Cyclin G2 Dysregulation in Human Oral Cancer

Yong Kim, Satoru Shintani, Yohko Kohno, Rong Zhang, and David T. Wong

School of Dentistry and Dental Research Institute, University of California at Los Angeles, Los Angeles, California; Department of Oral and Maxillofacial Surgery, Ehime University School of Medicine, Ehime, Japan; and Department of Oral Pathology, School of Dentistry, Showa University, Tokyo, Japan

ABSTRACT

Using expression microarray, we have previously shown that human cyclin G2 (hCG2) is significantly down-regulated in laser capture microdissected oral cancer epithelia. Western analysis showed detectable hCG2 protein in normal (2 of 2) but not in malignant (4 of 4) oral keratinocyte cell lines. Immunohistochemistry analysis done on oral cancers showed that normal oral mucosa (100%, 12 of 12) and 69.1% (47 of 68) of dysplastic oral epithelium expressed readily detectable hCG2 in the nuclei. However, only 11.1% of oral cancer epithelia (14 of 126) showed mild hCG2 nuclear staining. Interestingly, of the oral cancers devoid of nuclear hCG2 (112 cases), 58 cases (52%) showed cytoplasmic hCG2 immunostaining, whereas the other 54 cases (48%) exhibited neither nuclear nor cytoplasmic hCG2 staining. In vitro functional study by ectopic restoration of hCG2 expression in the human malignant squamous cell carcinoma (SCC) line SCC15 resulted in a significant inhibition of cellular proliferation (P < 0.001) and colony formation (P < 2 × 10−5) with increased population of G0 phase and decreased in S phase (P < 0.01). Furthermore, stable down-regulation of hCG2 by short interference RNA-based gene silencing in immortalized normal oral keratinocytes resulted in enhanced cell growth with increase in S and prominently in G2 phase. Because hCG2 has been implicated as a negative regulator in cell cycle progression, our results support that hCG2 dysregulation may play an important role in epithelial transformation and the early stages of human oral cancer development.

INTRODUCTION

Although molecular progression models exist for oral carcinogenesis, the precise molecular targets and pathways remain unclear (1). Chromosomal structural alterations are associated with dysplasia (9p21, 3p21, and 17p13), carcinoma in situ (11q13, 13q21, and 14q31), and invasive carcinoma (4q26-28, 6p, 8p, and 8q; refs. 2, 3). Recently, there has been an explosion of gene expression studies associated with oral/head and neck carcinogenesis; however, few have been consistently identified or functionally implicated with the oral cancer phenotype. Certain gene alterations have a stronger association with oral cancer development. Among these include p16INK4a, cyclin D1, and p53, with 80%, 30%, and 50% involvement in head and neck cancers, respectively (3). All three are located in chromosomal regions linked to oral cancer development: 9p21 (p16INK4a), 11q13 (cyclin D1), and 17p13 (p53). Several molecular events, such as altered expression of p120CTAP1 and retinoic acid receptor-β, have been associated with oral cancer development that has no obvious structural chromosomal change (4, 5). Likely, there are several genes/pathways yet to be identified as having a true causal relationship with oral cancer development. Identification of potentially novel diagnostic and therapeutic targets is now being addressed by DNA hybridization arrays (6).

Cyclin G2 (CG2) is a newly identified homologue of cyclin G1 and exhibits 60% nucleotide sequence identity and 53% amino acid sequence identity with cyclin G1, which was identified as one of the transcriptional targets of p53 (7, 8). Murine CG2 has been shown to be a cytoplasmic protein, whereas cyclin G1 is largely localized to the nucleus (9). CG2 contains a protein destabilizing PEST-rich sequence and a potential Shc phosphotyrosine binding site, implying its cell cycle dependent temporal level. Cyclin G1 is constantly expressed throughout the cell cycle, whereas CG2 expression peaks in the S phase. Despite their significant sequence homology, cyclin G1 and G2 are believed to have distinct cellular functions. CG2 has been implicated in negative selection of self-reactive lymphocytes, apoptosis, and also in DNA damage repair (10). Growth inhibitors, such as transforming growth factor β1 or dexamethasone, significantly induce CG2 mRNA expression. Further studies with B-cell antigen receptor-mediated cell cycle arrest have shown that CG2 may be a key negative regulator of cell cycle progression (11). A recent study by Bennin et al. (12) showed that CG2 inhibits cell cycle progression through interaction with PP2A. Ectopic expression of CG2 induces the formation of aberrant nuclei and cell cycle arrest. The mechanism of CG2 regulation and cellular function in carcinogenesis is poorly understood, and its dysregulation during oral carcinogenesis has not been described previously. Ito et al. (13) have recently reported decreased expression of CG2 in papillary carcinoma of the thyroid. It has also been shown that the expression of CG2 is down-regulated in estrogen-treated human breast cancer cells, implying its possible antiproliferative function (14). More recently, CG2 mRNA levels were shown to be elevated in G0, reduced as cells enter the cell cycle, and elevated again at the late S and G2-M phases (15). It was also shown that the expression of CG2 mRNA could be regulated by the FoxO family of forkhead transcription factors (15).

Using expression microarray, we recently identified down-regulation of CG2 expression is associated with the development of human oral cancers (6). Herein, we report the validation results showing down-regulation of CG2 in oral cancer cell lines and dysregulation of CG2 in oral cancer tissues by immunohistochemical analysis. Furthermore, we investigated the functional role of CG2 in epithelial cell growth examined in vitro. These data present the first evidence that alteration of CG2 expression can be functionally associated with oral/head and neck cancer development, and CG2 may serve as a molecular target for diagnostic and therapeutic applications.

MATERIALS AND METHODS

Materials. Cell culture media (DMEM, DMEM-F12, and KSF M) and cell culture supplies including antibiotics were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from HyClone (Logan, UT). The LipofectAMINE transfection reagent was from Invitrogen and FuGene 6 was from Roche Diagnostics (Indianapolis, IN). All restriction enzymes used were from New England Biolabs (Beverly, MA). The anti-CG2 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the anti-FLAG antibody was from Sigma-Aldrich (St. Louis, MO). The secondary antimonium IgG-horseradish peroxidase and enhanced chemiluminescence reagent was purchased from Amersham (Piscataway, NJ). The antigen-horseradish peroxidase antibody was from Sigma-Aldrich. Reagents used for quantitative PCR and reverse transcription (RT)-PCR were from Bio-Rad ( Hercules, CA) and Perkin-Elmer (Boston, MA). The CCK-8 kit was pur-
chased from Alexis Biochemicals (San Diego, CA), and propidium iodide was purchased from Sigma-Aldrich. RNaseA was obtained from Calbiochem (San Diego, CA), and all of the other chemical reagents were purchased from Sigma-Aldrich.

**Northern and RT-PCR Analysis.** Human tissues preserved in RNAlater (Ambion, Austin, TX) or actively growing cell cultures were used to isolate total RNA by using RNeasy kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. For Northern analysis, equal amount of total RNA was used and probed with CG2 cDNA as described previously (16). RT-PCR was done for CG2 (forward, 5'-GAGACAGCTGAGACCTCTCC-3'; and reverse, 5'-CCCTGCTTGTGGCCTGCTC-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-AGTCCACTG-GCCGTCTCACC-3'; and reverse, 5'-TGGTTCACACCCATGAC GAA-3') as follows: 1 μg of total RNA in 9 μL is mixed with 1 μL of 10 mmol/L deoxyxynucleoside triphosphate and 1 μL of oligo(dG)3-7 and heated for 5 minutes at 65°C. After chilling on ice for 3 minutes, 4 μL of 5X first-strand buffer, 2 μL of 0.1 mol/L DTT, and 2 μL of 20 units/μL RNase inhibitor was added. After 2 minutes incubation at 42°C, 1 μL of SuperscriptII RT (5 units/μL) was added and incubated for 50 minutes at 42°C, 15 minutes at 72°C, and soaked at 4°C. For PCR amplification, 1 μL of RT product was mixed with 5 μL of 10X PCRII buffer, 3 μL of 25 mmol/L MgCl₂, 2.5 μL of 10 mmol/L deoxyxynucleoside triphosphate, 1 μL of primer mix (10 μmol/L each), and 0.25 μL of Taq (5 units/μL) in 50 μL of reaction mixture. After 5’ heating at 94°C, amplification was carried out for 35 cycles in 94°C 45 seconds; 60°C 45 seconds; and 72°C 2 minutes.

**Western Analysis.** Cell lysate was isolated and homogenized in 10 volumes of radioimmunoprecipitation assay buffer [10 mmol/L Tris-HCl (pH 7.5) 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% SDS; 1% NP40; and 1% sodium deoxycholate-containing protease inhibitor] and heated at 100°C for 10 minutes. After centrifugation, the supernatant was applied to Immobilon-P PVDF membranes (Millipore). Membranes were blocked with 5% bovine serum albumin (BSA) for 60 minutes at RT and incubated overnight at 4°C with primary antibodies. Membranes were washed with 1X TBST (TBS with 0.1% Tween-20) and incubated with HRP-conjugated secondary antibodies for 1 hour at RT in a shaking incubator. Membranes were finally washed with TBST, and proteins were visualized by enhanced chemiluminescence substrate (Amersham Life Science, Arlington Heights, IL). Loading of protein was normalized by reprobing with a GAPDH antibody (Sigma-Aldrich). The resulting bands were quantitated with NIH Image software.

**Immunohistochemistry Analysis.** Four-micron sections were cut from the specimens, deparaffinized in xylene, and rehydrated in graded alcohol. Endogenous peroxidase was blocked by incubating tissue sections in 0.1% hydrogen peroxide in absolute methanol for 20 minutes. Before applying the antihuman CG2 antibody, sections were heated in an autoclave for 15 minutes in citrate buffer (pH 6.0). The CG2 antibody was used at 1:500 dilution, and the incubation time was 30 minutes at RT. Immunohistochemistry was done with the Envision system (DAKO, Carpinteria, CA). Peroxidase activity was visualized by applying diaminobenzidine chromogen, containing 0.05% hydrogen peroxide. Sections were counterstained with methyl green, dehydrated, and mounted. Negative control staining was carried out by substituting nonimmune serum for primary antibodies. Immunostaining was evaluated by three independent observers. For each section, 10 high-power fields were chosen, and a total of at least 1,000 cells were evaluated. The results were expressed as the percentage of positive cells counted. To confirm the reproducibility, 25% of the slides were chosen randomly and scored twice. All duplicates were similarly evaluated.

**Cloning of the Full-length Human CG2 (hCG2) Coding Sequence.** On the basis of the published sequence (17), we have cloned the full-length hCG2 coding region with a PCR-based approach. OKF4 human normal oral keratinocyte total RNA was primed with an oligo(dG)3-7 and reverse transcribed to synthesize the first strand of cDNA with SuperScriptII RT (Invitrogen). The resulting cDNA was then subjected to PCR amplification by using a primer set specific for hCG2 coupled with proper restriction site sequences (EcoR 1 and XbaI) to facilitate subcloning (forward, 5’GGCAAT- T7AATGAGATTTGAGGCGAG3; and reverse, 5’GCTCTGACG- TAAGATGGAAGCAGTGTT3). The PCR product was subsequently subcloned into the pFLAG-CMV2 cloning vector (Sigma), and selected clones were sequenced by automated capillary sequencing (ABI 3100; UCLA Department of Human Genetics DNA Sequencing Core Facility, Los Angeles, CA). Our CG2 clone has 99% nucleotide homology with previously reported studies, including the 1035 bp open reading frame encoding the 345 AA protein of M, ~40,000 (17). There was one silent mutation (T>C substitution) found at +117 that does not alter the putative amino acid sequence.

**Generation of CG2 Short Interfering RNA (siRNA) Constructs for Gene Silencing.** Several hCG2 siRNA target sequences were designed and cloned into pSilencer 2.1-U6 hygro and pSilencer 3.1-H1 hygro vectors (Ambion). Clones were selected and verified by DNA sequencing. Plasmid DNA for transfection was prepared by using QiaGen EndoFree Maxi kit (Qiagen), and test transfection was carried out with HeLa cells. The results were analyzed by RT-PCR, and two different siRNA target sites for the hCG2 gene were selected (sihCG2.1 (-570 -> -588: AAGTCGGCAACTCCGACTC) and sihCG2.2 (+750 +/- 768: ATGCGTACGGCAGATTCTT)).

**Cell Culture and Transfection Studies.** For ectopic overexpression of hCG2 in SCC15, the cells were grown in DMEM/F12 mixed with a conditioned medium from NIH3T3 cell (1:1). A day before transfection, the cells were plated on a 60-mm dish and transfected by using Lipofectin combined with Plus reagent according to the manufacturer’s protocol (Invitrogen) with a minor modification. After 5 hours transfection, the cells were fed with fresh complete medium and incubated for 48 hours before analysis. Downregulate hCG2 by siRNA in immortalized human normal oral keratinocytes OKF6tert1 (provided by Dr. Jim Rheinwald from Harvard Medical School, Boston, MA), the cells were grown and maintained in KSMF medium (25 μg/mL bovine pituitary extract, 0.4 mmol/L CaCl₂, 0.5 ng/mL epidermal growth factor, 1% penicillin, and 0.1% streptomycin). A day before transfection, the cells were plated on a 60-mm dish in the same medium without antibiotics. The pSiilencer siRNA construct (sihCG2.1 or sihCG2.2) was transfected by using FuGene 6 transfection reagent according to the manufacturer’s instructions (Roche Diagnostics Corp.). Stable sihCG2 cell lines along with control lines were established by selecting transfecants with 50 μg/mL hygromycin B (Invitrogen) for 3 weeks. The resulting clones were maintained in 1 μg/mL hygromycin B.

**Cellular Growth and Fluorescence-activated Cell Sorter Analysis.** For cell growth analysis, cells were seeded on a 96-well plate, and the cellular proliferation was measured by using the CCK-8 kit (Alexis Biochemical Corp.) following manufacturer’s instructions with a minor modification as follows. Briefly, on the day of assay, the cells were fed with fresh medium and incubated for 1 hour at 37°C. The CCK-8 assay reagent (10% of the cell culture medium) was added to each well and mixed gently. After 1 hour incubation at 37°C, 1% SDS (1 of 10 volume of the culture medium) was added, and the A₄₅₀ was measured. All of the assays were done in triplicate, and the growth rate was presented as the fold-increase over the day 1 value. For colony forming analysis, transfectantly transfected cells were harvested after 48 hours transfection and plated on a 60-mm dish at 50 cells per dish. After a 14-day incubation, colonies were stained with methylene blue, washed with deionized water, and counted. For cell cycle profiling by fluorescence-activated cell sorter (FACS) analysis (UCLA Janis V. Giorgi Flow Cytometry Core Facility, Los Angeles, CA), the transiently or stably transfected cells from 100-mm dishes were harvested by trypsinization and washed twice with PBS. After resuspending in 500 μL of ice-cold PBS, the cells were fixed by mixing with 5 mL of cold 95% EOH and kept at 4°C overnight. The cells were pelleted and washed twice with PBS/1% fetal bovine serum and finally resuspended in 800 μL of PBS/1% fetal bovine serum. The cell suspension was mixed with 100 μL of 10X propidium iodide [500 μg/mL in 38 mmol/L sodium citrate (pH 7.0): Sigma] and 100 μL of boyled RNaseA [10 mg/mL in 10 mmol/L Tris-HCl (pH7.5): Calbiochem]. After incubating for 30 minutes at 37°C, the cell cycle profile was analyzed by FACS analysis.
RESULTS

Expression Microarray Analysis on Human Oral Cancers Showed Down-regulation of CG2. In an attempt to establish a molecular gene expression profile in oral/head and neck cancer and concurrently to identify specific gene sequences the expression of which are differentially regulated in oral cancer, we have previously done expression microarray analysis on laser capture microdissected normal and oral cancer tissues (6, 18). Through extensive analyses of Affymetrix GeneChip microarray data, we have identified about 600 genes that are associated with oral cancer. These include oncogenes, tumor suppressors, transcription factors, xenobiotic enzymes, metastatic proteins, differentiation markers, and also genes that have not been implicated in oral cancer (6). Additional validation of the expression microarray data led us to select genes that are linked to cell cycle regulation. Fig. 1 shows that hCG2 is significantly down-regulated (4 of 5 cases; P < 0.0375) in oral cancer tissues. It should be noted that we later found that the case number 2 was a bony invasive variant of oral squamous cell carcinoma (SCC) and thus clinically behaves differently. CG2 was initially identified as a homologue of cyclin G1, and it has been implicated in the regulation of G2-M phase. The growth inhibitory effect of CG2 has also been validated in cultured oral keratinocytes. Establishing cell model system is important and valuable for the functional analysis and also to understand the mechanism of altered gene expression. To confirm dysregulation of CG2 in oral cancer cells, human normal oral keratinocytes and malignant SCC lines were cultured in serum-free KSF medium. Total RNA was prepared from growing normal oral keratinocytes (OKB2 and OKF4) and malignant SCC lines (SCC13, 15, and 25) and analyzed for CG2 mRNA expression by semi-quantitative RT-PCR. The result showed that CG2 mRNA expression is dramatically decreased in SCC lines compared with normal oral keratinocytes (Fig. 2B). We also examined the level of CG2 protein by Western analysis. Total protein lysate was prepared from normal and malignant oral keratinocytes and subjected to immunodetection of CG2. As shown in Fig. 2C, normal oral keratinocytes (OKF4 and OKF6) express readily detectable and comparable levels of CG2, whereas all of the malignant oral SCC lines (SCC4, 15, 66, and 105) do not express detectable levels of CG2 protein. To examine the in vivo expression and cellular localization of CG2 protein in oral cancer tissues, immunohistochemistry was done on 12 normal human oral mucosa, 68 epithelial dysplasia (mild to severe), and 126 oral cancer tissues obtained from patients. There was strong CG2 staining in the nuclei in normal oral mucosa, but there was a progressive loss of nuclear CG2 staining and a transition to cytoplasmic localization of CG2 as cancer progressed (Fig. 3). This finding differs from the previously reported cytoplasmic localization of murine CG2 (9, 19). However, those findings were based on the in vitro ectopic expression...
of the exogenous recombinant murine CG2 in cultured cells, which could be different from the in vivo environment. As summarized in Table 1 and depicted in Fig. 3B, all of the normal oral mucosa examined (100%, 12 of 12) and 69.1% (47 of 68) of the dysplastic oral epithelium expressed readily detectable CG2 in the nuclei. However, only 11.1% of oral cancer epithelia (14 of 126) showed mild CG2 nuclear staining. Thus, 88.8% of oral cancers (112 of 126) showed loss of nuclear CG2. Interestingly, of the oral cancers devoid of nuclear CG2 (112 cases), 58 cases (52%) showed cytoplasmic CG2 immunostaining, whereas the other 54 cases (48%) exhibited neither nuclear nor cytoplasmic CG2 staining (Fig. 3B). These data jointly suggest that CG2 expression is dysregulated during human oral cancer development. There is a progressive loss of CG2 expression during oral carcinogenesis and an altered cellular localization from nuclear in normal cells to cytoplasmic to no expression in cancer cells. Because CG2 has been implicated as a negative regulator in cell cycle progression, our results suggest that CG2 dysregulation may play a role in the early stages of human oral cancer development.

Ectopic Expression of CG2 in Oral SCC Line Inhibited Growth and Colony Formation with Increased G1 Phase Cells. Previous studies on possible cellular functions of CG2 have shown that ectopic expression of murine CG2 in HEK293 and CHO cell lines induced growth arrest in G1 phase with decreased population in S and G2 phases after 21 hours post-transfection (12). It was also reported that ectopic expression of CG2 resulted in aberrant and fragmented nuclei. After longer times in culture, a broad G1–S–phase distribution and alteration in G2–M phase profile suggestive of possible aneuploidy were observed (12). Overexpression of hCG2 in HeLa cells also resulted in reduced colony formation (20). As an indirect way of assessing the functional consequences of down-regulation of CG2 in oral cancers, hCG2 was ectopically overexpressed in a SCC line (SCC15) that does not show expression of endogenous hCG2. After transiently transfecting the pFLAG-CMV2–hCG2 into SCC15 cells along with a control null vector, the hCG2 transfectants were analyzed by cell proliferation analysis, colony forming assay, and FACS analysis and compared the results to control transfectants. Western analysis was used to confirm the expression of the exogenous FLAG-tagged hCG2. Total cell lysate was prepared from transiently transfected cells and immunoblotted with anti-CG2 antibody (Santa Cruz Biotechnology). As shown in Fig. 4A, the expression of recombinant FLAG-hCG2 was detected by anti-CG2 or anti-FLAG antibody

### Table 1

<table>
<thead>
<tr>
<th>CG2 Staining</th>
<th>Normal (n = 12)</th>
<th>Mild (n = 28)</th>
<th>Moderate (n = 19)</th>
<th>Severe (n = 23)</th>
<th>SCC (n = 126)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>12(100%)</td>
<td>24(85.7%)</td>
<td>14(73.7%)</td>
<td>9(39.1%)</td>
<td>14(11.1%)</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>1(5.3%)</td>
<td>3(13.0%)</td>
<td>58(46.0%)</td>
</tr>
<tr>
<td>Negative</td>
<td>0(0%)</td>
<td>4(14.3%)</td>
<td>4(21.0%)</td>
<td>11(47.8%)</td>
<td>54(42.9%)</td>
</tr>
</tbody>
</table>

Note: Data was analyzed by using StatView software (Cary, NC) and P values were determined by using χ² test.

* P = 0.000022
† P = 0.00271

Fig. 3. Immunohistochemical analyses of CG2 expression in human oral cancers. A. The in vivo expression of CG2 was examined by immunohistochemistry with anti-CG2 antibody (Santa Cruz Biotechnology). Normal human oral mucosa and dysplastic oral epithelia expressed detectable CG2 in the nuclei. But CG2 expression was greatly reduced in cancer tissues. For negative control, the slide was stained with secondary antibody only. B. The percentage of positive cells in each case was semiquantitatively evaluated into one of the following five groups: (a) immunoreactivity completely absent (negative, 0%); (b) <5%; (c) <25%; (d) <50%; and (e) up to 100%. In the present study, cases showing >5% of nuclear positive cells were defined as "nuclear positive." And cases showing >5% of cytoplasmic positive cells were defined as "cytoplasmic positive." The result was examined by Fisher’s exact test. Normal epithelia and dysplasia: P = 0.0300, dysplasia versus SCC: P < 0.0001. (OSCC, oral SCC)

Fig. 4. Transient ectopic overexpression of CG2 in SCC line SCC15 inhibited cellular proliferation and colony formation. A. The oral SCC line SCC15 that does not show expression of endogenous CG2 was transiently transfected with pFLAG-hCG2 (Lane 3) along with control construct (Lane 1: no transfection and Lane 2: pFLAG). Western analysis showed overexpression of FLAG-hCG2 detectable by both anti-hCG2 and anti-FLAG antibody. B. The cellular proliferation of transiently transfected cells was monitored by using the Cell Counting kit (CCK-8, Alexis Biochemical), and overexpression of hCG2 in SCC15 cells induced significant inhibition (P < 0.001) of cell growth compared with null transfectant (pFLAG). C. pFLAG-hCG2 transfectant showed greatly reduced colony forming cells compared with the null transfectant (P < 2 × 10⁻⁵). Bars, ±SD.
only in cells transfected with the pFLAG-hCG2 plasmid (Lane 3). Note that endogenous hCG2 was not detectable in SCC15. The level of hCG2 achieved by transient transfection was ~3-fold higher than an endogenous level of CG2 in normal oral keratinocytes. The effect of hCG2 restoration in the growth of SCC15 cells is shown in Fig. 4B. Overexpression of FLAG-hCG2 in SCC15 greatly reduced cell proliferation rates (P < 0.001; Fig. 4B) and also induced a floating cell population, suggesting that CG2 overexpression may induce apoptosis. We also measured clonogenicity of the transient transfectants, and ectopic expression of CG2 resulted in significantly reduced formation (P < 2 × 10⁻⁶) of methylene blue stained colonies as shown in Fig. 4C. To examine if ectopic expression of CG2 induced apoptosis, we measured percentage of apoptotic cell population at 24 and 48 hours post-transfection by using Annexin V-EGFP FACS analysis. Consistent with the observation of increased floating cells in hCG2 transfectant, we observed significantly induced apoptosis after hCG2 transfection compared with null vector transfection (P = 0.00053 at 24 hours and P < 0.0001 at 48 hours; Fig. 5A). Also, to examine if ectopic expression of CG2 altered the cell cycle profile in SCC15 cells, we determined the cell cycle positions of the transient transfectants at 24 and 48 hours post-transfection. Compared with control transfectants, hCG2-overexpressing cells showed a decrease in G1 phase and an increase in G1 phase at 24 hours post-transfection (P = 0.01), and the effect was more dramatic at 48 hours post-transfection (P = 0.007; Fig. 5B). These data supports the growth inhibitory function of CG2.

Reduction of CG2 Expression in Human Normal Oral Keratinocyte Resulted in an Increased Cellular Proliferation. Our initial expression microarray analysis and additional follow-up studies showed that there is a significant down-regulation of CG2 expression in both mRNA and protein level in oral cancer tissues and squamous cell carcinoma lines. Therefore, it will be important to examine the effect of down-regulation of CG2 level in normal oral tissues or cell lines. As a way to address this, we examined the effect of down-regulation of CG2 in normal oral keratinocytes. The siRNA was used to specifically down-regulate CG2 in an immortalized human normal oral keratinocyte (OKF6tert1) cell line. OKF6tert1 was established by expressing hTERT in normal oral keratinocyte (OKF6) to rescue cells from senescence (21). OKF6tert1 cells express readily detectable levels of CG2. The hCG2 siRNA sequences (sihCG2) were designed by using the Ambion target finder web-based program, and their specificity was analyzed by NCBI BLAST search. Selected hCG2 siRNA sequences were prepared by DNA synthesis (MWG Biotech Inc., High Point, NC) and subsequently cloned into pSilencer 2.1-U6 hygro and pSilencer 3.1-H1 hygro vectors (Ambion). The effects of chosen siRNA sequences were initially tested by transiently transfecting them into HeLa cells. Reduction of hCG2 mRNA was evaluated by semiquantitative RT-PCR and Northern analysis (data not shown). The selected clones from hCG2.1 siRNA construct were additionally analyzed by Western analysis with anti-CG2 antibody (Santa Cruz Biotechnology) to assess the extent of gene silencing by siRNA. As shown in Fig. 6A, compared with control clones [vector alone (V) or negative siRNA with random scrambled sequence (N)], sihCG clones showed various reduced levels of hCG2 expression (from 30 to 70% reduction after normalized against β-actin; P < 0.05). Clones with significant reduction of hCG2 were selected, and the effect of hCG2 down-regulation on cell growth was examined by cellular proliferation assay. As shown in Fig. 6B, the sihCG2 clones showed increased rate of cellular proliferation (1.3- to 1.5-fold; P < 0.03) compared with siRNA negative control clones. We have additionally examined the cell cycle profiles of these sihCG2 clones by DNA FACS analysis. As shown in Fig. 6C, the sihCG2 clones showed significant alteration in cell cycle profile (P < 0.003). There was a decrease in G1 phase (17 to 36% decrease compared with controls) along with increase in S phase (18 to 33% increase compared with controls) and prominent increase in G2 phase (0.9- to 2.6-fold increase compared with controls). Taken together, these data strongly support that CG2 plays a physiologic role in epithelial cell growth and cell cycle control, and alteration of its expression could significantly affect normal cellular

Fig. 5. Induced apoptosis and cell cycle arrest by hCG2 in SCC15 cells. A. Transient ectopic expression of CG2 induced significant apoptosis in SCC15 cells after 24 hours (*, P = 0.00053) and 48 hours (**, P < 0.0001). B. Cell cycle profiling by FACS analysis showed that overexpression of hCG2 in SCC15 resulted in an increase in G1 population at 24 hours (*, P = 0.01) and 48 hours post-transfection (**, P = 0.007). The data were analyzed by using paired student’s t test. Bars, ±SD.
physiology. Furthermore, our data supports that down-regulation of CG2 is a functionally important event in cellular transformation of oral epithelial cells.

**DISCUSSION**

Cancer biology is governed by alterations of complex pathways and cellular programs. Many mechanisms potentially important to the management of the cancer patient remain undiscovered. The dysregulation of the molecular events governing cell cycle control is emerging as a central theme of oral carcinogenesis (22). Regulatory pathways responding to extracellular signaling or intracellular stress converge on the cell cycle apparatus. Abrogation of mitogenic and antimitogenic response regulatory proteins, such as the retinoblastoma tumor suppressor protein (pRB), cyclin D1, cyclin-dependent kinase 6 (CDK6), and cyclin-dependent kinase inhibitors (p21WAF1/CIP1, p27KIP1, and p16INK4a), occur frequently in human oral cancers (22). Cellular response to metabolic stress or genomic damage through p53 and related pathways that block cell cycle progression are also altered during oral carcinogenesis. In addition, new pathways and cell cycle regulatory proteins, such as p12CDK2-AP1, are being discovered (4, 5).

By using expression microarray on laser capture microdissected-normal and oral cancer tissues, we have identified CG2 as a potentially important molecule in the development of oral cancer. As a follow-up validation process, we have confirmed the dysregulation of CG2 in malignant oral SCC lines in vitro and also down-regulation of CG2 expression in vivo in oral cancer tissues. More importantly, the functional significance of differential expression of CG2 in epithelial cell growth has been addressed. Data presented in this report strongly support that down-regulation of CG2 is a functionally significant event in epithelial transformation and may have biological significance in the early development of oral cancer. Dysregulation of CG2 in cancers has been emerging recently; however, the functional significance of dysregulation of CG2 in cancer development has never been addressed. CG2 may control cellular growth to maintain proper balances in G2-M phase of cell cycle. As shown by Bennin et al. (12), if CG2 is involved in proper control and maintenance of mitosis and cytokinesis, the alteration of CG2 will result in genomic instability from accumulation of aberrant DNA content (Fig. 7). Especially under the circumstances that CG2 is altered to be down-regulated, there will be a loss of growth control combined with alteration in downstream interactions with other cellular partners, such as phosphatases or kinases. This will result in cellular transformation through altered mitosis/cytokinesis.

It remains to be tested if down-regulation of CG2 in vivo will eventually have a significant effect on tumor growth. Our data showing a growth inhibitory function of CG2 in a human malignant oral SCC line set the stage for animal tumor model study to evaluate the in vivo effect of CG2 on tumor regression. At this point, it is not known mechanistically how CG2 expression is down-regulated in oral cancers. Future studies should be directed toward identification of any genomic alterations of CG2 (Ccn2) gene that is located at chromosome 4q21.22, investigation on the gene regulation mechanisms of CG2, and also the cellular mechanisms of CG2 function. Preliminary genomic loss of heterozygosity analysis done on a panel of normal and malignant oral keratinocytes has implied that there is a comparable degree of genomic alteration at the Ccn2 locus. In addition, based on our findings that CG2 mRNA level is differentially regulated in oral cancers in vitro and in vivo, we are investigating a possible mechanism of down-regulation of CG2 in human SCC lines at the transcriptional and post-transcriptional levels. To answer how dysregulation of CG2 renders cellular growth inhibitory effect, we are now identifying CG2 interacting cellular partners.

The multistep process of oral carcinogenesis likely involves functional alteration of cell cycle regulatory members combined with escape from cellular senescence and apoptotic signaling pathways (23). Detailing the molecular alterations and understanding the functional consequences of the dysregulation of the cell cycle apparatus in the oral cancer cells may uncover novel diagnostic and therapeutic approaches. Our data strongly suggest that CG2 be one of the promising candidate genes.

**REFERENCES**


Cyclin G2 Dysregulation in Human Oral Cancer

Yong Kim, Satoru Shintani, Yohko Kohno, et al.

*Cancer Res* 2004;64:8980-8986.

Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/24/8980

Cited articles This article cites 20 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/24/8980.full.html#ref-list-1

Citing articles This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/64/24/8980.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.