Tumor Suppressor Activity of CCAAT/Enhancer Binding Protein α Is Epigenetically Down-regulated in Head and Neck Squamous Cell Carcinoma

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Abstract

Tumor suppressor CCAAT enhancer binding protein α (C/EBPα) is a transcription factor involved in cell cycle control and cellular differentiation. In a recent study, microarray expression profiling on head and neck squamous cell carcinoma (HNSCC) samples identified significant C/EBPα down-regulation, correlating with poor prognosis. However, the mechanisms of C/EBPα down-regulation remained elusive. C/EBPα has been previously found to provide an antiproliferative role in lung cancer, and our laboratory showed that its down-regulation involves epigenetic mechanisms. This prompted us to investigate the involvement of epigenetics in down-regulating C/EBPα in HNSCC. Here, we show that C/EBPα is down-regulated in HNSCC by loss of heterozygosity and DNA methylation, but not by gene mutation. We found a consistently methylated upstream regulatory region (−1,399 bp to −1,253 bp in relation to the transcription start site) in 68% of the HNSCC tumor samples, and DNA demethylation using 5-aza-2′-deoxycytidine treatment was able to significantly restore C/EBPα mRNA expression in the HNSCC cell lines we tested. In addition, C/EBPα overexpression in a HNSCC cell line (SCC22B) revealed its ability to provide tumor suppressor activity in HNSCC in vitro and in vivo. In conclusion, we showed for the first time not only that C/EBPα has tumor suppressor activity in HNSCC, but also that it is down-regulated by DNA promoter methylation. [Cancer Res 2007;67(10):4657–64]

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world (1). It is primarily caused by exposure to alcohol and tobacco products, which influence major pathways of cell proliferation (2). Surgery is done only in cases of locally advanced HNSCC, and following surgical treatment, many HNSCC patients are prone to relapse and ultimately systemic dissemination (3). Neck dissection followed by chemotherapy may allow substantial regional control, but very few patients achieve long-term remission (4). Therefore, early detection and better disease prediction via genetic and epigenetic biomarkers become crucial.

Epigenetic modifications, especially in the form of DNA methylation, have emerged as a relevant factor in the disease progression of HNSCC. Despite the fact that global DNA methylation screens showed relatively lower levels of promoter methylation in HNSCC as compared with other malignancies (5), several epigenetically silenced cancer genes have been described in HNSCC. These include secreted frizzled-related protein family genes (6), LHX6 (7), p16 (8), TCF21 (9), p53 (10), and members of the Fanconi anemia/BRCα1 pathway (11). However, none of the epigenetically regulated genes have been identified as poor prognostic markers in HNSCC (12).

In a recent study, microarray expression profiling was done on 40 HNSCC tumor samples to identify gene expression differences correlating with poor prognosis (13). CCAAT enhancer binding protein α (C/EBPα) was one of the genes analyzed, and the authors found 78% (31/40) of the HNSCC tumor samples had C/EBPα down-regulation compared with the expression in matched normals (13). Furthermore, a significant correlation was found between C/EBPα down-regulation and patients with extensive lymph node metastasis (13). Decreased C/EBPα expression could contribute to the tumor cells’ characteristics of uncontrolled proliferation and loss of differentiation. This has already been shown in acute myelogenous leukemia (AML), where mutations in the coding region of C/EBPα have been found to relieve the protein of its ability to block cell cycle progression (14). In addition, C/EBPα provides an antiproliferative role in lung cancer (15), and we have recently shown that its down-regulation is conducted by epigenetic mechanisms (16). Therefore, we hypothesized that C/EBPα might play a role in tumor and metastatic suppression in HNSCC.

Materials and Methods

Patient samples and cell lines. Frozen tumor tissues and adjacent normal tissue from HNSCC patients were attained from the Ohio State University Medical Center via the Cooperative Human Tissue Network. Surgery was done on all patients at the Ohio State University Medical Center. All sample collections were done according to the NIH guidelines and under a protocol approved by the Institutional Review Board of the Ohio State University. Control samples were collected from morphologically normal tissue located at least 3 cm from the tumor margin. Histopathologic evaluation was done on all samples for verification. The seven different established human HNSCC cell lines used in the study (SCC4, SCC5, SCC8, SCC11B, SCC17A5, SCC22B, and SCC25) were kindly provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). Cell lines were maintained in DMEM with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin antibiotics.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

http://cancerres.aacrjournals.org/content/67/10/4657.full.pdf+html

References


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**Immunohistochemistry.** Immunohistochemical staining was done as previously described (16), including three tissue microarrays containing HNSCC normal, tumor, or metastatic samples. Tissue microarrays contained triplicates of each primary tumor or metastatic tissue sample. The tumor array contained 48 patient samples, the metastatic tissue array contained 34 samples (shared in the tumor array), and the normal array contained 47 matching adjacent normal samples. Scoring was done using the following index: 0, no staining; 1, faint staining in <10% cells; 2, light staining in 10% to 50% of cells or medium staining in <10% cells; 3, light staining in all cells or dark staining in 10% cells; 4, medium staining in 10% to 50% of cells; 5, medium staining in all cells or dark staining in 10% to 50% of cells; 6, dark staining in all cells. HNSCC tumor or metastatic tumor samples had to contain an index of at least two scores below its paired normal to be considered down-regulated. One tumor sample was without a matching normal sample.

**Migration assay.** Approximately 400 μL of 10% FBS DMEM was plated in a 24-well plate. Millicell Culture Plate Inserts (Millipore) were placed into each well, and 5,000 or 20,000 cells were suspended in 300 μL DMEM without FBS. As a control, chambers with no cells were prepared similarly. After 20 h, the media was aspirated, and chambers washed with Dulbecco's phosphate-buffered saline (PBS). About 250 μL of 0.05% crystal violet 3.7% formaldehyde was placed in the upper and outer chambers. Cells were allowed to incubate for 30 min; then the crystal violet was removed, and the wells were washed with water. After allowing 15 min for drying, 80 μL of 3.7% crystal violet was again taken for absorbance reading at 570 nm. Approximately 250 μL of 80% methanol was placed inside the chamber, incubated in a shake for 30 min at room temperature, and 100 μL was again taken for absorbance at 570 nm. Percent migration was determined by the following calculation: 100 × (absorbance migrated cells/absorbance background) + (absorbance migrated cells absorbance background) + (absorbance nonmigrated cells absorbance background).

**5-Aza-2′-deoxycytidine treatment.** HNSCC cell lines were incubated for 96 h with 3 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC, Sigma), with medium and drug changed daily. Treated cells were harvested for analysis after the procedure. Cells were suspended in TRIzol for RNA isolation or Laemmeli buffer [62.5 mmol/L Tris-Cl (pH, 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 300 mmol/L 2-mercaptoethanol] for Western analysis.

**RNA isolation and cDNA synthesis.** RNA was isolated according to the manufacturer's protocol. DNase treatment was done on 1 to 2 μg of RNA by using the manufacturer's protocol.
adding 2 units of DNaseI (Invitrogen), 1 μL DNase buffer, and 0.4 μL RNase Out (Invitrogen) for 15 min at room temperature. About 1 μL EDTA was then added to the mix for 10 min at 65°C, followed by an incubation on ice for 5 min. cDNA synthesis was done by the following reaction: 2 μL random hexamers and 1 μL deoxyribonucleotide triphosphates (10 mmol/L) for 5 min at 65°C then 2 min at 4°C; 2 μL of 10× buffer, 4 μL MgCl₂, 2 μL DTT; and 1 μL RNase Out was added for 2 min at 25°C, 100 units of Superscript II (Invitrogen) for 50 min at 42°C, 15 min at 70°C, then transferred to 4°C.

Real-time PCR. Quantitative C/EBPα mRNA expression was measured using SYBR Green 1 (Bio-Rad) in an iCycler (Bio-Rad). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control gene. iCycler conditions and reverse transcription-PCR (RT-PCR) primers are listed in Supplementary Data. We additionally did PCR on DNase-treated, "−" (without the reverse-transcriptase enzyme) RT samples to ensure that no DNA contamination was present in the RNA extract (given the fact that C/EBPα is an intronless gene). No amplification of PCR product was seen in these samples, indicating the absence of contaminating genomic DNA in the DNase-treated RNA extracts.

Sequenom methylation analysis. Quantitative DNA methylation analysis was done by Sequenom, Inc. About 1 μg of HNSCC patient or cell line DNA was bisulfite treated, in vitro transcribed, cleaved by RNase A, and subjected to matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry analysis to determine methylation patterns, as previously described (17).

Chromatin immunoprecipitation assay (ChIP). Chromatin immunoprecipitation (ChIP) was done according to the Upstate Cell Signaling Solutions Protocol. Quantitative PCR for the ChIP assay was done with 3 μL of ChIP eluate and primers surrounding the promoter binding sites. Promoter enrichment was assessed by normalization of threshold crossing of the samples compared with the threshold crossing of the negative control (no antibody).

Plasmid constructs and oligos. The pBABE-C/EBPα construct was generously provided by Dr. Golkan Hotamisligil (Harvard University, Boston, MA) and previously described (18). The pBS retroviral vector from Origene was used for stable silencing experiments, shRNA oligo sequences are listed in Supplementary Data. The sequence targeted for stable silencing was borrowed from a previous publication (19).

Transfections. Stable transfections were done as previously described (9). The target cells were collected, counted, and suspended in Laemmeli buffer for Western blot confirmation of silencing 120 h postinfection. For the transient silencing experiment, 1.6 × 10⁵ C/EBPα-overexpressing SCC22B cells were plated per well in a six-well plate. The transfections were done in duplicates. The negative control transfection mix contained 100 μL EC-R buffer (Qiagen), 14.4 μL RNAfect (Qiagen), and DMEM + 10% FBS. The small interfering RNA (siRNA) transfection mixture contained 4.8 μL 20 μmol/L siRNA stock, 95.2 μL EC-R buffer (Qiagen), 14.4 μL RNAfect (Qiagen), and DMEM + 10% FBS, for a final concentration of 30 nmol/L. The transfection media was left on the cells for 72 h. Then, the cells were

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**Figure 2.** C/EBPα expression analysis for methylated HNSCC patient samples and cell lines. A. Northern analysis was done on six HNSCC normal/tumor patient pairs. Left to right, samples were loaded on the blot in order of increasing methylation (according to MassARRAY). Top, hybridization with C/EBPα probe; bottom, GAPDH. Quantification by ImageQuant of the C/EBPα mRNA expression is shown beneath each lane. Each sample was normalized to its GAPDH, and the C/EBPα expression of each tumor was normalized to its matching normal, which was set as 1. The average methylation according to MassARRAY is listed below the expression quantification. B, bisulfite sequencing analysis was done on SCC17AS cells with and without 5-aza-dC. The diagram is drawn to scale; arrow, transcription start site; the region tested for methylation via bisulfite sequencing is indicated on this diagram (spanning from −1,423 to −1,121 bp). Bisulfite sequencing was done by comparing the sequences with 100% and 0% methylated DNA sequence (i.e., CG or TG at CpG sites, respectively). ○, unmethylated CpGs; ●, methylated CpGs. Each row represents an individual clone. C, RT-PCR analysis was done on RNA isolated from the treated or untreated HNSCC cells (SCC11B, SCC22B, and SCC25), subjected to DNase treatment and cDNA synthesis. Each C/EBPα expression level was normalized to its internal control (GPI expression). The SCC cell line with the lowest endogenous C/EBPα expression, SCC25, was set to 1 for comparison. *, P < 0.0177; **, P < 0.0083; ***, P < 0.0010. D, Western analysis was done on SCC11B or SCC17AS cells with and without treatment. Cells were isolated, suspended in Laemmeli buffer, and loaded on a 12% PAGE gel. Upon transfer, the blot was probed with C/EBPα (sc-61) and α-tubulin (control for loading) antibodies.
trypsinized, counted, and suspended in Laemmli buffer for Western analysis.

**Colony formation assay.** Colony formation assay was done as previously described (9).

**Growth curve.** Cells were counted using a Cell Coulter Counter. About \(1 \times 10^5\) cells were plated in triplicates on six-well culture plates. Cells were then permitted to grow 3 days before taking counts, after which cells were washed, trypsinized, and counted every 24 h for 4 days. For each count, 500 \(\mu\)L of trypsin was added to each well for 3 min, after which the cells were suspended in 2 mL of PBS, and 500 \(\mu\)L was counted using a Coulter Counter.

**Combined bisulfite restriction analysis.** Genomic DNA (1 \(\mu\)g) in a volume of 30 \(\mu\)L was denatured by NaOH (final concentration, 3 mol/L) for 30 min at 37°C. The denatured DNA was then treated with 30 \(\mu\)L of 10 mmol/L hydroquinone and 520 \(\mu\)L of 3 mol/L sodium bisulfite at 50°C overnight. The primers used to amplify the bisulfite-treated DNA are as follows: BSCEBP-region1R \(5\prime\)-TTTTGTAGTGTGAAGTTATG-3\prime; BSCEBP-region1F \(5\prime\)-AAACCTAAAACCCCTTAAATA-3\prime. The PCR conditions were initiated with a denaturing step of 95°C for 10 min followed by 36 cycles of 96°C for 30 s, 59°C for 30 s, and 72°C for 30 s and were concluded with 72°C for 10 min. The PCR products were purified with a purification kit (Qiagen) and incubated with BstUI at 60°C for 4 h. Digested DNA was then size fractionated via PAGE to detect the methylation status.

**Bisulfite sequencing.** Promoter region of bisulfite-treated HNSCC cell lines and patient samples were analyzed for methylation as previously described (16).

**Loss of heterozygosity analysis.** A total of 122 HNSCC patient adjacent normal, stroma, and epithelial tumor samples were subjected to loss of heterozygosity (LOH) analysis using D19S433 and D19S245 markers. Sequencing of the PCR products showed whether the tumor sample had homozygosity, retention of heterozygosity, or loss of heterozygosity when compared with normal.

**Northern blot analysis.** The Northern blot analysis was done as previously described (16), but instead, with 8 \(\mu\)g of RNA. Six pairs of HNSCC normal/tumor patient samples (also included in the MassARRAY methylation analysis) were loaded onto the blot in order of increasing methylation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. ImageQuant was used to determine C/EBP\(\alpha\) expression in tumor compared with its matching normal (which was set as 1).

**Western blot analysis.** The Western blots were done as previously described (16). After C/EBP\(\alpha\) detection, the blot was stripped with Bio-Rad stripping buffer in 37°C for 5 min and reprobed with a 1:500 dilution of \(\alpha\)-tubulin antibody (1:3,000 secondary mouse antibody). A 1:250 dilution was used for involucrin (Biomedia) and a 1:3,000 secondary rabbit.

**Mutation analysis.** The coding region of C/EBP\(\alpha\) was amplified in 49 HNSCC tumor samples and 10 HNSCC cell lines using four sets of primers. Primer sequences and conditions were previously described (20). Mutation screening was done by comparing the resulting sequences with the known normal sequence on BLAT.

**Animals and in vivo assay.** Three- to four-week-old male nude mice (Taconic, NCRNU-M) were maintained in a pathogen-free environment. Injections were done as previously described (9). Tumors were palpable after 17 and 26 days from the 8.5 and 3 million cell injections, respectively. Volumes were calculated by the following formula: \(V = (L \times W^2)/6\). Statistical analysis. The statistical significance of the results was calculated by unpaired Student’s \(t\) test, and \(P < 0.05\) was considered to be statistically significant.

**Results**

**C/EBP\(\alpha\) down-regulation in HNSCC.** Based on the previous publication by Roepman et al. (13), which showed that C/EBP\(\alpha\) is among the transcripts significantly down-regulated in poor-prognosis HNSCC patients, we first wanted to validate decreased C/EBP\(\alpha\) expression levels in our HNSCC patient samples via immunohistochemistry analysis. Strong C/EBP\(\alpha\) expression was detectable in epithelial cells in 39/47 (83%) of the normal tissue sections, but it is down-regulated in 33/48 (69%) of the tumor samples and 23/34 (68%) of the metastatic tumor samples (Fig. 1A and Supplementary Table S1). About 52.2% (12/23) of the paired metastatic and tumor samples shared similar levels of down-regulation, 43.5% (10/23) of the metastatic samples showed even greater down-regulation than the matched tumor samples, and 4.3% (1/23) of the tumors showed greater down-regulation than the matched metastatic samples (Supplementary Table S1). **C/EBP\(\alpha\) deletion in HNSCC.** We next investigated possible ways for C/EBP\(\alpha\) down-regulation, first evaluating the genetic mechanisms. We assessed the incidence of deletions in this genomic region in laser-captured microdissected (LCM) HNSCC
Epigenetic Down-regulation of C/EBPα in HNSCC

A. C/EBPα overexpression in vivo. A, the tumors analyzed were from the only mouse in a previous experiment that developed tumors from both cell types (SCC22B cells and SCC22B cells overexpressing C/EBPα). Tumors are shown to scale adjacent to their immunohistochemistry. Tumor tissues were stained with C/EBPα antibody (sc-61). Positive staining is evidenced by brown nuclear staining. Background cytoplasmic staining is negative. Magnification, ×400. W, pBABE-transfected SCC22B; C, SCC22B cells overexpressing C/EBPα. B, the 10 mice injected with pBABE-transfected SCC22B cells (left flank) and C/EBPα-overexpressing SCC22B cells (right flank). C, average tumor volume of the SCC22B cells versus the average tumor volume from the SCC22B cells overexpressing C/EBPα is plotted. Volumes are calculated by the following formula: \( V = \frac{(L \times W^2)}{4} \). * \( P < 0.009385 \).

B. Figure 4. C/EBPα methylation from \(-1,399\) bp to \(-1,253\) bp in HNSCC tumors and re-expression with 5-aza-dC treatment. Recently, our laboratory has shown that C/EBPα is epigenetically regulated in lung cancer (16). Therefore, we investigated epigenetic regulation of C/EBPα, a gene associated with a dense CpG island, in HNSCC. To first locate the region of methylation, we required a broad methylation analysis of the upstream and promoter-proximal regions of C/EBPα. Therefore, we did quantitative DNA methylation analysis by using the MassARRAY technique that combines in vitro transcription, RNase A cleavage, and MALDI-TOF mass spectrometry on HNSCC DNAs (17). This strategy provided information on upstream region 1 (\(-1,450\) to \(-1,120\) bp), promoter-proximal region 2 (\(-580\) to \(-297\) bp), and promoter-associated region 3 (+164 bp to +379 bp). All three of these regions are encompassed within a CpG island (i.e., the sequence contains a GC content of \(\geq 50\%\) over a length >200 bp). The MassARRAY analysis of region 1 showed an average of 25% methylation in the 28 HNSCC tumor samples, an average of 10% methylation in the five HNSCC adjacent normal samples, and an average of 87% methylation in the six HNSCC cell lines. Lower methylation seen in the HNSCC tumor samples compared with the HNSCC cell lines is most likely due to the potential for contaminating normal tissue in the tumor specimens. Virtually no methylation was seen in regions 2 and 3 (Fig. 1B). Interestingly, the sequence of the methylated region 1 is extremely conserved between humans, mice, rats, dogs, and opossums according to University of California–Santa Cruz Genome Browser on the March 2006 assembly. This sequence may provide promoter or enhancer activity, which is supported by previous promoter studies that have revealed increasing promoter activity by including more upstream sequence (16, 21). Subsequent to the broad methylation analysis, we desired to further define where DNA methylation is occurring in this region. Although MassARRAY allows a large-scale methylation analysis, information on some CpG sites is lost due to the nature of the technique (i.e., cleavage products of the same size are indistinguishable from one another). In contrast, bisulfite sequencing provides methylation data on each CpG within the sequenced region. Therefore, we did bisulfite sequencing on four tumor samples (and their adjacent normals) that had shown methylation in the MassARRAY analysis. Bisulfite sequencing revealed consistent methylation in all four tumor samples in upstream region 1 not seen in their corresponding normal tissues (or “germ line DNA”; Fig. 1C). These tumor samples showed decreased C/EBPα mRNA expression (compared with adjacent normal tissue DNAs) by quantitative RT-PCR (data not shown). Furthermore, two of the four samples had been included on the tissue microarray for the immunohistochemistry analysis, which revealed significant C/EBPα down-regulation in these samples (Supplementary Table S1). This bisulfite sequencing data closely correlates with the broad methylation analysis (Fig. 1B), and it reveals that methylation exists only in upstream region 1.

To correlate the incidence of LOH and methylation, 23 samples that exhibited LOH at D19S245 (the marker closest to the C/EBPα locus) were also tested for C/EBPα promoter methylation in the upstream region 1. Combined bisulfite restriction analysis revealed methylation in 65% of these samples (15/23; data not shown).
Bisulfite sequencing was done on five of these tumor samples, which showed partial methylation ranging from ~15% to 50% (Supplementary Fig. S1).

We next wanted to verify if C/EBPα promoter methylation is capable of decreasing C/EBPα expression. We did Northern analysis on six normal/tumor HNSCC patient pairs that had shown increasing methylation according to the MassARRAY analysis. Quantification of the Northern analysis revealed a strong correlation between increasing promoter methylation and decreased mRNA expression (Fig. 2A). Quantitative RT-PCR on 18 normal/tumor HNSCC patient pairs also revealed a correlation between increasing methylation (according to the MassARRAY) and decreased C/EBPα mRNA expression (Supplementary Fig. S2). Three HNSCC cell lines that all exhibited high amounts of methylation in the upstream C/EBPα sequence (SCC11B, SCC22B, and SCC25; Fig. 1B) were exposed to 3 μmol/L of the demethylating drug 5-aza-dC for 96 h to see if demethylation allows C/EBPα re-expression. After treatment, cells were harvested and analyzed for changes in C/EBPα methylation and expression compared with the untreated cells. Bisulfite sequencing on SCC17AS cells before and after 5-aza-dC treatment revealed a decrease in upstream C/EBPα methylation (93–56%; Fig. 2B). In addition, quantitative RT-PCR and Western blot analysis revealed an increase in C/EBPα expression. SCC11B, SCC22B, and SCC25 all showed a significant increase in C/EBPα mRNA expression (P < 0.018, P < 0.008, and P < 0.001, respectively; Fig. 2C), and Western blot analysis on two HNSCC cell lines (SCC11B and SCC17AS) showed an increase in C/EBPα protein expression (Fig. 2D), which suggests epigenetic control of the gene.

C/EBPα overexpression provides tumor suppressor activity in vitro and in vivo in HNSCC. To assess the potential of C/EBPα for tumor suppressor activity in HNSCC, we overexpressed C/EBPα in a HNSCC cell line (SCC22B). After protein overexpression was confirmed via Western analysis (Fig. 3A), growth curve analysis and colony formation assays were done. In comparison to the pBABE-transfected cells and SCC22B untransfected cells, C/EBPα transfectants exhibited a significant decrease in proliferation (21 h versus 50 h average doubling time, respectively) and colony formation (Fig. 3B and C). Next, we used a migration assay to investigate the ability of C/EBPα to inhibit metastasis. The migration assay showed a significant decrease in migration of C/EBPα transfectants compared with the pBABE transfectants (Fig. 3D).

To validate the in vitro tumor suppressor activity, we did in vivo analysis using nude mice. We executed two separate in vivo experiments using 3 and 8.5 million cells for the injections. The experiment using 3 million cells yielded 6 out of 10 mice with tumors from the wild-type SCC22B cells, and one of these also had a tumor from the C/EBPα-pBABE-transfected SCC22B cells (data not shown). We did immunohistochemistry on the tumors of this mouse that formed tumors from both cell types. As expected, this analysis revealed increased C/EBPα expression in the tumor from the C/EBPα-overexpressing SCC22B cells (Fig. 4A). The experiment using 8.5 million cells yielded 10 out of 10 mice with tumors from the pBABE-transfected SCC22B cells. However, none of these mice formed tumors from the C/EBPα-pBABE–transfected SCC22B cells (Fig. 4B and C).

C/EBPα re-expression allows differentiation in HNSCC. One of the main functions of C/EBPα is cellular differentiation. With differentiation-inducing agents, C/EBPα expression has been shown to increase in keratinocytes (22). Keratinocytes are a major cell type of the epidermis and account for ~90% of epidermal cells, allowing them to serve as the normal cell population control for HNSCC. Increased C/EBPα expression leads to elevated involucrin expression (a squamous cell differentiation marker in keratinocytes; ref. 22). Therefore, to check whether demethylation and increased C/EBPα expression induces differentiation, we used an involucrin antibody that showed increased involucrin expression subsequent to demethylation treatment. Therefore, this
analysis suggests a correlation between increased C/EBPα expression and increased involucrin expression (Fig. 5A). Furthermore, C/EBPα binding within the involucrin promoter was previously found to be required for its transcription (23), so we investigated whether increased involucrin expression is a direct effect from C/EBPα re-expression. To do this, we did ChIP analysis on SCC22B and SCC17AS cells with and without 5-aza-dC treatment. Quantitative PCR was then done on the ChIP eluate, using primers surrounding the C/EBPα binding site in the involucrin promoter. This revealed a significantly greater amount of involucrin promoter pull-down following 5-aza-dC treatment (Fig. 5B). This suggests that C/EBPα re-expression allows C/EBPα to bind to the involucrin promoter and activate its transcription. Although we did not exclude the possibility for methylation of involucrin, it is very unlikely that it is directly controlled by methylation [since it contains very few CGs (8 in 1,500 bp upstream of the TSS) and lacks a CpG island].

Furthermore, differences in cell morphology were seen through microscopic evaluation in C/EBPα-overexpressing cell lines. The C/EBPα-overexpressing SCC22B are much larger in size compared with the untransfected SCC22B cells (possibly due to accumulation of granules and/or vacuoles, which are often increased in differentiated cells; ref. 15). Using previously accepted criteria for differentiation determination (i.e., spindle formation; ref. 15), C/EBPα-overexpressing cells evidence a higher frequency of differentiated cells than the untransfected SCC22B cells (16% versus 5%, respectively; Fig. 5C).

Discussion

C/EBPα, a gene involved in cellular differentiation and cell cycle control (24), has been found to exhibit tumor suppressor activity in non–small cell lung cancer (15) and AML (14). More recently, a publication revealed C/EBPα activity in non–small cell lung cancer (15) and AML (14). More recently, a publication revealed C/EBPα activity in non–small cell lung cancer (15) and AML (14). Moreover, differences in cell morphology were seen through microscopic evaluation in C/EBPα-overexpressing cell lines. The C/EBPα-overexpressing SCC22B are much larger in size compared with the untransfected SCC22B cells (possibly due to accumulation of granules and/or vacuoles, which are often increased in differentiated cells; ref. 15). Using previously accepted criteria for differentiation determination (i.e., spindle formation; ref. 15), C/EBPα-overexpressing cells evidence a higher frequency of differentiated cells than the untransfected SCC22B cells (16% versus 5%, respectively; Fig. 5C).

In our study, we have found for the first time not only that C/EBPα overexpression provides tumor suppressor activity (and metastasis inhibition) in vitro and in vivo in HNSCC, but also that it is epigenetically down-regulated by upstream methylation. It seems surprising that we found no methylation around the transcription start site and core promoter, but this supports previous findings of C/EBPα epigenetic regulation in lung cancer (16). Therefore, it may be assumed that upstream methylation is capable of decreasing expression, but the core promoter remains unmethylated due to a requirement of basal expression for tumor cell viability (16). Deletions within chromosome 19 have not been previously discovered in HNSCC (26), and very few cases (i.e., RASSF1A and TCF21) have been documented thus far (9, 27) as having frequent co-occurrence of both DNA methylation and LOH in HNSCC, as we witnessed with C/EBPα (although there are several examples in other cancer types; refs. 28–32).

The findings of this study are of critical importance because HNSCC often has a fatal clinical course with very little chance of recovery following operation (4). Currently, there are relatively few good markers for poor-prognosis HNSCC detection. Loss of BRCA2 correlates with a significant decrease in survival time, and certain chromosomal deletions (i.e., 10q and 14q) were found to be associated with poor prognosis (33–35). Increased gene expression [i.e., NBS1, FGFβ, Ki-67, HER2, matrix metalloproteinase 2 (MMP2) and MMP9, and cyclin D1] has been shown to indicate the aggressiveness of the disease (36–41), and elevated alpha B-crystallin expression has been found to be a more sensitive marker for HNSCC recurrence than the combination of plasminogen activator-inhibitor-1 (PAI-1) and urokinase-type plasminogen activator (42). However, there is still not a DNA methylation marker for poor prognosis (12). DNA methylation occurs early in carcinogenesis, making it a good early indicator (43). Consequently, the discovery of C/EBPα down-regulation in poor-prognosis patients (13), combined with our findings of epigenetic regulation of the gene, has great importance for detection of HNSCC. This suggests that there may be great promise for using DNA methylation as a biomarker in clinical screening, which has already been proposed in other cancer types (42–45). This could ultimately lead to better detection (either by immunohistochemistry or by quantitative DNA methylation analysis) and treatment of the disease, increasing the rate of survival (44). However, C/EBPα methylation in HNSCC should be validated in a large sample set before pursuing its diagnostic potential (42). Furthermore, C/EBPα methylation in other tumor types (besides lung cancer and HNSCC) is worthy of investigation and could yield increased prognostic value and therapeutic intervention for other patients with this epigenetic alteration.

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