The (CCTTT)n Microsatellite Polymorphism in the Nitric Oxide Synthase 2 Gene May Influence Bladder Cancer Pathogenesis

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Abbreviations and Acronyms

LL = long-long

ML = intermediate-long

MM = intermediate-intermediate

NO = nitric oxide

NOS = NO synthase

PCR = polymerase chain reaction

SL = short-long

SM = short-intermediate

SS = short-short

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Study received Karolinska Institutet ethical committee approval.

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Purpose: We analyzed whether the NOS2 promoter microsatellite (CCTTT)n polymorphism influences bladder cancer pathogenesis.

Materials and Methods: We genotyped 359 patients with bladder cancer in a population based cohort and 164 population controls by DNA fragment analysis and sequencing. Genotypes were combined with information on tumor stage, grade and stage, grade progression and cancer specific death. Clinical followup was 5 years.

Results: We divided (CCTTT)n alleles into short—10 or fewer, intermediate—11 or 12 and long—13 or greater repeats. Patients homozygous for 13 or longer (CCTTT)n repeats were at decreased odds ratio for bladder cancer ($p = 0.010$). However, after illness developed they were at 3-fold increased hazard ratio for stage progression ($p = 0.062$) and 4-fold increased hazard ratio for death from bladder cancer ($p = 0.056$). We discovered what is to our knowledge a previously undescribed polymorphism at position 23105343 (C/T). There was no difference in frequency between bladder cancer cases and population controls for this polymorphism. No associations were found between tumor stage, grade or stage and grade progression. However, patients with bladder cancer with the heterozygous CT genotype were at 3-fold increased hazard ratio of death from cancer ($p = 0.011$).

Conclusions: Nitric oxide can induce proliferation or apoptosis depending on the cellular context. Results suggest that the (CCTTT)n NOS2 microsatellite may influence bladder cancer risk and aggressiveness. This polymorphism may have an impact on disease pathogenesis, possibly by affecting intracellular nitric oxide levels.

Key Words: urinary bladder; urinary bladder neoplasms; polymorphism, genetic; nitric oxide synthase; microsatellite repeats

BLADDER cancer is the fifth most common cancer in the Western world, currently accounting for up to 5% of all new cancers.[1] Clinically and molecularly bladder cancer is a heterogeneous disease with pathogenesis further complicated by multiple recurrences and possible oligoclonality.[2,3] Bladder cancer risk depends on environmental exposures, eg smoking, and genetic susceptibility. The genetic influence on bladder cancer risk is estimated to be 31%.[4] However, for molecular information to predict prognosis and treatment outcomes, and distinguish patients with tumor who are at risk for local progression or micrometastasis we must further understand the mechanisms of bladder cancer pathogenesis.[5]

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NO is an important biological messenger with antimicrobial and antitumor activities.[6] NO is formed by the oxidation of L-arginine to L-citrulline, a reaction catalyzed by 3 *NOS* isoforms present in most tissue.[7] Two *NOS* isoforms, including *NOS1* or neuronal *NOS* and *NOS3* or endothelial *NOS*, are continuously expressed, calcium dependent and produce NO in pmol concentrations for seconds. The third isoform, *NOS2* or inducible *NOS*, is inducible and produces NO in nmol concentrations for days. *NOS2* over expression was reported in several human cancers.[8,9] In the bladder we previously noted that *NOS2* is mainly up-regulated in malignant epithelium,[8,10] suggesting that it may contribute to cancer development and progression.

Regulation of the human *NOS2* gene is intricate, and induction and expression vary depending on cell type and induction signal. The gene promoter is one of the largest, most complex promoters known today, spanning 16 kb upstream of the gene initiation site.[11] Also, the *NOS2* gene, including the promoter, is a highly polymorphic locus. A number of studies indicate a disease association with *NOS2* polymorphisms. The (CCTTT)n promoter microsatellite polymorphism at −2.6kb (GenBank® X97821) was suggested to influence gastric cancer,[12] malaria,[13–15] type 1 diabetes[16,17] and bladder cancer.[18] In luciferase in vitro assays using reporter constructs altered induction of gene expression was noted for different repeat lengths of this (CCTTT)n polymorphism.[17,19] Thus, there is a high possibility of gene-environment interaction influenced by genetic variants.

In a population based bladder cancer material with 5-year clinical followup we analyzed polymorphisms in the *NOS2* gene and identified whether the (CCTTT)n promoter polymorphism of *NOS2* influences bladder cancer pathogenesis.

MATERIALS AND METHODS

Participants

Our study population was drawn from a population based material of 563 of all 721 newly diagnosed patients (78%) with bladder cancer in Stockholm County, Sweden, from 1995 to 1996, as previously described in detail.[2] Venous blood was collected at a later time point and was available in the 359 study patients. Blood was drawn into ethylenediaminetetraacetic acid tubes and immediately frozen at −80C until DNA extraction. Until use stock and aliquoted DNA was kept at −80C and −20C, respectively. Only DNA extracted from blood[20] was used for genotyping.

Clinical followup, available in 95% of patients in the study cohort, was calculated as the time from date of diagnosis to last clinical evaluation or death from bladder cancer or another cause (median 54 months). Recorded parameters were the number and date of diagnosed recurrent tumors, grade/stage progression, lymph node or distant metastasis, therapy type and cause of death. Tumor stage was assessed according to the Hall and Prout modified TNM system.[21] For tumor grading we used the WHO 1999 malignancy grading system.[22]

The 164 population controls[23] were from the same geographic area as patients with bladder cancer and most were white. Age distribution was similar in patients with bladder cancer and controls (median 71 years, range 33 to 96 and median 68, range 30 to 89, respectively). Informed written consent was obtained from all patients. The study was approved by the Karolinska Institutet ethical committee.

Study Design

We genotyped the (CCTTT)n microsatellite in the promoter (−2.6 kb) by DNA fragment analysis. Sequencing served as the quality control to verify amplified sequence authenticity. Patients were coded by numbers and the scientist performing genotyping was blinded to clinical parameters. We discovered what is to our knowledge the previously unidentified genetic variation (C/T) at chromosome 17q12, position 23105343, which was genotyped by sequencing.

DNA

Fragment analysis. PCR primers were designed with Primer3 software (http://frodo.wi.mit.edu/primer3/). The forward primer was labeled with 6FAM™. PCR products were generated in 13 μl reactions containing 1 × AmpliTaq Gold® PCR buffer, 2.2 mM $MgCl_2$, 0.11 mM dinucleoside triphosphate, 0.3 μM primer and 0.02U AmpliTaq Gold DNA polymerase. Thermal conditions were 95C for 10 minutes, 45 × (94C for 30 seconds, 50C for 30 seconds and 72C for 1 minute) and 72C for 15 minutes. PCR product (1.0 μl) was mixed with 9 μl Hi-Di™ Formamide and 0.5 μl GeneScan™ 500 LIZ® Size Standard, heated for 3 minutes at 95C, cooled on ice and analyzed using ABI Prism® 3730 Genetic Analyzer. Primary data were analyzed with GeneMapper®, version 4.0.

In previous studies (CCTTT)n alleles were divided into 2 groups, that is short and long based on the number of repeats with a cutoff between 11 and 12.[13,15,18] We divided repeat lengths into 3 groups, including short—8 to 10, intermediate—11 or 12 and long—13 or greater (CCTTT) repeats, providing the 6 genotypes SS, SL, SM, MM, ML and LL. Division was based on the distribution of allelic frequencies (fig. 1). The 2 most common repeats, 11 and 12, attained greater than 20% frequency and the genotype combination 11/12 was the most common one in our material at 14%. Including the large intermediate group in the short or the long group may mask possible effects of the other alleles. We used the SS genotype as the referent for OR and HR statistical calculation.

Sequencing. DNA sequencing was done to verify genotyping results in 149 cases and 46 controls chosen randomly. Concordance between methods was 98% on fragment analysis. PCR products for sequencing were generated as described. Sequencing reactions were done with the BigDye® Terminator Cycle Sequencing Kit in 5 μl, containing PCR product pretreated with ExoSAP-IT® and a sequencing primer. PCR conditions were 96C for 1 minute, followed by 30 × (96C for 10 seconds, 50C for 5

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