical tumour cells, the down-regulation of CDC27 may not be associated with this pathway, as down-regulation could lead to apoptosis, which might result in better response to radiotherapy, which is contrary to our findings. Zhou and Rigaud used DD for identifying changes in gene expression to low dose radiation (4 Gy) in human lymphoblasts. The study showed that Cdc16 (another subunit of the APC) mRNA was down-regulated. A similar down-regulation was also observed after oxidative stress with H$_2$O$_2$.

One hypothesis we propose is that, following radiation-induced DNA damage, in tumour cells which down-regulate CDC27, there could be a cell cycle arrest/delay, which could allow repair of the damage, enabling them to survive. Conversely, tumour cells, which have not shown down-regulation of CDC27, may continue to cycle without repairing their damaged DNA, which could trigger apoptosis. The mechanism, by which the down-regulation of CDC27 occurs in the tumours is not known. The possibility of DNA methylation-mediated silencing of the gene cannot be ruled out.

In conclusion, we have shown that CDC27 when down-regulated in cervix cancers is associated with poorer response to radiation treatment. However, in view of the small number of patients studied, the value of CDC27 protein as a predictive marker for radio-response in cervix cancer needs to be further evaluated prospectively in a larger patient population.

Acknowledgement

This study was supported by a grant from the Dept. of Atomic Energy, Government of India. The ABI 310 genetic analyzer was a kind donation from the Chennai Willingdon Corporate Foundation, Chennai.

We would like to thank Dr. Helen Hurst, Cancer Research UK, Molecular Oncology Unit, ICRF at Hammersmith Hospital, London for her critical comments on the manuscript.

References