Increased Expression of the Polycomb Group Gene, EZH2, in Transitional Cell Carcinoma of the Bladder

Jay D. Raman,1 Nigel P. Mongan,2 Satish K. Tickoo,3 Stephen A. Boorjian,1 Douglas S. Scherr,1 and Lorraine J. Gudas2

Abstract

Purpose: The Polycomb group gene, EZH2, functions as a transcriptional repressor involved in gene silencing. Amplification of EZH2 has been reported in several malignancies, including prostate, breast, and lymphoma. We evaluated EZH2 mRNA and protein expression in bladder specimens from patients and the EZH2 mRNA expression in five bladder cancer cell lines.

Experimental Design: EZH2 mRNA expression was assessed by reverse transcription-PCR (RT-PCR) in 38 bladder tissue specimens. We also evaluated 39 bladder cancer specimens for EZH2 protein expression using immunohistochemistry with affinity-purified antibodies to human EZH2. In addition, five human bladder cancer cell lines were analyzed by RT-PCR for EZH2 mRNA expression.

Results: Five of 14 (36%) nontumor bladder specimens versus 21 of 24 (88%) bladder tumors showed EZH2 mRNA expression (P = 0.003). All of the invasive tumors (10 of 10) had detectable EZH2 mRNA expression, compared with 11 of 14 (79%) superficial tumors. In addition, EZH2 mRNA expression was noted in 100% (16 of 16) of high-grade bladder tumors versus 50% (4 of 8) of low-grade tumors (P = 0.01). EZH2 protein expression, meanwhile, was increased in neoplastic tissue compared with nontumor urothelium (78% versus 69% of nuclei, P < 0.005). There were no differences in EZH2 protein levels between superficial and invasive tumors. High-grade tumors had increased EZH2 staining compared with normal urothelium (78% versus 68%, P < 0.005), whereas low-grade lesions did not. Four of five human bladder cancer cell lines expressed high levels of EZH2, whereas only low levels were detected in one cell line.

Conclusions: We report a significant increase in EZH2 expression in transitional cell carcinoma of the bladder compared with normal urothelium. These data suggest that similar to other human malignancies, increased EZH2 expression correlates with oncogenesis of the bladder.

The Polycomb group genes of Drosophila melanogaster are negative regulators of gene expression (1). They are required for the stable repression of homeotic selector genes and other developmentally regulated genes (2, 3). Polycomb group proteins are believed to function by forming multimeric protein complexes that modify chromatin structure and repress target gene expression (4). Although Polycomb group proteins are repressors of gene expression, the trithorax proteins activate genes (5). Both families of genes are required for the stable transmission of gene expression patterns to progeny cells throughout development (6). Dysregulation of this transcriptional regulatory system, by either inappropriate gene activation or repression, may contribute to oncogenesis (7). Indeed, aberrant expression of both Polycomb group and trithorax proteins has been reported in human malignancies (8, 9).

Several human homologues of the Drosophila Polycomb group genes have been identified. The enhancer of Zeste homologue 2 (EZH2) gene is the human homologue of the Drosophila Polycomb group gene, enhancer of Zeste E(z) (10). EZH2 (also known as ENX-1) was initially isolated during a search for proteins that interact with Vav, a proto-oncogene that plays a critical role in hematopoietic signal transduction (11). There is increasing evidence that overexpression of the EZH2 gene occurs in a variety of human malignancies. EZH2 amplification was first reported in hematologic malignancies, including myeloid tumors, Hodgkin’s lymphoma, non-Hodgkin’s lymphoma, and mantle cell lymphoma (12–15). EZH2 was subsequently shown to be involved in the progression of prostate cancer and was reported to be a marker that distinguishes indolent malignancy from more lethal disease (16, 17). Most recently, EZH2 has been identified as a marker of aggressive breast cancer and a promoter of neoplastic transformation of breast epithelial cells (18).

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Received 5/11/05; revised 9/14/05; accepted 9/19/05.

Grant support: NIH grants R01 DE10389 and R01CA097543 (L.J. Gudas) and AACR Cancer Research Foundation of America postdoctoral fellowship in cancer prevention (N.P. Mongan).

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doi:10.1158/1078-0432.CCR-05-1047
Bladder cancer is the fourth most common cancer among men in the United States. It has been estimated that there were between 50,000 and 60,000 new cases of bladder cancer and ~12,000 deaths attributable to bladder cancer in 2003 (19). Transitional cell carcinoma (TCC), which refers to cancers arising from the transitional epithelium of the bladder, accounts for >90% of bladder cancers (20). Approximately 70% to 80% of transitional cell carcinomas of the bladder present as superficial, non-muscle-invasive tumors (Ta, Tis, and T1) that are associated with a high risk of recurrence (70%) but a low risk of progression (10-20%) to muscle invasion. The remaining 20% to 30% of cases of TCC, which present with tumor involvement of the bladder muscle (T2), perivesical fat (T3), or adjacent organs (T4), are collectively known as invasive tumors (20).

Gene amplification plays an important role in the development and progression of bladder cancer. More than 30 independent genomic loci have been identified that harbor such DNA amplifications (21, 22). There is little information, however, regarding EZH2 gene expression and its potential association with bladder carcinogenesis. Weikert et al. have reported that EZH2 mRNA expression levels correlates with histologic grade and invasiveness of bladder cancer (23). Two groups have reported overexpression of the E2F3 transcription factor, which has been reported to modulate the expression of EZH2 via binding sites on the EZH2 promoter (24), in a subset of patients with bladder cancer, and have postulated that such amplification may be a facilitating mechanism in bladder carcinogenesis (25, 26). Neither of these studies, however, directly assessed EZH2 expression.

Here then, we evaluated the mRNA and protein expression of the transcriptional repressor EZH2 in nontumor and malignant human bladder tissue specimens. We additionally compared EZH2 expression between superficial versus invasive and high-grade versus low-grade tumors. Finally, we examined the expression of EZH2 mRNA in five human bladder cancer cell lines.

Materials and Methods

Cell lines and culture conditions. Five bladder cancer cell lines (HTB 1, HTB 2, HTB 3, HTB 9, and HTB 1376) were purchased from the American Type Culture Collection (Rockville, MD). The HTB 2 (RT-4) cell line is derived from a well-differentiated transitional cell papilloma, and HTB 9 (5637) is from a moderately well-differentiated transitional cell carcinoma of the bladder. The HTB 1 (J82) and HTB 1376 cell lines are from high-grade, invasive transitional cell carcinomas (27, 28). The HTB 3 (SCaBER) cell line originated from an invasive squamous cell carcinoma of the bladder (27). Cultures were maintained at 37°C, 10% CO2 in DMEM supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% FCS. Cells were plated at a density of 1 x 10⁶ per plate on 100-mm tissue culture plates and were allowed to attach and grow for 24 hours. Total cellular RNA was then extracted as described below. Two independent experiments were done for each cell line.

Patient tissue collection. We obtained 38 bladder specimens from 27 patients treated for transitional cell carcinoma of the urinary bladder by one physician (D.S.S.) at the New York Presbyterian Hospital/Weill Cornell Medical Center between March 2003 and November 2004. Tissue samples were procured either at the time of transurethral resection or radical cystectomy. In 11 patients, samples (n = 22) were obtained from the visible tumor as well as from grossly uninvolved adjacent bladder urothelium. Six of these patients had superficial tumors, whereas five patients had invasive bladder tumors. Thirteen additional bladder tumor specimens were obtained from patients with either superficial (n = 8) or invasive (n = 5) bladder cancer. Finally, three bladder specimens were obtained from patients with prostate cancer undergoing radical retropubic prostatectomy. These three patients had no previous or current diagnosis of TCC; thus, these specimens were considered representative of normal urothelium. Tumors were classified and staged based on final reports on the tissues submitted to the Department of Pathology, according to the 1997 American Joint Committee on Cancer/Union International Centre le Cancer Tumor-Node-Metastasis classification (29). Superficial tumors were considered to be tumors confined to the bladder epithelium (Ta and Tis) or extending into the lamina propria (T1). Invasive tumors were characterized by tumor involvement of the muscularis propria (T2) or beyond. Tumors were graded using the 1998 WHO/International Society of Urologic Pathology classification of papillary neoplasm of low malignant potential, low-grade urothelial carcinoma, and high-grade urothelial carcinoma (30). Tissues were either immediately frozen at ~70°C or placed into RNAlater (Ambion, Austin, TX) at ~20°C. The Institutional Review Board of the New York Presbyterian Hospital approved tissue procurement. Patient and tumor demographics are listed in Table 1.

RNA isolation and semiquantitative reverse transcription-PCR. Total cellular RNA was extracted from cell lines using the TRizol reagent (Invitrogen, Carlsbad, CA). Thirty-eight bladder tissue specimens were collected at the Department of Pathology, University of Pennsylvania, Philadelphia, PA, and University of Washington School of Medicine, Seattle, WA. Total RNA was isolated from 5 mm thick sections of tissue using TRIzol reagent (Invitrogen). Sample RNA was quantified using a fluorometric assay. Two independent experiments were done for each cell line and tissue sample.

Table 1. Demographics of patients and tumor characteristics of tissue specimens included in the RT-PCR and immunohistochemical analysis of EZH2

<table>
<thead>
<tr>
<th>Patients analyzed</th>
<th>Patients analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>for EZH2 mRNA expression</td>
<td>by RT-PCR immunohistochemistry</td>
</tr>
<tr>
<td>(27 patients, 38 specimens)</td>
<td>(30 patients, 39 specimens)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Mean (range)</th>
<th>68 (48-86)</th>
<th>65 (43-88)</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>20</td>
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</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Pathologic tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0 (nontumor)</td>
<td>14</td>
<td>0*</td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Tis</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>3</td>
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<td>T2</td>
<td>9</td>
<td>8</td>
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<td>T3</td>
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<td>T4</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Superficial tumors</td>
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<tr>
<td>(Ta + Tis + T1)</td>
<td>14</td>
<td>27</td>
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<tr>
<td>Invasive tumors</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(T2 + T3 + T4)</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Pathologic tumor grade</td>
<td></td>
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<tr>
<td>Low malignant potential</td>
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<tr>
<td>Low grade</td>
<td>8</td>
<td>6</td>
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</tr>
<tr>
<td>High grade</td>
<td>16</td>
<td>33</td>
<td></td>
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</table>

*All tissue blocks with bladder tumors contained adjacent, nontumorous urothelium.
ground using a mortar and pestle in the presence of TRIzol. First-
strand cDNA was synthesized from 1 μg of total RNA by reverse
transcription with Superscript II or III Reverse Transcriptase (Invi-
trogen) at 42°C for 60 minutes, and the synthesized cDNA was
diluted to 200 μL with sterile, ultrapure water. Oligonucleotide
primes were designed to amplify the EZH2 cDNA product. Primers
were designed to span intron-exon boundaries, thus preventing
amplification of any contaminating genomic DNA. The EZH2 primers
(Genbank accession no. NM_004546 and NM_152998) 5'-CTGCA-
GCA TGTCTGCTGAAATGAAG-3' (forward) and 5'-CCCACATCTC
AGGGCATCACGCACC-3' (reverse) generated a 289-bp product.
Conditions for PCR on the patient samples consisted of 95°C for 5
minutes followed by cycles of 94°C for 30 seconds, 61°C for 30
seconds, and 72°C for 45 seconds. Each PCR contained ~40
ng of each oligonucleotide primer, 2 μL of cDNA, 2.5 × 10^{-2} unit Taq
polymerase and accompanying 1× buffer (Invitrogen), 1.5 mmol/L
MgCl₂, and 0.2 mmol/L deoxynucleoside triphosphates. Reverse
transcription-PCR (RT-PCR) analysis of EZH2 expression was opti-
mized by comparison of the results of 35, 33, and 28 cycles of
PCR for each sample. The PCR was found to be beyond the linear
range for both 35 and 33 cycles of PCR. Qualitative differences in
EZH2 expression detected by 28 and 30 cycles of PCR were similar.
Thus, 30 cycles were found optimal for presentation. To confirm that
30 cycles of PCR were within the linear range for the cDNA samples,
template dilutions of a random selection of samples were prepared
and examined using 30 cycles of PCR. Representative results of such
an experiment are illustrated in Fig. 1B. PCR amplification of GAPDH
was done to confirm the integrity of cDNA. A 472-bp fragment of the
GAPDH cDNA (Genbank accession no. NM_002046) was generated
using cDNA-selective oligonucleotide primers (31), sense primer
5'-AGGCTCTTTCCCTACAC-3' and the antisense primer 5'-GAGG-
CATGCATGATGATGTG-3'. These primer pairs were designed to have
low homology with the 17 known GAPDH pseudogenes (31). The
primer pairs were evaluated using in silico PCR analysis (http://
genome.ucsc.edu/), and RNA, which had not been transcribed, was
tested to detect any amplification from contaminating genomic DNA in
the cDNA preparation (Fig. 1B). Template dilutions and/or differing
numbers of PCR cycles were used to determine that PCR analysis was
done within the linear range (e.g., Fig. 1B). Negative control PCRs
using reverse osmosis–grade water in the place of template were
incorporated in every PCR experiment. PCR products were separated on
a 1.5% agarose gel and stained with ethidium bromide. The identity of the
DNA product was confirmed by comparison of the PCR-amplified
DNA to the predicted fragment size, as well as automated sequencing
of the PCR product and comparison with the known DNA sequences of
the target gene. PCR experiments were repeated in triplicate on the
patient samples with similar results.

Immunohistochemistry. Thirty-nine paraffin-embedded tissue speci-
mens were obtained from 30 patients treated for bladder cancer at the
New York Presbyterian Hospital between July 2002 and June 2003.
Five-micrometer tissue sections were cut from the patient blocks and
deparaffinized in Histo-Clear (National Diagnostics, Atlanta, GA)
followed by rehydration in a graded series of ethanol. Antigen
removal was done with heat with the Antigen Unmasking Solution (Vector
Laboratories, Burlingame, CA) in a pressure cooker for 8 minutes.
A 3% solution of H2O2 was used to quench the endogenous peroxidase
activity (15 minutes of incubation). Slides were initially blocked with
1.5% goat serum for 30 minutes. This was followed by incubation with
the affinity-purified, polyclonal rabbit anti-human EZH2 antibody
(Zymed, San Francisco, CA) diluted 1:500 in 1.5% goat serum for 1 hour at room temperature and then with 100 μL horse-
radish peroxidase–conjugated goat anti-rabbit secondary antibody
(SuperPicture, Zymed) for 30 minutes at room temperature.

Immunostaining with this commercially available EZH2 antibody has
previously been validated (16, 18). Color was developed with the 3, 3'-
diaminobenzidine chromogen substrate followed by counterstaining
with hematoxylin (Vector Laboratories). The negative control normal
and tumor sections were treated identically to all other sections, with
the exception that 1.5% normal goat serum was used in place of the primary
antibody.

The immunohistochemical expression of the EZH2 protein was
scored quantitatively by a single pathologist (S.K.T.) using a previously
validated scoring system for EZH2 expression (16, 18). This system
scores nuclear EZH2 expression by recording the percentage of nuclei
staining positive for the EZH2 protein, irrespective of staining intensity.
In this study, all slides were reviewed under high power magnification,
and a minimum of 1,000 cells in each of the tumors and adjacent,
nontumorous urothelium were counted. Recording the percentage of
nuclei staining positive for the EZH2 protein scored nuclear EZH2
expression. Independent persons did the immunohistochemical stain-
ing and scoring analyses.

Statistical analysis. Excel 2000 (Microsoft, Redmond, WA) software
and SAS for Windows, version 9.1 (SAS Institute, Cary, NC) were used
to perform all statistical calculations with P < 0.05 considered
statistically significant. The χ² test with the Yates correction factor
was used to compare EZH2 mRNA expression in the non tumor and
tumor samples. It was also used to assess differences in mRNA
expression in superficial versus invasive and low-grade versus high-
grade tumors. Comparison of EZH2 protein staining between non-
tumor and tumor specimens, as well as between the grade and stage of
tumors, was done using the two-tailed, unpaired Student’s t
test.
Increased Expression of EZH2 in Bladder Cancer

Results

Reverse transcription-PCR analysis of EZH2 expression in nontumor bladder tissue versus bladder tumors. Thirty-eight bladder specimens obtained from 27 patients were evaluated for EZH2 mRNA expression (Table 1). Eleven patients had both tumor and adjacent nontumor specimens obtained, whereas the remaining 16 patients had unpaired samples procured (three nontumor, eight superficial TCC, and five invasive TCC).

Results from the semiquantitative RT-PCR analysis on paired samples from nine patients are shown in Fig. 1A. EZH2 mRNA expression was noted in four of nine (44%) nontumor bladder tissue specimens compared with nine of nine (100%) bladder tumor specimens. In all four nontumor bladder specimens where EZH2 mRNA expression was detected, the levels were qualitatively lower than that in adjacent tumor samples. Furthermore, there were no qualitative differences in EZH2 expression between the superficial (n = 5) and invasive tumors (n = 4).

Semi-quantitative RT-PCR analysis on the 16-unpaired samples is shown in Fig. 2. EZH2 mRNA expression was noted in one of three (33%) nontumor bladder specimens, five of eight (63%) superficial tumors, and five of five (100%) invasive tumors. Once again, there were no qualitative differences in EZH2 expression between the superficial and invasive tumors.

EZH2 mRNA expression in all 38 bladder samples is summarized in Table 2. Overall, 36% (5 of 14) of nontumor bladder specimens showed EZH2 mRNA expression compared with 88% (21/24) of bladder tumors (χ² = 8.7, P = 0.003). All (10 of 10) invasive tumors showed EZH2 expression versus 11 of 14 (79%) superficial tumors (χ² = 0.9, P = 0.35) and 5 of 14 (36%) nontumor specimens (χ² = 7.7, P = 0.005). There was a trend towards differential EZH2 mRNA expression between superficial tumors and nontumor specimens (χ² = 3.6, P = 0.056). In addition, EZH2 mRNA expression was noted in 100% (16 of 16) of high-grade bladder tumors versus 50% (4 of 8) of low-grade tumors (χ² = 6.4, P = 0.01).

Immunohistochemical analysis of EZH2 protein expression in nontumor urothelium versus bladder tumors. Thirty-nine bladder specimens obtained from 30 patients were evaluated for EZH2 protein expression (Table 1). Twenty-seven of these specimens were from superficial tumors, whereas 12 were from invasive lesions. Thirty-three tumors were high grade, and the remaining six tumors were low grade.

Representative immunostaining of the specimens is shown in Figs. 3 and 4. There was strong nuclear staining evident in both the nonneoplastic and tumors urothelial cells. There was a significant increase in the percentage of nuclei, which stained in the tumor specimens compared with nontumor, adjacent urothelium (Fig. 3A). For tumors of similar pathologic stage, high-grade lesions (Fig. 4C) had increased EZH2 staining compared with nontumor urothelium, whereas low-grade tumors (Fig. 4B) did not. Both superficial (Ta/Tis/T1) and invasive (T2/T3/T4) bladder tumors showed increased staining compared with normal urothelium. There was no difference in staining, however, when comparing superficial tumors involving the lamina propria (T1) tumors (Fig. 4A) to invasion lesions extending into the muscularis propria (T2; Fig. 4B) or perivesical fat (T3; Fig. 4C).

EZH2 protein expression is summarized in Table 3. Overall, EZH2 protein expression was increased in neoplastic tissue compared with nontumor urothelium (78% versus 69%; P < 0.005). Both superficial (78% versus 69%, P < 0.005) and invasive (79% versus 69%, P < 0.005) tumors had increased staining compared with adjacent nontumor urothelium; however, there was no difference in EZH2 protein expression between the superficial and invasive groups (P = 0.72). Moreover, high-grade tumors had increased EZH2 staining compared with nontumor urothelium (78% versus 69%, P < 0.005), whereas low-grade lesions did not (78% versus 72%, P = 0.41).

Tumors analyzed for EZH2 mRNA expression were obtained from patients treated between March 2003 and November 2004. Conversely, tumors analyzed by immunohistochemistry were obtained retrospectively from archival tissue in our Department of Pathology collected between July 2002 and June 2003. Thus, most of the tumors analyzed by immunostaining were different.

Table 2. Summary of RT-PCR for EZH2 mRNA expression in bladder tissue specimens

<table>
<thead>
<tr>
<th>Pathology of specimen</th>
<th>No. specimens</th>
<th>No. expressing EZH2 (%)</th>
<th>χ²</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontumor</td>
<td>14</td>
<td>5 (36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial tumors</td>
<td>14</td>
<td>11 (79)</td>
<td>3.6</td>
<td>0.056*</td>
</tr>
<tr>
<td>Invasive tumors</td>
<td>10</td>
<td>10 (100)</td>
<td>7.7</td>
<td>0.005</td>
</tr>
<tr>
<td>Total tumors (Superficial + Invasive)</td>
<td>24</td>
<td>21 (88)</td>
<td>8.7</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*Comparison between EZH2 expression in superficial tumors versus nontumor bladder specimens.
†Comparison between EZH2 expression in invasive tumors versus nontumor bladder specimens.
‡Comparison between EZH2 expression in all tumors versus nontumor bladder specimens.
from tumors analyzed by RT-PCR. However, two patients had samples that were evaluable both by RT-PCR and immunohistochemistry. One patient had a high-grade, T2 invasive bladder tumor with increased $EZH2$ mRNA expression (Fig. 1A, lane 8) and increased immunostaining (82% versus 62%) in the tumor compared with adjacent nontumor urothelium. The second patient had a low-grade, Ta superficial tumor with similarly increased $EZH2$ mRNA expression (Fig. 1A, lane 3) and increased immunostaining (72% versus 64%) in the tumor compared with nontumor.

$EZH2$ mRNA expression in bladder cancer cell lines. $EZH2$ mRNA expression was assessed by reverse transcription-PCR in five human bladder cancer cell lines (Fig. 5). High levels of $EZH2$ mRNA were detected in four bladder cancer cell lines (HTB 1, HTB 3, HTB 9, and HT 1376), whereas low levels were detected in one cell line (HTB 2). Consistent with data from patient samples, the lowest $EZH2$ mRNA expression occurred in the cell line (HTB 2) derived from a well-differentiated, superficial papilloma. Conversely, tumors derived from invasive squamous cell carcinoma (HTB 3), moderately differentiated TCC (HTB 9), and poorly differentiated, invasive TCC (HTB 1 and HT1376) all expressed higher levels of the $EZH2$ message.

Discussion

A variety of genetic changes have been implicated in the transformation from normal bladder epithelium to TCC. Genetic events associated with the development of invasive TCC include decreased expression of the tumor suppressor genes $Rb$ and $p53$ (32, 33), as well as increased expression of epidermal growth factor receptor (34, 35) and the $H-ras$ (36) and $c-met$ (37) proto-oncogenes. Genomic hybridization and loss of heterozygosity studies have further identified other regions of consistent chromosomal gain or loss that may be additional sites of oncogenes and tumor suppressor genes involved in bladder carcinogenesis (38).

The Polycomb group proteins form part of the gene regulatory mechanism that determines cell fate during both normal and pathogenic development (39). Polycomb group proteins function as large multimeric protein complexes. To date, two separate subsets of Polycomb group complexes (PRC1 and PRC2) have been described in humans (40). PRC1, which is proposed to be involved in the maintenance of gene repression, contains the BMI-1, RING1, HPH1, HPH2, and HPC2 Polycomb group proteins (41–43). PRC2, which is thought to initiate repression, consists of the $EZH2$, EED, and SUZ proteins (39, 40). Altered expression of several of these Polycomb group proteins has been implicated in carcinogenesis. In murine models, overexpression of HPC2, RING1, and BMI-1 has been associated with anchorage-independent growth, cellular transformation, and malignant cellular degeneration (43–45).

$EZH2$ gene amplification has recently been characterized in a variety of human cancers, including several hematologic malignancies, prostate cancer, and breast cancer (12–16, 18). Furthermore, Varambally et al. showed increased $EZH2$ expression in invasive and metastatic prostate cancers and found that $EZH2$ levels are a predictor of poor outcome in clinically localized disease. Mechanistically, they showed that...
EZH2 overexpression in high-grade and invasive bladder cancer specimens (23). We found here that increased expression when comparing low-grade and high-grade tumors relies upon epigenetic modifications on specific histone tails that are inherited through cell divisions (39, 47). Histone lysine methylation and histone deacetylation represent two such covalent modifications, which can regulate gene expression and chromatin function (48). The EZH2 family of proteins contains the SET domain, which has recently been shown to harbor an intrinsic histone lysine methyltransferase activity (49–51). One proposed mechanism of Polycomb group gene–mediated repression involves PRC2-associated histone deacetylases removing the acetylation of histone H3, thereby allowing histone methyltransferases (such as EZH2) to methylate specific lysine residues on the histones (39, 52). Indeed, previous work has shown that gene silencing mediated by EZH2 requires an intact SET domain and recruitment of histone deacetylase activity (16). Furthermore, in vitro EZH2-mediated cell invasion has been inhibited by the histone deacetylase inhibitors SAHA and TSA (18). Such observations suggest that inhibition of either histone deacetylation or histone methyltransferase activity may be novel future therapeutic alternatives for TCC of the bladder.

Differences. Another possibility is that overexpression of EZH2 may contribute variably to oncogenesis in different malignancies. For example, although EZH2 expression correlates with invasiveness and metastatic progression in breast and prostate cancer, it may function as an initiator of oncogenesis in bladder cancer. Future work to characterize specific downstream gene targets of EZH2 will continue to define the functional role of this transcriptional repressor in bladder cancer.

The regulatory mechanism of the Polycomb group proteins relies upon epigenetic modifications on specific histone tails that are inherited through cell divisions (39, 47). Histone lysine methylation and histone deacetylation represent two such covalent modifications, which can regulate gene expression and chromatin function (48). The EZH2 family of proteins contains the SET domain, which has recently been shown to harbor an intrinsic histone lysine methyltransferase activity (49–51). One proposed mechanism of Polycomb group gene–mediated repression involves PRC2-associated histone deacetylases removing the acetylation of histone H3, thereby allowing histone methyltransferases (such as EZH2) to methylate specific lysine residues on the histones (39, 52). Indeed, previous work has shown that gene silencing mediated by EZH2 requires an intact SET domain and recruitment of histone deacetylase activity (16). Furthermore, in vitro EZH2-mediated cell invasion has been inhibited by the histone deacetylase inhibitors SAHA and TSA (18). Such observations suggest that inhibition of either histone deacetylation or histone methyltransferase activity may be novel future therapeutic alternatives for TCC of the bladder.

<table>
<thead>
<tr>
<th>Specimen pathology</th>
<th>No. specimens</th>
<th>% Nuclei expressing EZH2 protein</th>
<th>Nontumor</th>
<th>Tumor</th>
<th>P</th>
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<tbody>
<tr>
<td>Overall</td>
<td>39</td>
<td></td>
<td>69.1 ± 14.2</td>
<td>78.1 ± 9.3</td>
<td>&lt;0.005*</td>
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<tr>
<td>Superficial (Ta/Tis/T1)</td>
<td>27</td>
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<td>69.4 ± 14.6</td>
<td>77.8 ± 8.3</td>
<td>&lt;0.005†</td>
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<tr>
<td>Invasive (T2/T3)</td>
<td>12</td>
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<td>68.5 ± 13.7</td>
<td>78.7 ± 9.7</td>
<td>&lt;0.005†</td>
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<tr>
<td>Low grade</td>
<td>6</td>
<td></td>
<td>72.4 ± 11.4</td>
<td>77.5 ± 8.3</td>
<td>0.41‡</td>
</tr>
<tr>
<td>High grade</td>
<td>33</td>
<td></td>
<td>68.5 ± 14.8</td>
<td>78.2 ± 9.5</td>
<td>&lt;0.005‡</td>
</tr>
</tbody>
</table>

*Comparison of EZH2 expression in all tumors versus adjacent nontumor bladder specimens.
†Comparison of EZH2 expression in superficial tumors versus adjacent nontumor bladder specimens.
‡Comparison of EZH2 expression in invasive tumors versus adjacent nontumor bladder specimens.
§Comparison of EZH2 expression in low-grade tumors versus adjacent nontumor bladder specimens.
||*

Fig. 5. Expression of EZH2 mRNA by RT-PCR in five human bladder cancer cell lines. Cells were cultured, RNA was isolated, and RT-PCR was done by techniques described in the Materials and Methods. This experiment was done in triplicate with similar results. Representative of one experiment.

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In summary, our data show a significant increase in EZH2 expression in bladder cancer compared with nonneoplastic bladder epithelium. We suggest that inhibition of EZH2 expression may be a novel strategy for TCC therapy. Future studies will further define the role of EZH2 in the initiation and progression of bladder cancer and the efficacy of proposed therapeutic agents.

Acknowledgments

We thank the perioperative staff (Kira Borkina, Clara Díaz, Aijun Li, and Neela Guzman) at the NewYork Presbyterian Hospital for assistance in procuring tissue specimens. Dr. Elizbeth Hoek and Yi Fong for assistance on the immunohistochemistry; the Gudas, Scher, and Nanus laboratories for scientific discussions; and Karl Ecklund for editorial assistance.

References


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