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PII: S0022-5347(13)00173-0
DOI: 10.1016/j.juro.2013.01.078
Reference: JURO 9890

To appear in: The Journal of Urology
Accepted date: 23 January 2013


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HYPERMETHYLATION OF THE POLYCOMB GROUP TARGET GENE PCDH7 IN BLADDER TUMORS FROM PATIENTS OF ALL AGES
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Running title: Hypermethylation in early bladder carcinogenesis

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Word count abstract: 251
Word count: 2202

Keywords: Urinary Bladder Neoplasms; Methylation; Adolescent; Polycomb Group Proteins
Abstract

Purpose: Bladder tumors of patients <20yr have a low incidence of genetic and epigenetic aberrations typically found in older patients. One of the most common epigenetic aberrations in human malignancies is DNA hypermethylation. Polycomb group (PcG) complexes play an important role during lineage choices in embryogenesis and their target genes are 12 times more likely to be methylated than non-PcG target genes. We hypothesized that methylation of PcG target genes is an early event in urothelial carcinogenesis and thus might be observed in young patients.

Materials and Methods: Patients (n=167) were stratified according to age into four groups: <20yr (n=14), 20-40yr (n=48), 40-60yr (n=47) and >60yr (n=58). Five PcG target genes identified by Kandimalla et al (MEIS1, ONECUT2, OTX1, PCDH7 and SOX21) were selected for methylation analysis. Methylation ratios were calculated by using the unmethylated and methylated signal. The outcome represented the fraction of methylated cells within one tumor. Genes with similar methylation ratios in all age groups were considered as potential bladder cancer initiating candidates.

Results: Three genes showed higher methylation ratios in tumors from older patients: ONECUT2 (p<0.001), SOX21 (p<0.001) and OTX1 (p<0.001). MEIS1 displayed similar methylation ratios in all groups. However the median methylation ratio was low. PCDH7 exhibited similar median methylation percentages in all age categories, i.e. <20yr 54%; 20-40yr 59%; 40-60yr 59%; >60yr 67% (p=0.1).

Conclusions: Tumors from young patients showed less methylation for most markers. PDH7 showed high methylation ratios in all age categories and could therefore play an important role in early urothelial carcinogenesis.
1. Introduction

Bladder cancer (BC) is a disease of the elderly with a peak incidence in the sixth decade of life and only 1-2.4% of all cases are under the age of 40. BC in patients younger than 20 years is even more uncommon with reported incidence rates of only 0.1-0.4%. Conflicting results have been published regarding clinical outcome of these young patients. Some studies have observed a similar disease course in both young and older patients, while other studies have reported a more favorable clinical outcome in younger patients with less recurrences and disease progression. This discrepancy may be caused by the wide variation in the definition of a young patient; this ranges from <20 years to <40 years.

Patients with BC <20 years have mainly been described in smaller studies, including case reports and therefore bladder carcinogenesis in young patients is not well defined. It is still unclear whether BC arising in younger patients proceeds through the same molecular pathways as those seen in their older counterparts. Interestingly, tumors of patients <20 years are predominantly of low stage and grade with a lack or much lower incidence of epigenetic and genetic aberrations typical of bladder cancer in elderly patients. Since BC patients <20yr seem to be a biologically distinct group, we decided to investigate the underlying mechanism of bladder carcinogenesis in young patients.

Genetic and epigenetic aberrations play an important role in the formation of many carcinomas. The most common and best characterized epigenetic abnormality in human malignancies is DNA hypermethylation. Previous studies have reported polycomb group (PcG) target genes to be more frequent targets of aberrant silencing by DNA methylation than non-PcG target genes. These genes are targets for PcG complexes, which are the determining factor in cell lineage choices during
embryogenesis. These proteins are needed to maintain the correct identities of stem cells, progenitor cells and differentiated cells. According to current hypotheses, deregulated repression of PcG target genes results in the accumulation of a population of cells which are not able to respond to differentiation signals, leading to loss of cell identity and ultimately to cancer.

Since it has been shown that this aberrant methylation of PcG target genes plays an important role in the formation of many carcinomas,\textsuperscript{14} we hypothesized that PcG target gene hypermethylation might be an early event in bladder carcinogenesis and that these epigenetic aberrations might therefore also be observed in tumors of young patients. In order to define epigenetic characteristics more specifically, we divided patients into four different age categories i.e. <20 years, 20-40 years, 40-60 years and >60 years.
2. Materials and Methods

2.1 Patient population and tissue collection

Patients 167 patients were included in this study and were divided according to age into four different age categories, i.e. <20 years (n=14), 20-40 years (n=52), 40-60 years (n=47) and >60 years (n=58). Patients <20yr, treated between 1991 and 2009 for UCC, were retrieved from the PALGA-Database (The nationwide registry of histο-and cytopathology in the Netherlands). Tissue blocks from patients 20-40yr were collected from the pathological archive of the Leiden University Medical Centre, Leiden and from our own archive. Tumors from patients above the age of 40 were randomly included from our own pathological archive. These patients were all treated between 1990 and 2012. Urines from 35 healthy controls >50yr were retrieved from a previous study\textsuperscript{15} and served as the equivalent of normal urothelium.

Tissue for DNA extraction was obtained by manual dissection from formalin-fixed, paraffin-embedded (FFPE) blocks, containing tumor areas that were selected by pathological examination of the corresponding histological slides. Tissue slides were deparaffinised with xylene and ethanol and DNA was isolated using the DNeasy Tissue kit (Qiagen, Hilden, Germany), according to manufacturers’ protocol.

2.2 Gene panel selection

Five PcG target genes were selected from the findings of a genome-wide methylation study in bladder cancer.\textsuperscript{16}

Genes were selected based on two selection criteria. Firstly, genes with the highest average delta beta and beta ratio were selected. The beta-value could be interpreted as the percentage methylation at a certain CpG-site. The selected genes had the highest discrepancy in methylation between cancer and urine from healthy controls.
Secondly, the genes were down-regulated in bladder cancer according to the Oncomine Database™.

2.3 Methylation analysis
Methylation analysis was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Irvine, California, USA) according to the manufacturer’s protocol. Briefly, DNA was treated with sodium bisulfite, followed by bisulfite-specific PCR for the five regions of interest. For each PCR reaction a DNA input of 20ng and a PCR primer concentration of 20 pM was required. After PCR, a Single Nucleotide Primer Extension (SNuPE) analysis was performed, using primers that annealed to the PCR product adjacent to the cytosine of interest. SNuPE probes were extended with a labelled dideoxynucleotide and the products were analyzed on an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems), with the label indicating the presence or absence of a methylated cytosine. For each gene, the methylation ratio was calculated by dividing the height of the methylated peak by the sum of the height of the methylated and unmethylated peaks. Primer and probe concentrations are given in table 1.

2.4 Statistical Analysis
Data analysis was performed using the Statistical Package for the Social Sciences 17.0 (SPSS Statistics 17.0). The Kruskal-Wallis Test was used to determine the difference in methylation ratios between the age groups. Results were considered statistically significant if p<0.05.
3. Results

3.1 Patient and tumor characteristics

A total of 167 patients with a primary bladder tumor were included in this study. Patients were divided into four different age groups; 14 patients in the group <20yr, 48 patients in the group 20-40yr, 47 in the 40-60yr patient group, and 58 patients in the age group >60yr. Patient and tumor characteristics are depicted in table 2. 130/167 (78%) patients were male. Male:female ratio was 3:1 in the highest three age groups. In the youngest age group more males were affected than females, with a male:female ratio of 13:1. Tumors in all age groups were predominantly of low stage (pTa; 65%) and grade (G1-2; 78%). Tumors in the youngest age group were of significantly lower stage and grade compared to the >60yr group (p<0.05).

3.2 Gene panel selection

Based on the selection criteria described above (see materials and method), five PcG target genes were selected i.e. MEIS1, ONECUT2, OTX1, PCDH7 and SOX21. Beta values of cancer DNA in relation to beta values of urine DNA from healthy controls were derived from data from a genome-wide methylation study\(^\text{15}\) and are depicted in Figure 1. The selected PcG target genes had the highest beta-ratio and delta-beta values; these genes had the highest discrepancy in methylation between cancer DNA and urine DNA from healthy controls (Figure 1).

3.3 Methylation analysis

Methylation analysis was performed for each selected gene. Since we were looking for an initiating event in bladder carcinogenesis, genes with equal methylation ratios in all four age groups were of interest. ONECUT2, OTX1 and SOX21 showed a
significant difference in methylation ratios between the four age groups; \textit{ONECUT2} (p<0.001), \textit{OTX1} (p<0.001) and \textit{SOX21} (p<0.001). As shown in figure 2A and 2B \textit{ONECUT2} and \textit{OTX1} had significantly less methylation in the <20yr group compared to the three older age groups. \textit{SOX21} (figure 2C) had significantly higher methylation ratios in the >60yr age group compared to the three younger age groups. \textit{MEIS1} and \textit{PCDH7} showed similar methylation ratios in all four age groups, p-value 0.17 and 0.10 respectively. However the median methylation ratios for \textit{MEIS1} were low in all age groups (<20yr: 0.06, 20-40yr: 0.24, 40-60yr: 0.06 and >60yr: 0.18, figure 2D). \textit{PCDH7} showed high median methylation ratios in all four age groups as depicted in figure 3. Although, the median methylation ratio did increase with age, i.e. <20yr: 0.44, 20-40yr: 0.60, 40-60yr: 0.61 and >60yr: 0.71. We therefore decided to perform additional methylation analysis for \textit{PCDH7} in 35 urine samples from healthy controls (age >50yr). The amount of methylation in the healthy controls was significantly less than that in the four patient groups (median 0.22; p<0.001).
4. Discussion

Previous findings suggest that BC in patients <20 years comprise a biologically distinct group. These tumors seem to be genetically stable, they lack the typical genetic aberrations found in older patients and are often of low stage and low grade. In this study we aimed to elucidate bladder carcinogenesis in young patients by investigating the methylation status of five PcG target genes. We divided the included patients into four different age categories, i.e. <20yr, 20-40yr, 40-60yr and >60yr. Overall, tumors from young patients had substantially less methylation for most of the markers compared to tumors from patients in the older age groups. Only PCDH7 showed high median methylation ratios in all four age groups.

To our knowledge, hypermethylation in patients <20yr has only been investigated in one previous study. Owen et al. studied the methylation status of eight genes that were already known to be associated with bladder carcinogenesis, i.e. TNFRSF25, EDNRB, WIF1, APC, BCL2, MGMT, Cyclin D2 and E-Cadherin. They analyzed tumors from 12 patients under the age of 20 and compared the methylation results with those of tumors in patients aged 20-45 years and in patients aged above 45 years. Most markers had significantly less methylation in the youngest group. Only TNFRSF25 and cyclin D2 showed similar methylation rates in all age groups. However, overall methylation rates for cyclin D2 were low in all three age groups. Methylation rates for TNFRSF25 were high, suggesting a role in early development of bladder tumors. Yet, the authors did not include negative controls in their study. In a previous study, we found high methylation of TNFRSF25 in urine DNA from healthy controls, raising the hypothesis that TNFRSF25 does not play an important role in urothelial tumorigenesis.
Furthermore, since TNFRSF25 and cyclin D2 are not regulated by PcG target genes, it was beyond the scope of our study to investigate their methylation rates.

We found PCDH7 with similar methylation ratios in all age groups. We therefore suggest that PCDH7 is an interesting candidate in early bladder tumorigenesis. PCDH7, also known as BH-PCDH, is a gene located on the p-arm of chromosome 4 and is a member of the protocadherin family, which is a subgroup of the cadherin superfamily. Protocadherins are predominantly expressed in the brain and they function as cell-cell recognition molecules.\textsuperscript{19, 20} There is less known about the function of the PCDH7 gene in bladder cancer. In a gene-profiling study Sanchez-Carbayo et al found significant downregulation of PCDH7 in bladder cancer by investigating tissue from 52 normal urothelium and 105 bladder tumors.\textsuperscript{21} Similarly, Djyrskøt et al analyzed 60 bladder tumors and also found PCDH7 underexpression.\textsuperscript{22} In our study we did not investigate PCDH7 gene expression, but only studied DNA-hypermethylation. However aberrant DNA-hypermethylation often results in gene silencing and this should be confirmed by functional studies.

The major limitations in this study were the small number of tumor samples from patients in the youngest age group and the fact that these samples were collected from the national pathological database. Since the database only provides anonymous pathological information, we were not able to complete follow-up of these patients with regard to disease recurrence and progression. \textit{Neither are we able to follow these patients over time.}

In conclusion, bladder cancer patients aged <20yr seem to form a clinically and molecularly distinct group. Epigenetically, most markers showed lower methylation rates in patients <20yr. Only PCDH7 showed similar high methylation ratios in all age groups and therefore might play a role in early urothelial
carcinogenesis. Future studies with larger patient populations may provide more insight into the role of epigenetic silencing of Polycomb target genes in tumorigenesis.
Acknowledgements

The authors wish to thank the PALGA-foundation for their help with patient selection and mediating in collecting tissue from the different hospitals.
References

Figure legends

Figure 1. Methylation ratios per gene in bladder cancer compared to urine from healthy controls. Data is derived from a genome wide methylation study (Kandimalla et al, *Eur Urol*, 2011)

Figure 2. Methylation ratios by patient age for A. *ONECUT2* (P<0.001), B. *OTX1* (P<0.001), C. *SOX21* (P<0.001) and D. *MEIS1* (P=0.17). Boxplots indicate 25% to 75% quartile range. Horizontal lines indicate median.

Figure 3. Methylation ratios by patient age for *PCDH7*. Methylation ratios were not significantly different in the four age groups (p=0.10) Median of age groups are 0.44; 0.60; 0.61 and 0.71, respectively. There was significantly less methylation in the normal controls compared to the patient groups (median 0.22; p<0.001). Boxplots indicate 25% to 75% quartile range. Horizontal lines indicate median.
### Table 1. SNuPE primers and probes

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<th>Primer</th>
<th>Sequence (5' &gt; 3')</th>
<th>Size (bp)</th>
<th>Strand</th>
<th>UM</th>
<th>M</th>
<th>μM</th>
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<td>MEIS1</td>
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<td></td>
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<td>ONECUT2</td>
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<td>PCDH7</td>
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<tr>
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<td>C</td>
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</table>

BSP = Bilsulfite Specific PCR  
UM = Unmethylated  
M = Metylated

### Table 2. Patient and tumour characteristics (n=167)

<table>
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<tr>
<th>Variable</th>
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<th>&lt;20 yr n (%)</th>
<th>20-40 yr n (%)</th>
<th>40-60 yr n (%)</th>
<th>&gt;60 yr n (%)</th>
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<td>37 (77)</td>
<td>37 (79)</td>
<td>44 (76)</td>
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<td>10 (21)</td>
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<td>108 (65)</td>
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<td>5 (11)</td>
<td>8 (14)</td>
<td>21 (12.5)</td>
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<td>-</td>
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<tr>
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<td>pTx</td>
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<td>2 (4)</td>
<td>1 (2)</td>
<td>4 (2)</td>
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</table>
Figure 1

The figure shows a bar chart comparing the mean methylation ratio of genes across urine healthy controls and cancer samples. The genes compared are MEIS1, ONECUT2, OTX1, PCDH7, and SOX21. The y-axis represents the mean methylation ratio, while the x-axis lists the gene names. The chart indicates a higher methylation ratio in cancer samples compared to urine healthy controls for most genes, with SOX21 showing a particularly notable difference.
Figure 2

(A) ONECUT2

(B) OTX1

(C) SOX21

(D) MEIS1

The box plots illustrate the distribution of methylation ratios across different age groups (<20 yr, 20-40 yr, 40-60 yr, >60 yr) for the genes ONECUT2, OTX1, SOX21, and MEIS1. Each box represents the interquartile range, with the central line indicating the median. The whiskers extend to show the range of the data, excluding outliers. Outliers are indicated by individual points outside the whiskers.
Figure 3

Box plot showing the methylation ratio across different age groups (<20 yr, 20-40 yr, 40-60 yr, >60 yr) and controls for PCDH7.