Overexpression of Cdc20 leads to impairment of the spindle assembly checkpoint and aneuploidization in oral cancer

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Defects in the spindle assembly checkpoint are thought to be responsible for an increased rate of aneuploidization during tumorigenesis. Despite a plethora of information on the correlation between BUB-MAD gene expression levels and defects in the spindle checkpoint, very little is known about alteration of another important spindle checkpoint protein, Cdc20, in human cancer and its role in tumor aneuploidy. We observed overexpression of CDC20 in several oral squamous cell carcinoma (OSCC) cell lines and primary head and neck tumors and provide evidence that such overexpression of CDC20 is associated with premature anaphase promotion, resulting in mitotic abnormalities in OSCC cell lines. We also reconstituted the chromosomal instability phenotype in a chromosomally stable OSCC cell line by overexpressing CDC20. Thus, abnormalities in the cellular level of Cdc20 may deregulate the timing of anaphase promoting complex (APC/C) in promoting premature anaphase, which often results in aneuploidy in the tumor cell.

Introduction

Genetic instability is thought to play a key role in the multistep process of tumorigenesis and has been broadly categorized into microsatellite instability (MIN) and chromosomal instability (CIN) (1,2). It has been observed that CIN often leads to a cellular phenotype with aberrant chromosome number (aneuploidy), which is a hallmark of cancer (3,4). A growing body of evidence suggests that defects in the spindle assembly checkpoint (SAC), a surveillance mechanism crucial for proper segregation of chromosomes during mitotic cell division, is responsible for an increased rate of aneuploidization during tumorigenesis (5–7). To maintain genome integrity, precise partitioning of chromosomes from one mother cell to two daughter cells occurs during mitosis. When an error is detected in the sister chromatid segregation process or any spindle damage occurs, a mitotic metaphase-to-anaphase checkpoint is activated, which pauses the cell cycle until all of the kinetochores are attached to both poles of the bipolar spindle and equal allocation of chromosomes is assured (8,9). Genetic studies in yeast and mammals have implicated at least seven genes, including BUB1, BUBR1 (BUB1B), BUB3, MAD1 (MAD1L1), MAD2 (MAD2L1), MAD3 and CDC20 in mitotic spindle checkpoint function (8,10). Proteins encoded by these genes function in ensuring orderly chromosome segregation during mitosis (11). Although the details of how these complexes work remain to be fully understood, it is generally agreed that the function(s) of one or more of these genes must be compromised for spindle checkpoint abrogation to occur (5,7).

Initial attempts to identify somatic mutations in the SAC genes leading to aberrant protein function in human malignancies were not very encouraging. Mutations in the BUB1, BUBR1 and BUB3 genes were identified in several cancers, albeit at a very low frequency (12–15). Similar attempts to identify mutations in the MAD2 gene also were unsuccessful (13). However, several reports have now documented differential expression of the BUB1, BUB3, BUBR1, MAD1 and MAD2 genes in various human tumors and cell lines (1,16–21). Reduced expression of MAD1, MAD2, BUB1 and BUBR1 has been found in different human cancers (18,20–23), and heterozygous MAD2, BUB3 or BUBR1 disruptions in mice result in partially deregulated checkpoint protein levels, an impaired spindle checkpoint and aneuploidy (24–26). In a recent study, stable partial downregulation of MAD1 led to functional inactivation of the spindle checkpoint and gross aneuploidy (22). In contrast, overexpression of MAD2, BUB1, BUBR1 and BUB3 in primary gastric tumors was observed, without any correlation with tumor aneuploidy (1,16,17).

Despite a plethora of information on the correlation between the BUB-MAD gene expression level and defects in the SAC, very little is known about alteration of another important SAC protein, Cdc20, in human cancer and its role in tumor aneuploidy. Upon attachment of all of the kinetochores to their respective spindle poles, Cdc20 activates the anaphase promoting complex (APC/C) to initiate anaphase (7,27). Cdc20 is usually kept sequestered during prophase and metaphase by Mad2 and BubR1/Bub3 in the form of a mitotic checkpoint complex (MCC) (28,29). This diffusible MCC serves as a wait-anaphase signal from the kinetochore and prevents premature anaphase progression. Thus, an abnormality in the cellular Cdc20 level or in its function may deregulate APC activation and promote premature anaphase, which often results in aneuploidy in both daughter cells (5,20,30). It has been reported that overexpression of CDC20 in Saccharomyces cerevisiae...
abolishes the mitotic arrest caused by microtubule-disrupting agents (31). It has also been suggested that besides MCC-mediated sequestration, the spindle checkpoint also reduces Cdc20 to below a certain threshold level to ensure complete inhibition of Cdc20 before anaphase (32). A recent meta-analysis of cancer microarray data identified CDC20 as one of the highly expressed genes in undifferentiated human cancer tissues (33). However, the importance of Cdc20 expression in human cancer is unknown.

Head and neck squamous cell carcinomas (HNSCC) and its subset, oral squamous cell carcinoma (OSCC), are characterized by complex, non-random genomic alterations (34,35). These include, but are not limited to, aneuploidy, translocations, insertions, deletions and amplifications (6,34,36,37). Several reports have documented mitotic malfunction in HNSCC primary tumors and oral cancer cell lines, including aberrant centrosome numbers (38), anaphase bridges (38) and multipolar mitotic divisions (38,39). It has been suggested that the ongoing instability in chromosome number and structure are consistent features of primary head and neck tumors and oral cancer cell lines owing to underlying genetic instability (6). Although mitotic abnormalities consistent with CIN due to defects in chromosomal segregation have been described, the underlying genetic defect responsible for such phenotypes in all HNSCC has not been elucidated. Recently, Quintyne et al. (39) showed that one cause of multipolar spindles in HNSCC and other tumor types is overexpression of the NUMA protein. In the present study, we report overexpression of CDC20 in most of the oral cancer cell lines and primary HNSCC tumors analyzed. For the first time, we demonstrate a potential link between CDC20 overexpression and aneuploidy in oral cancer. We also provide evidence that, similar to S.cerevisiae, overexpression of CDC20 in a chromosomally stable oral cancer cell line can impair SAC function.

Materials and methods

Cell culture, mitotic arrest and transfection

UPCI OSCC cell lines were developed in Dr Susanne M.Gollin’s lab (University of Pittsburgh, Graduate School of Public Health, Pittsburgh, USA). All other cell lines excepting MDA-MB 231, HuH7, Bu925 and HC116 were obtained from National Center for Cell Science, Pune, India. MDA-MB 231 and Bu925 cell lines were kind gifts from Dr Ashok K. Giri (Indian Institute of Chemical Biology, Kolkata, India). HC116 cell line was kindly provided by Dr Sudit Mukhopadhyay (Baylor College of Medicine, Texas, USA). HuH7 cell line was a gift from Dr Chiao Shih (University of Texas, Galveston, USA). HeLa cells were cultured in DMEM medium supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin-G, 100 μg/ml streptomycin, Invitrogen, Life Technologies, Carlsbad, CA, USA). Various OSCC cell lines (UPCL:SCC036, UPCL:SCC084, UPCL:SCC104, UPCL:SCC131 and UPCL:SCC136) were grown in MEM supplemented with 10% fetal calf serum, non-essential amino acids, 1% 1-glutamine and antibiotics (100 U/ml penicillin-G, 100 μg/ml streptomycin) at 37°C in a humidiﬁed incubator under a 5% CO2 atmosphere. For mitotic arrest, 300 ng/ml of nocodazole (Sigma, St Louis, MO, USA) was added to the cell cultures and incubated for various times as described in the text. Trypan blue staining was performed to check the viability of the nocodazole-treated cells. The cell viability was found to be ~80–90% for all cell lines. Purified GFP-Cdc20 expression plasmid DNA (2 μg) was transiently transfected into UPCL:SCC084 or HeLa cells using Lipofectamine (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol.

RNA extraction, cDNA preparation and real-time polymerase chain reaction (PCR) analysis

Freshly operated primary HNSCC tumors along with their corresponding non-neoplastic adjacent normal tissues were collected from the patients before they received any treatment from Chittaranjan National Cancer Institute, Kolkata and Thakurpukur Cancer and Welfare Home, Kolkata. All tumors were histopathologically diagnosed as squamous cell carcinoma (SCC). Histological analysis also revealed that the adjacent normal tissues were unaffected by tumor cells owing to field cancerization. Before sample collection, the written consent was taken from the patients. The institutional review committee on research using human subject of Indian Institute of Chemical Biology, Kolkata, cleared the project after due deliberations. Total cellular RNA was isolated from the various cancer cell lines, primary HNSCC tumor and adjacent normal tissue of the corresponding patients as control using TRIZOL reagent (Invitrogen, Life Technologies) according to manufacturer’s protocol. One microgram of DNaseI-treated RNA was reverse-transcribed with random hexamer primer using an Ambion Reverse Transcript Kit (Ambion, USA) in a total volume of 20 μl and stored at −20°C until use. Four microliters of each cDNA was subjected to real-time PCR analysis using the SYBR Green Q-PCR Kit (Finnzymes, USA) in the iCycler (Q Thermal Cycler, Bio-Rad, Herfordshire, UK), and the following cDNA primers were used: MAD2-S: GGA AGC GCG TGC TTT TGT TT; MAD2-A5: TCT TTC AGT TGC TTT ACC ACA; CDC20-S: CGC CAA CGC ATC CCA CAG; CDC20-A5: CAG GTT CAA AGC CGA GGC. Melting curve analysis was performed to verify the purity of the amplified products. Gene expression levels were normalized to β-actin by calculating the ΔCT [ΔCT = CT (test gene) – CT (β-actin)]. Differences in expression between tumor and normal tissues were calculated as (ΔMCCT) = ΔACT (tumor tissue) − ΔACT (normal tissue)). Similarly, for cancer cell lines, Δ(ΔCt) was calculated using the average ΔCt value of two different normal tissues as controls (gastric and oral). The fold difference in expression was calculated using the formula 2−ΔΔCt. Fold difference < 0.5 was considered as under-expression, whereas >1.5-fold increase was considered to be overexpression (18,40–42). For each cell line and tumor normal tissue ΔCT was determined from three independent experiments.

Determination of mitotic index (MI)

All the cell lines were treated with nocodazole for various time periods (as mentioned in respective cases) and one set was left untreated. This was followed by treatment with colchicine for 2 h before harvesting. Metaphase spreads were prepared from the colchicine-treated cells according to the standard protocol followed by Giemsa staining. Frequencies of mitotic cells were counted among 200–300 cells each time and three times for each cell line to calculate MI.

Cell synchronization and cell cycle analyses

Cells were synchronized by starvation for 48 h, using their respective culture media without serum or antibiotics, followed by addition of complete media with (300 ng/ml) or without nocodazole. At the respective time points, ~106 cells were harvested, washed twice and resuspended in 0.5 ml of cold phosphate-buffered saline (PBS). Cells were fixed by adding 1 ml of cold 100% ethanol dropwise into the sample while vortexing gently, and then incubated at 4°C for at least 24 h until fluorescence-activated cell sorter (FACS) analysis. After fixation, cells were pelleted, washed once with PBS and resuspended in 1 ml PBS containing 20 mg/ml propidium iodide (Sigma) and 200 μg/ml RNase (Sigma). Cells were incubated at room temperature for 30 min and then analyzed by FACS (Flow Cytometer-BDLSR, Becton Dickinson, USA). Dead cells and debris were excluded from the analysis using forward and side scatter plots [43], http://www.proimmune.com/FC.htm.

Immunoblotting and immunofluorescence

For immunoblot analysis, cells were lysed with SDS sample buffer, and total protein was resolved in 10% SDS–PAGE and transferred to PVDF membrane (Millipore). Mad2, Cdc20, Cyclin B1 and α-tubulin proteins were detected with their protein-specific antibodies (anti-Mad2 and anti-Cdc20 antibodies from Santa Cruz Biotechnology, USA, and anti-α-tubulin antibody from Sigma, and Cyclin B1 from ID Labs Ontario, USA) and visualized by chemiluminescence (Amersham, USA) after treatment with HRP-conjugated secondary antibody (Santa Cruz Biotechnology). For the normal control in the western blot analysis, oral epithelial cells were collected from an oral brushing from a normal individual and treated similarly to the cultured cells. Densitometry scan of the western blot data was done and all the values were normalized by dividing with corresponding α-tubulin expression levels. Fold change was calculated by dividing each ratio with that of the normal control. To confirm GFP-Cdc20 expression in UPCL:SCC084 and HeLa by immunofluorescence, transfected cells were observed after 48 h under a fluorescence microscope (Olympus BX-40, USA).

Analysis of multinucleated cells

For assessment of the presence of multiple nuclei within one cell, cells were treated with nocodazole (300 ng/ml) for the respective time periods. Multinucleated cells were then fixed with MeOH and stained with the
α-tubulin (Sigma) specific antibody followed by detection with FITC conjugated secondary antibody (Sigma). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 mg/ml). Stained cells were visualized under the fluorescence microscope, and at least 150–200 cells were counted from each slide to determine the percent of multinucleated cells. Cells with more than two nuclei were defined as multinucleated cells. This experiment was repeated four times, and the average of these four individual values has been recorded.

Chromosome enumeration and fluorescence in situ hybridization (FISH)
Metaphase spreads were prepared by treating cells with colchicine for 3 h before harvesting, followed by Giemsa staining using a standard protocol, and observed under light microscope. For chromosome counts, 20–30 such spreads were counted from each slide and the mean value and modal chromosome number (M) were calculated. Average modal deviation (AMD) (F) was calculated by taking the average of the differences between each individual value and modal number (M). For the chromosome condensation assay, 25–40 metaphase spreads were counted from each slide under the microscope and frequency was calculated.

Interphase FISH was carried out with centromeric probes for chromosomes 1, 2, 3, 6 and 9 individually. SpectrumOrange™-labeled centromeric probes, CEP1 and CEP2, were purchased from Vysis (Downers Grove, IL, USA). Centromeric probes for chromosomes 3, 6 and 9 were prepared by nick translation of centromeric clones pAE06-68, pEDZ6, pEG9, respectively (a generous gift from Dr. Mariano Rocchi, Dip. di Genetica e Microbiologia, Bari, Italy), using SpectrumOrange™-labeled dUTP and a nick translation kit from Vysis according to the manufacturer’s protocol. Slides containing fixed interphase nuclei were denatured in 70% formamide in 2x SSC buffer at 75°C for 5 min. Subsequently, probe DNA was denatured at 75°C for 5 min and applied to the slides, followed by incubation at 37°C overnight. After hybridization, samples were washed in 2x SSC (pH 7.2)/0.1% NP-40 (Sigma) at 73°C for 2 min and 0.4x SSC (pH 7.2)/0.3% NP-40 at room temperature for 1 min. Nuclei were then counterstained with DAPI (Sigma) with antifade compound. FISH signals were counted using a fluorescence microscope (Olympus BX-40) equipped with a triple band-pass filter. At least 150–200 nuclei were counted from each sample to determine the percent aneuploidy. Nuclei with more than two fluorescent signals for each chromosome were defined as aneuploid.

Cyclin B1 degradation assay
Four different cell lines (HeLa, HCT116, UPCI:SCC084 and UPCI:SCC131) were synchronized by standard double thymidine block and released in G1/S in the presence and absence of nocodazole. Cells were harvested at different timepoints during mitosis in lysis buffer followed by western blot analysis with anti Cyclin B1 antibody (BD Labs) and anti-α-tubulin antibody (Sigma). The synchronized initiation of mitosis was assigned as 0 h and other timepoints were calculated accordingly. Cyclin B1 levels were normalized with α-tubulin and plotted with respect to time to obtain the Cyclin B1 degradation pattern. Similarly, HCT116 and UPCI:SCC084 were transfected with a Cdc20 expression vector followed by a similar analysis as above.

Statistical analysis
Mean fold expression values of clusters of cell lines obtained from real-time PCR analysis were compared using ANOVA. A Student's t-test was done to analyze the statistical significance of aneuploidy and multinucleation. To test the statistical significance of a chromosome condensation defect, a P-value was calculated by Fisher’s exact test as mentioned. All these calculations were done using freely available software from the URL, http://www.graphpad.com/quickcalc/.

Results
Expression of Cdc20 varies significantly in OSCC cell lines and primary HNSCC tumors
We examined the level of Cdc20 mRNA in 11 human tumor cell lines of various origins and 10 oral cancer cell lines. Additionally, 10 pairs of matched primary HNSCC tumors and adjacent normal tissues were also analyzed for Cdc20 expression. Table I shows the fold overexpression or underexpression values for the Cdc20 mRNA in different tumor cell lines and primary tumor tissues. ANOVA of the fold value data showed that the cell lines and tumor tissues showing Cdc20 overexpression are statistically significant (P < 0.00001). It is evident that the majority of the tumor cell lines and primary tissues exhibited overexpression of Cdc20 mRNA. Overexpression of the Cdc20 mRNA was found to be present in 70% of the OSCC cell lines and 80% of other tumor cell lines (Table I and Figure 1A). Thus, Cdc20 overexpression was widespread in both OSCC and other tumor cell lines. Analysis of the level of Cdc20 mRNA in primary HNSCC tumors was consistent with the cell line results. Seventy percent of the primary tumors exhibited overexpression of the Cdc20 mRNA and only 10% showed decreased expression (Table I and Figure 1A). To calculate the fold overexpression/underexpression in solid tumors, matched normal adjacent tissues were considered as normal controls. However, in the case of tumor cell lines, the fold overexpression/underexpression was calculated against the average ΔCT of one oral and another gastric tissue as normal controls. Both of these normal tissues had similar ΔCT values. Since the same normal ΔCT value was subtracted from all the individual ΔCT values of the cell lines to calculate their relative fold differences, we propose that it does not affect the comparative representation of fold values. We have also confirmed the differential expression pattern of the Cdc20 gene by analyzing the corresponding Cdc20 protein levels in five OSCC cell lines (UPCI:SCC036, UPCI:SCC084, UPCI:SCC104, UPCI:SCC131, and UPCI:SCC136), HCT116, HeLa and normal oral epithelial tissue (Figure 1B). Each bar of the histogram shows the corresponding fold values of protein expression with respect to that of normal, and the trend is quite consistent with the relative mRNA levels (Figure 1B). These studies provide independent evidence for overexpression of Cdc20 gene in primary HNSCC tumor, OSCC cell lines and other tumor cell lines. We also noted that another mitotic specific gene Cyclin B1 did not show any significant difference in steady state protein levels in different tumor cell lines (Figure 1C). This result suggests that the observed difference in Cdc20 protein levels in these cell lines was not due to the difference in number of mitotic cells present in the asynchronous culture of these cell lines.

Cdc20-overexpressing cell lines exhibit defective SAC function
Overexpression of Cdc20 in both primary HNSCC tissues and OSCC cell lines prompted us to find any possible link with

| Table I. Fold expression of Cdc20 mRNA as measured by real-time PCR |
|--------------------------|------------------|------------------|---------------|--------------|
| OSCC cell lines          | Fold values*     | Other tumor cell lines | Fold values*  | Primary HNSCC tumors | Fold values* |
| UPCI:SCC003              | 22.6             | HeLa              | 32.0          | #3111         | 0.23         |
| UPCI:SCC036              | 0.0              | Hep2              | 32.0          | #6545         | 0.35         |
| UPCI:SCC066              | 7.5              | C33A              | 2.5           | #6904         | 1.74         |
| UPCI:SCC084              | 1.3              | MDA-MB 231        | 0.4           | #4388         | 2.64         |
| UPCI:SCC103              | 7.5              | Siha              | 239           | #3900         | 19.7         |
| UPCI:SCC104              | 32.0             | Huh7              | 45.3          | #2281         | 59.7         |
| UPCI:SCC105              | 9.8              | ME180             | 0.4           | #2748         | 1.74         |
| UPCI:SCC131              | 14.9             | MCF7              | 3.2           | #3440         | 27.9         |
| UPCI:SCC136              | 1.2              | Bu925             | 2.8           | #3592         | 1.62         |
| KB                       | 4.3              | U397              | 111           | #6939         | 24.2         |

*To calculate the fold values of the cell lines the ΔCT value of normal tissue (average of one oral and another gastric) was considered as reference. In case of solid tumors, ΔCT values of matched normal adjacent tissues were considered as references. Expression levels were categorized as overexpression (>1.5-fold), underexpression (<0.5-fold) and normal (0.5- to 1.5-fold).
SAC function of these cell lines. To check the efficiency of SAC function the MI of different OSCC cell lines was determined following treatment with nocodazole (a spindle depolymerizing drug used to activate SAC function) for different time periods (Figure 2A). In the case of the Cdc20-overexpressing cell lines (UPCI:SCC104, UPCI:SCC131 and HeLa), mitotic indices increased initially, but came down with increasing time of nocodazole exposure. On the other hand, the Cdc20-low cell lines (UPCI:SCC036, UPCI:SCC084, UPCI:SCC136 and HCT116) showed higher MI compared with Cdc20-high group, which increased gradually with the increase in time of nocodazole treatment and sustained a maximum value at least up to 36 h. This result suggests that the Cdc20-overexpressing cell lines may have an inherent defect in their SAC; these cells cannot arrest efficiently after nocodazole treatment.

To gain better insight into the SAC defect in the high CDC20-expressing cell lines, we carried out cell cycle analyses of synchronized oral UPCI:SCC cell lines with and without nocodazole treatment. Figure 2B shows the cell cycle distribution of all six cell lines treated with nocodazole for 24 h, cells were arrested in the mitotic phase, as indicated by a major 4C DNA content peak for each cell line (Figure 2B, panel +Noc, 24 h). Different cell lines exhibited rather similar behavior up to this point, but prolonged exposure to nocodazole revealed a distinct difference in cell cycle distribution of the high CDC20-expressing cells (Figure 2B, panel +Noc, 48 h). Although most cell lines showed an 8C DNA content peak after 48 h of nocodazole treatment, indicating the formation of multinucleated cells by checkpoint adaptation (44), strikingly, the high Cdc20-expressing cell lines (UPCI:SCC104, UPCI:SCC131 and HeLa) showed an additional 2C peak, which is an indication of an escape from the mitotic arrest and resumption of the cell cycle, even under conditions of spindle depolymerization as a result of the nocodazole treatment. Thus, this result further emphasizes the defective SAC function in the Cdc20-overexpressing cell lines.

Overexpression of Cdc20 is correlated with abnormal chromosome number and multinucleation in OSCC cell lines

It is known that defective SAC function leads to abnormal cellular phenotypes like aneuploidy, polyploidy and multinucleation (5,22). Thus, we examined these phenotypes in Cdc20-overexpressing OSCC cell lines. To examine the extent of irregularity in chromosome number, interphase FISH was carried out on OSCC cell lines expressing low (UPCI:SCC036, UPCI:SCC084, UPCI:SCC136) or high levels of CDC20 (UPCI:SCC104 and UPCI:SCC131) using centromeric probes for chromosomes 1, 2, 3, 6 and 9. Cells with more than two centromeres as revealed by any one of the centromeric probes were considered as aneuploid (Figure 3A). Cells showing more aneuploidy by CEP probes also exhibited higher modal number of chromosomes by chromosome enumeration, reflecting an increase in total chromosome number in the cell (see below). Table II shows the percentage of aneuploid cells determined by each probe for the five different OSCC cell lines and the HeLa cell line. It is evident from Table II that under stressed (+Noc) as well as normal growth conditions (−Noc), the average percent aneuploidy of all chromosomes was higher for the high CDC20 OSCC cell lines than for the low CDC20 cell lines. Next, we pooled the values of average percent aneuploidy for each group of OSCC cell lines (low CDC20 group: UPCI:SCC036, UPCI:SCC084, UPCI:SCC136 and high CDC20 group: UPCI:SCC104, UPCI:SCC131) and calculated the group average. The group average of percent aneuploidy...
for the CDC20-high group of OSCC cell lines was found to be significantly higher than the CDC20-low group of OSCC cell lines under stressed condition (+Noc, $P = 0.0015$, Table II). Similarly, the higher aneuploidization of the high CDC20-expressing cell lines was also found to be statistically significant when cells were grown in the absence of nocodazole ($P = 0.0209$, Table II). A similar comparison revealed that the increase in aneuploidization upon nocodazole treatment was not significant ($P = 0.2671$) for the CDC20-low group [25.6 (2.9) versus 28.4 (2.4), Table II], whereas the CDC20-high group showed a significant increase ($P = 0.0063$) in aneuploidization [35.3 (0.42) versus 50.8 (1.7), Table II] after nocodazole treatment. It is interesting to note that the HeLa cells, which have a high endogenous CDC20 level, also showed a very high level of aneuploidization.

Inherent fidelity of SAC function for each of the cell lines was reflected through missegregation of chromosomal DNA
due to abnormal spindle formation (Figure 3B), which ultimately led to multinucleation, by escaping the SAC (Figure 3C). We analyzed the frequency of multiple nuclei in these cells in the presence or absence of nocodazole. As shown in Figure 3D, each of the low CDC20-expressing cell lines exhibit a lower frequency of multinucleated cells than the high CDC20-expressing cell lines (compare UPCI:SCC036, UPCI:SCC084 and UPCI:SCC136 with UPCI:SCC104 and UPCI:SCC131) in both the nocodazole-treated and nocodazole-untreated conditions. As expected, HeLa cells, which had a high endogenous level of Cdc20, also exhibited a high frequency of multinuclear cells in the presence of nocodazole (Figure 3D). We observed a significant increase in frequency of multinuclear cells upon nocodazole treatment in both the Cdc20-high and Cdc20-low groups of cells (Figure 3E). However, comparison between the two groups revealed an insignificant increase in multinucleation under normal growth conditions (−Noc), which became significant after nocodazole treatment (Figure 3F). These results further support the presence of defective SAC function and inefficient cell cycle arrest in the high CDC20-expressing cell lines. Thus, enhanced Cdc20 level in these cells can lead to aberrant chromosome numbers and polyplidization through multinucleation.
Table II. Percent aneuploid cells determined by FISH analysis

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>(%) Aneuploid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean Gr Mean&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH probes</td>
<td>CEP1</td>
<td>CEP2</td>
</tr>
<tr>
<td>−Noc&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPCI:SCC036</td>
<td>51.6</td>
<td>27.7</td>
</tr>
<tr>
<td>UPCI:SCC084</td>
<td>39.0</td>
<td>21.0</td>
</tr>
<tr>
<td>UPCI:SCC136</td>
<td>49.6</td>
<td>28.3</td>
</tr>
<tr>
<td>UPCI:SCC104</td>
<td>73.2</td>
<td>28.9</td>
</tr>
<tr>
<td>UPCI:SCC131</td>
<td>79.7</td>
<td>26.2</td>
</tr>
<tr>
<td>HeLa</td>
<td>81.5</td>
<td>47.6</td>
</tr>
<tr>
<td>+Noc&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPCI:SCC036</td>
<td>59.2</td>
<td>31.1</td>
</tr>
<tr>
<td>UPCI:SCC084</td>
<td>43.8</td>
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<tr>
<td>UPCI:SCC136</td>
<td>54.9</td>
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</tr>
<tr>
<td>UPCI:SCC131</td>
<td>96.3</td>
<td>53.0</td>
</tr>
<tr>
<td>HeLa</td>
<td>93.3</td>
<td>63.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent of aneuploid cells were determined by counting number of nuclei containing more than two centromeres as revealed by any given centromeric probe among 150–200 nuclei counted. Depending on Cdc20 levels, UPCI OSCC cell lines were categorized into two groups as Cdc20-low (UPCI:SCC036, UPCI:SCC084, UPCI:SCC136) and Cdc20-high (UPCI:SCC104, UPCI:SCC131).

<sup>b</sup>Noc: nocodazole.

<sup>c</sup>Gr Mean: average of the mean percent aneuploidy for each group of cell lines.

Table III. Chromosome enumeration of metaphase spreads

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>–Noc&lt;sup&gt;c&lt;/sup&gt;</th>
<th>+Noc (24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (M)</td>
<td>AMD (F/SD)</td>
</tr>
<tr>
<td>UPCI:SCC084 − (Cdc20)</td>
<td>49.7</td>
<td>0.7(3.7)</td>
</tr>
<tr>
<td>UPCI:SCC084 + (Cdc20)</td>
<td>52.7</td>
<td>2.3(10.6)</td>
</tr>
<tr>
<td>UPCI:SCC131 − (Cdc20)</td>
<td>59.7</td>
<td>1.7(6.7)</td>
</tr>
<tr>
<td>HeLa − (Cdc20)</td>
<td>72.7</td>
<td>1.8(7.2)</td>
</tr>
</tbody>
</table>

<sup>c</sup>AMD' and 'SD,' average modal deviation and standard deviation, respectively.

*x,' samples 'not done.'

Ectopic expression of Cdc20 impairs SAC function

The effect of overexpression of Cdc20 on SAC function was confirmed by ectopically expressing GFP-Cdc20 in chromosomally stable cell line UPCI:SCC084, which has a low level of endogenous Cdc20. Chromosome enumeration of UPCI:SCC084 revealed that it was near-diploid (total chromosome count: mean, 49.7; mode, 49) under normal growth conditions (Figure 5A and Table III, −Noc column) and that it was chromosomally stable is also evident from the low value of AMD \( (F) = 0.7 \). On the other hand, UPCI:SCC131 and HeLa, which expressed high levels of Cdc20, had near-triploid chromosome numbers, as revealed by mean and modal chromosome numbers (mean, 59.7; mode, 58 and mean, 72.7; mode, 76, respectively) and higher AMD values (1.7 and 1.8, respectively; Table III). Overexpression of GFP-Cdc20 in transiently transfected UPCI:SCC084 cells was confirmed by western blot analysis (Figure 4A).

Similar to the Cdc20-overexpressing cell lines, ectopic expression of Cdc20 in UPCI:SCC084 also resulted in a noticeably low value of MI in comparison with untransfected cells (Figure 2A, Table III). The impairment of SAC function in Cdc20-overexpressing UPCI:SCC084 cells was further examined by cell cycle analysis. Both mock-transfected UPCI:SCC084 and HeLa cell lines showed a normal distribution of cells with a major DNA peak at 2C under nocodazole-untreated conditions and a 4C peak after nocodazole treatment (Figure 4B). However, Cdc20-transfected cells showed an interesting phenomenon. The nocodazole-treated Cdc20-overexpressing UPCI:SCC084 cells showed an additional 2C peak along with the expected 4C peak, indicating partial impairment of checkpoint function (Figure 4B, +Cdc20 panel). The effect was more pronounced in HeLa cells, suggesting a severe SAC defect in the majority of the cells due to Cdc20 overexpression (Figure 4B).

Cells without CIN acquire the CIN phenotype upon Cdc20 overexpression

Ectopic expression of Cdc20 in UPCI:SCC084 resulted in cells with various types of mitotic defects. Frequency of cells containing prematurely separated sister chromatids, decondensed chromosomes (Figure 5B), multinuclei and extra copies of chromosomes (Figure 5C) increased significantly after ectopic expression of Cdc20. Defects in chromosome condensation were found to be significantly enhanced owing to Cdc20 overexpression \( (P = 0.0054, \text{Table III}) \) in the normally growing UPCI:SCC084 cell line, which was further enhanced upon spindle depolymerization \( (P = 0.0001, \text{Table III}) \). This effect was ultimately reflected through increases in both chromosome number and AMD values (Table III).

Impairment of SAC function in Cdc20-overexpressing UPCI:SCC084 cells was also examined by determining the frequency of multinucleated cells (Figure 5D). As observed earlier, UPCI:SCC084 cells having a low endogenous level of Cdc20 showed infrequent multinucleation, which increased substantially upon overexpression of Cdc20 \( (P = 0.001, \text{Figure 5D}) \). HeLa cells, which have a high endogenous level of Cdc20 and a higher propensity toward multinucleation, also exhibited a further increase in multinucleated cells upon ectopic expression of Cdc20 \( (P = 0.0043, \text{Figure 5D}) \). The effect of Cdc20 overexpression on multinucleation was again apparent when cells were forced to divide with a
depolymerized spindle resulting from nocodazole treatment for 24 h (Figure 5D). Thus, analysis of all types of SAC-regulated parameters supports our interpretation that ectopic expression of Cdc20 can lead to impairment of SAC function.

The SAC defect induced by Cdc20 overexpression is also reflected in Cyclin B1 degradation

The proteolysis of Cyclin B1 has been correlated with the onset of anaphase upon inactivation of the SAC (45). This proteolysis of Cyclin B1 is delayed by re-imposition of the SAC in the checkpoint proficient cells (45). We hypothesize that the observed SAC defect in Cdc20-overexpressing cells leads to premature anaphase promotion. To test the hypothesis, we measured the Cyclin B1 level in OSCC cell lines UPCI:SCC084 (high Cdc20) and UPCI:SCC084 (low Cdc20) during the metaphase to anaphase transition (one from each group). As a control, we also measured the Cyclin B1 level of HCT116 (low Cdc20) and HeLa (high Cdc20) cells. Under normal growth condition (−Noc) all four cell lines showed rapid degradation of Cyclin B1 at the onset of anaphase (Figure 6A, B, C and D). Upon imposition of SAC in these cells by nocodazole treatment, Cyclin B1

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Fig. 4. Induction of SAC defects by ectopic expression of Cdc20. (A) Western blot analysis of GFP-Cdc20-transfected (+lane) and mock-transfected (−lane) UPCI:SCC084 and HeLa cells. The endogenous α-tubulin level in both cell type is also shown. The histogram shows fold expression of both endogenous and ectopically expressed Cdc20 proteins. (B) FACS diagram of mock- or Cdc20-transfected UPCI:SCC084 and HeLa cells. M1 shows apoptotic cells and M2 shows normal G0/G1 cells (indicated as 2C), whereas M3 shows cells arrested at mitosis and/or polyploid cells.
degradation was found to be delayed only in the case of UPCI:SCC084 and HCT116 (Figure 6A and B), indicating efficient checkpoint function. On the other hand, the two Cdc20-overexpressing cell lines, UPCI:SCC131 and HeLa, failed to delay the degradation of Cyclin B1 even after nocodazole treatment (Figure 6C and D), suggesting inefficient SAC function in these cells.

The Cyclin B1 degradation was further monitored in chromosomally stable cell lines, UPCI:SCC084 and HCT116, upon ectopic expression of Cdc20. The Cyclin B1 degradation pattern was similar in both cell lines irrespective of their Cdc20 level under normal growth condition (–Noc) (compare Figure 6A with E and Figure 6B with F at –Noc condition), but interestingly, in both the cell lines, Cyclin B1 was degraded rapidly, even in presence of nocodazole upon ectopic expression of Cdc20 (Figure 6E and F). This early degradation of Cyclin B1 in nocodazole-treated cells indicates premature anaphase promotion caused by ectopic Cdc20 overexpression.

Discussion

The present report provides evidence for the overexpression of CDC20 in OSCC cell lines and primary HNSCC tumors. Several human tumor cell lines of various tissue origins also exhibited overexpression of CDC20. Thus, CDC20 overexpression might be a generalized phenomenon in human tumorigenesis. This study also confirms the cellular phenotype expected as a result of overexpression of Cdc20 in mammalian cells. We show that Cdc20-overexpressing OSCC cell lines exhibit different mitotic abnormalities, such as incomplete chromosome condensation, premature anaphase promotion, missegregation of chromosomes, multinucleation and aneuploidy. Further, we can induce mitotic abnormalities by ectopically overexpressing Cdc20 in a chromosomally stable OSCC cell line. It is known that Cdc20 overexpression results in premature transition from G1 to S phase in myeloid cells (46). Further, we showed that improper entry of Cdc20-overexpressing cells to the next cycle was by overriding not only the G1/S arrest (46) but also the SAC arrest, especially in the context of nocodazole-induced spindle depolymerization.

In this study, we analyzed different parameters, some of which are directly (MI, missegregation of chromosomes and premature anaphase promotion) and others indirectly (chromosome condensation defect, multinucleus formation and aneuploidy) related to SAC efficiency. MI, a measure of the fractions of cells in mitosis in a dividing cell population, was found to be noticeably low in Cdc20-high cell lines than the Cdc20-low or normal cell lines. FISH analysis of different OSCC cell lines showed a significantly higher level of aneuploidy in the Cdc20-high group than the Cdc20-low group, both in the presence and absence of nocodazole. The extent of aneuploidization due to nocodazole treatment was only found to be significant in the case of the Cdc20-high group. Thus, OSCC cell lines having high Cdc20 levels were inherently defective in SAC function, which became more apparent after spindle depolymerization by nocodazole. A similar conclusion could also be drawn from the multinucleation property, which is related to abnormal cytokinesis.
The Cdc20-overexpressing cells appeared to fail to restrict multinucleation as a result of their inherent SAC defect. Thus, premature sister chromatid separation due to Cdc20 overexpression not only leads to aneuploidy in the daughter cells but also cytokinesis failure leading to multinucleation (44). The inherent SAC defect of the Cdc20-overexpressing cells was also observed by cell cycle analysis after prolonged nocodazole treatment of the OSCC cell lines. The analysis showed a noticeable amount of premature anaphase promotion only in the Cdc20-high cells. Finally, the fidelity of the SAC was tested biochemically by Cyclin B1 proteolysis in both types of cell lines, which is a direct evidence of anaphase promotion in a mitotic cell. Unlike the Cdc20-low group, the Cdc20-high cell lines failed to sustain the Cyclin B1 level after nocodazole treatment, revealing an inefficient SAC. Thus, all of these results suggest frequent impairment of SAC function in the CDC20-overexpressing cell lines.

We next attempted to mimic the defective SAC phenotype seen in Cdc20-overexpressing cells by a reconstitution assay. Toward that end, we considered UPCI:SCC084 as a near-diploid and chromosomally stable cell line, because it showed not only a low value of AMD but also minimal deviation from proficient checkpoint function under normal growing conditions (−Noc, −Cdc20). Upon Cdc20 overexpression in this cell line, we observed all of the phenotypes associated with SAC impairment. The MI was found to be low and also to fall sharply owing to ectopic expression of Cdc20 in the UPCI:SCC084 cell line. Similarly, Cyclin B1 degradation assay of ectopically Cdc20-expressing cell lines resulted in early degradation of the protein level, indicating lack of SAC response. Thus, all the parameters measured indicate marked impairment of SAC function in Cdc20-overexpressing cells.

It is well known that APC/C activation by Cdc20 is essential for metaphase to anaphase transition; it initiates ubiquitination of securin in order to set separase free. Separase proteolytically degrades cohesin molecules, leading to the onset of anaphase. However, how Cdc20 activates the APC/C and how inhibitors like Mad2 inhibit the Cdc20-APC/C interaction upon spindle depolymerization are not clearly understood. Two possible models were proposed to explain the mechanism by which Cdc20 regulates APC: steric
hinderance and the kiss and run mechanism (47). The first model states that phosphorylation of multiple sites on APC by Cdk allows Cdc20 to associate and create binding sites for substrates like securin, which is to be ubiquitinated. The second model suggests that Cdc20 delivers and removes the catalytic enzymes, which modify APC for ubiquitination of its substrate (47). Inhibitors like Mad2 block Cdc20 from interacting with its substrate or activator in order to inhibit the APC activation procedure. So there must be a stoichiometric balance between Cdc20 and its inhibitors in order to initiate proper SAC arrest under physiological conditions. Thus, it is conceivable that overexpression of Cdc20 can tip the balance toward improper activation of APC/C, overriding the inhibitory effects of the Mad2 and Bub3/BubR1 molecules (48).

Mad2 underexpression or loss was found to be associated with similar SAC defects (20,21,49). We speculate that overexpression of Cdc20 would have a more deleterious effect on SAC function. It is known that not only Mad2 but other factors like Bub3/BubR1 also act as more potent inhibitors of Cdc20 function during SAC arrest. Thus, underexpression of Mad2 will have a partial effect on SAC deregulation. Secondly, during SAC arrest, cellular Cdc20 levels are decreased via degradation by the APC\(^{Cdc11}\) complex (32,49). So, overexpression of Cdc20 at the metaphase to anaphase transition forces premature anaphase promotion, leading to aberrant phenotypes, such as aneuploidy.

It has been proposed that for a gene to be called a CIN gene, it must satisfy two criteria. These include (i) unambiguous evidence for an alteration in an encoded protein’s expression or function in cancers compared with normal cells; and (ii) that reconstitution of that alteration confers CIN on a diploid or near-diploid cell in tissue culture (5). In the present report, we provide evidence that satisfies both criteria for the \(CDCC2\) gene. On the one hand, overexpression of \(CDCC2\) has been observed in many human cancer cell lines including most of the OSCC cell lines as well as HNSCC primary tumors examined. On the other hand, transient ectopic expression of Cdc20 conferred a CIN phenotype in an otherwise chromosomally stable OSCC cell line. Thus, our results support the prediction that the mitotic spindle checkpoint is one of the important points of failure in CIN and altered expression of genes involved in this pathway would be expected to contribute to a human cancer.

Supplementary material
Supplementary material is available online at http://www.carcin.oupjournals.org/.

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Conflict of Interest Statement: None declared.

References