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Constitutional short telomeres are strong genetic susceptibility markers for bladder cancer

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Lack of functional telomeres can cause chromosomal aberrations. This type of genetic instability may promote tumorigenesis. We have investigated the association between mean telomere length in buccal cells (assessed with quantitative real-time PCR) and bladder cancer risk in a case–control study. Patients with bladder cancer displayed significantly shorter telomeres than control subjects ($P = 0.001$). Median telomere length ratio was 0.95 (range 0.53–3.2) for cases and 1.1 (0.51–2.4) for controls. Moreover, the adjusted odds ratio (OR) for bladder cancer was significantly increased in the quartile with the shortest telomere length OR = 4.5 [95% confidence interval (CI) 1.7–12]. It is known that oxidative stress, alkylation or UV radiation increases shortening of telomeres. Therefore, we also analyzed whether environmental and genetic factors associated with DNA damage, i.e. smoking and polymorphisms in the genes involved in the metabolism of genotoxic carcinogens (*EPHX1*, *GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*, *NAT1*, *NAT2* and *NQO1*) or DNA repair (*APE1*, *NBS1*, *XPC*, *XPD*, *XRCC1*, *XRCC3* and *XRCC4*), could modify the association between telomere length and cancer risk. A clear effect of smoking and telomere length could be observed. Current smokers with short telomeres had more than six times as higher risk as non-smokers/former smokers with long telomeres (OR = 6.3, 95% CI 1.7–23). Lack of the biotransformation gene *GSTM1* and short telomeres were associated with OR = 6.5 (95% CI 2.4–18), whereas homozygous carriers of 312Asn in the DNA repair gene *XPD*, with short telomeres, displayed an OR of 17 (95% CI 1.9–150). However, no significant interaction for cancer risk could be proven for telomere length, smoking and susceptibility genotypes of metabolizing and DNA-repairing genes.

Introduction

Telomeres represent the ends of eukaryotic chromosomes. The telomeres are composed of a varying number of simple repetitive DNA-sequences, $(TTAGGG)_n$, as well as specialized nucleoproteins (1). More than 50 years ago, Barbara McClintock reported from the studies of *Zea mays* that chromosome ends lacking telomeres have a tendency to fuse (2,3). More recent studies from mice and yeast have confirmed that telomeres are crucial to maintain chromosomal stability (4), by prohibiting fatal incidents like fusion of chromosome ends, nucleolytic decay or aberrant recombination.

In most cells, telomeres shorten with each cell division, owing to incomplete replication of linear DNA molecules and the absence of a mechanism for elongation of telomeres (5–7). Thus, the permanence of the genetic material is lost little by little. When the telomeres reach a critical length, a signal for replicative senescence is initiated, with the result that the cell stops dividing and in some cases also undergo apoptosis (8). However, studies from mice show other effects of telomere shortening. Mice with shorter telomeres have an increased incidence of tumors, indicating that telomere shortening increases the cancer risk rather than preventing it (9). It has also been demonstrated that short telomeres may increase the risk of developing epithelial cancers by the formation of complex non-reciprocal translocations (10).

Replication is not the only factor to cause successive shortening of the telomeres. Several studies have demonstrated a rapid telomere shortening as a result of increased oxidative stress, alkylation and UV radiation (reviewed in ref. 11). The mechanisms for this phenomenon are not yet fully elucidated, but it is known that single stranded DNA breaks caused by oxidative or alkylating agents are less repaired in telomeric sequences than in other parts of the genome (12).

In peripheral blood lymphocytes, telomere length has been demonstrated to differ markedly among humans of the same age (13,14). We wanted to analyze if individuals with shorter telomeres would be at a higher risk for developing cancer, and furthermore, if exposure to genotoxic compounds or genotypes more susceptible to oxidative and alkylated DNA damage could modify this association. Therefore, we investigated in a case–control study whether telomere length, smoking and genotype for genes involved in biotransformation of carcinogens, as well as DNA repair, were associated with the risk of bladder cancer.

Materials and methods

Cases and controls

The study, approved by the Ethics Committee of Lund University, is based on bladder cancer patients from southern Sweden, enrolled during the years 1995–2000 when they were treated at the Lund and Helsingborg hospitals. The patients were diagnosed using the criteria from World Health Organization (15) classification of uroepithelial tumors, and the tumor staging was performed in accordance with the UICC tumor-node-metastasis system (16). Histopathological data were accessible for 90% of the patients. The patients, apart from one carrying an adenocarcinoma, were diagnosed with transitional cell carcinomas (TCCs). Twenty-four percent (13 tumors) of the TCCs were classified as well differentiated, 53% (29 tumors) as moderately differentiated and 24% (13 tumors) as poorly differentiated. Fifty-three percent (28 tumors) of the TCCs were papillary tumors limited to mucosa, 36% (19 tumors) extending into lamina propria, 11% (6 tumors) invading superficial muscle, whereas 11% (6 tumors) displayed carcinoma *in situ*. The control subjects were enrolled as a random sample from the Regional Population Registry during 1995–2000, frequency-matched with the case series with respect to sex, year of

Abbreviations: CI, confidence interval; OR, odds ratio; RERI, relative excess risk due to interactions; TCC, transitional cell carcinomas.

birth, year of enrolment in the study, as well as county of living. Information about life-long smoking status was obtained by structured face-to-face interviews conducted by two occupational health nurses during 2001–2002. Mouth wash samples were collected at the time of the interview. Originally, 98 cases and 303 controls were selected for this study. However, the study size was reduced, since 35 of the cases (36%) and 145 (48%) of the controls did not participate in the interview or the mouth wash sampling. The non-participants were those who could not be reached either by phone or by mail, were not interested in taking part in the study or felt too ill to participate. Thus, the case patients included 9 women and 54 men, with a median age of 69 years (range 35–85). The control group consisted of 37 women and 121 men, with a median age of 69 years (range 21–90).

DNA extraction

Buccal cells were collected through rinsing the mouth with 10 ml of Hexident (1 mg/ml; Ipex, Danderyd, Stockholm). The cells were spun down, resuspended in 3 ml TE-buffer and subsequently frozen at −80°C until DNA extraction. The samples were transferred to 96-well microtiter plates and DNA was extracted with QIAamp 96 DNA blood kit (Qiagen, Hilden, Germany) at the DNA/RNA genotyping Lab, SWEGENE Resource Center for Profiling Polygenic Disease, Lund University, Malmö, Sweden (http://www.swegene.org/polygenic_diseases). The DNA samples were then diluted with sterile water to 0.8 ng/μl and stored at −20°C until analysis.

Polymorphism selection

The polymorphisms were selected from genes involved in metabolism of agents suspected to be genotoxic carcinogens for bladder cancer [microsomal epoxide hydrolase I, *EPHX1*; (the glutathione *S*-transferases alpha 1, *GSTA1*; mu1, *GSTM1*; pi1, *GSTP1*; theta1, *GSTT1*;) *N*-acetyltransferases 1 and 2, *NAT1* and *NAT2*; as well as NAD(P)H dehydrogenase quinone 1, *NQO1*]. Moreover, polymorphisms in genes involved in different DNA repair pathways were selected (APEX nuclease 1, *APE1*; Nijmegen breakage syndrome 1, *NBS1*; xeroderma pigmentosum complementation group: group C, *XPC*; group D, *XPD*; X-ray repair cross-complementation group: group 1, *XRCC1*; group 3, *XRCC3*; and group 4, *XRCC4*). Candidate polymorphisms within genes of interest were identified through literature searches and from the National Center Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp). Polymorphisms chosen were primarily that the ones have been reported to have an association between genotypic and phenotypic changes. Furthermore, the polymorphisms should display high enough variant allele frequencies (usually >20%) allowing us to detect a 2-fold increase or decrease in relative risk. For *NAT1*, the alleles *4, *10, *14 and *15 were determined (nomenclature of the *NAT1* and *NAT2* alleles are presented at: http://www.louisville.edu/medschool/pharmacology/NAT.html), and the genotypes were categorized as fast (4*/10* or 10*/10*) or slow (remaining genotypes) acetylators (17,18). For *NAT2*, the alleles 4*, 5*, 6* and 7* were determined, and the carriers of at least one 4* allele were characterized as fast acetylators, whereas slow acetylators carried two slow alleles (5*, 6* or 7*) (19). The *EPHX1* genotypes were categorized as predicted enzyme activity categories, based on classification established by Benhamou *et al*. (20).

Genotyping of polymorphisms

Genotyping was performed on 63 cases and 158 controls. Owing to variations in the success rate of the genotype analyses, the number of analyzed individuals differs to some extent between the different polymorphisms. The polymorphisms for each gene as well as primers/probes used are presented in Tables I and II. The samples were genotyped for deletions of *GSTM1* and *GSTT1* with ordinary polymerase chain reaction (PCR) (21,22) with *NAT2* as an internal control. *NAT2**6 (rs1799930, refers to the accession number for polymorphisms in the SNP database at the National Center of Biotechnology Information) and *7 (rs1799931) were analyzed with restriction fragment analysis (21), whereas a Taqman-based assay was developed for *NAT2**5 (rs1801280). Polymorphisms of *GSTP1*, *GSTA1*, *XPD* rs1052559 and *APE1* were analyzed with Taqman-based assays as well, with the following conditions: the 25 μl reaction volume contained 1× Taqman Universal mix (Applied Biosystems, Foster City, CA), forward and reverse primers (for the concentration, see Table I), probes, as well as template 10–20 ng. The PCR was performed on a real-time PCR machine 7000 (ABI PRISM 7000, Applied Biosystems) with the conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min. The allelic discrimination was performed under 60°C for 1 min. The remaining polymorphisms (Table II) were analyzed with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom™, San Diego, CA) (23) at the DNA/RNA genotyping Lab, SWEGENE Resource Center for Profiling Polygenic Disease, Lund University, Malmö. The PCR primers and extension primers for these polymorphisms were designed using MassARRay Assay Design software (Sequenom; Table II). In the Taqman-based and ordinary

Table I. Primers, probes and PCR conditions, for genotyping of polymorphisms and telomere length analyses (ordinary PCR and real-time PCR)

Gene/polymorphism [nucleotide changes]	Forward primer (5′–3′)	Reverse primer (5′–3′)	Concentration forward/reverse (μM)	Vic-probe[a] (5′–3′)	Fam-probe[a] (5′–3′)	Concentration Vic/Fam (μM)
GSTM1/deletion	CTGCAATGTGTAGGGGGAAG	CTGGATTGTAGCAGATCATG	0.4/0.4	—	—	—
GSTT1/deletion	TTCCTTACTGGTCCTCACATCTC	TCACCGGATCATGGCCAGCA	0.4/0.4	—	—	—
NAT2/rs1799930 and rs1799931[b] [G/A] and [G/A]	GGAAGCTCCTCCCAGATGTG	GAGAGGATATCTGATAGCAC	0.4/0.4	—	—	—
NAT2/rs1801280 [T/C]	AACAAATACAGCACTGGCATGG	GGCTGCCACATCTGGGAG	0.9/0.9	CCGTCAGTGGTCAC	TGCCTGCAATGGT	0.2/0.2
GSTA1/rs3957356 [G/A]	GCTTTTCCCTAACTTGACCCTTCT	GTTAAACGCTGTCACCGTCCT	0.9/0.9	AGTGGGAGGGAACTA	AGTGGGAGAGAACTA	0.04/0.04
GSTP1/rs947894 [G/A]	CCCTGGTGGACATGGTGAA	CCTGGTGCAGATGCTCACAT	0.9/0.9	TGCAAATACGTCTCCCT	TGCAAATACATCTCCCT	0.2/0.2
APE1/rs3136820 [G/T]	GCTTTCCCTTTTCTTATAGTTTTTTATGC	AACGAGTCAAATTCAGCCACAAT	0.6/0.6	CTATAGGCGAGGAGGA	CTATAGGCGATGAGGAG	0.2/0.2
XPD/rs1052559 [A/C]	CCCTCTCCCTTTCCTCTGTTCT	CACTCAGAGCTGCTGAGCAATC	0.9/0.9	TCCTCTGCAGCGTC	ATCCTCTTCAGCGTCT	0.04/0.04
TEL	GGTTTTTGAGGGTGAGGGTGAGGGTGA GGGTGAGGGT	TCCCGACTATCCCTATCCCTA TCCCTATCCCTATCCCTA	0.27/0.9	—	—	—
36B4	AGCAAGTGGGAAGGTGTAATCC	CATCAGTACCCCATTCTATCATCAAC	0.3/0.9	—	—	—

[a]Polymorphic base underlined. All probes are MGB-probes.
[b]Accession number for polymorphisms in the SNP database of National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/entrez/query. fcgi?db=snp.

Table II. Primers (5′–3′) for genotyping with MALDI-TOF mass spectrometry (Sequenom)

Gene/polymorphism[a] [nucleotide exchanges]	1st PCR	2nd PCR	Extension primer
NBS1	ACGTTGGATGCTTTC	ACGTTGGATGACGTC	CTGAATTCCTGA
rs1805794 [G/C]	AATTTGTGGAGGCTG	CAATTGTAAAGCCAG	AAGCAGTT
XRCC3	ACGTTGGATGGCTGT	ACGTTGGATGTGGAA	CTGCTCAGCTCA
rs861539 [C/T]	GAATTTGACAGCCAG	GGCACTGCTCAGCTC	CGCAGC
XRCC3	ACGTTGGATGACTTGC	ACGTTGGATGGGACC	TGACCAGCATAGAC
rs1799796 [A/G]	TTCTTATTCACACAC	TGTCCTGTGGGGACAGC	ACAGC
EPHX1	ACGTTGGATGACTTCAT	ACGTTGGATGAAACT	TCAGCAAGGGC
rs2234922 [A/G]	CCACGTGAAGCCC	CGTAGAAAGAGCCGG	TTCGGGGTA
EPHX1	ACGTTGGATGTTGACTG	ACGTTGGATGCTGGC	AGTCTTGAAGT
rs1051740 [T/C]	GAAGAAGCAGGTG	GTTTTGCAAACATAC	GAGGGT
XPD	ACGTTGGATGACGGAC	ACGTTGGATGAGGCG	ACCCTGCAGCA
rs1799793 [G/A]	GCCCACCTGGCCAA	GGAAAGGGACTGGG	CTTCGT
XRCC1	ACGTTGGATGAGGATA	ACGTTGGATGTAAGG	TCGGCGGCTGC
rs25487 [G/A]	AGGAGCAGGGTTGG	AGTGGGTGCTGGACT	CCTCCC
XPC	ACGTTGGATGGCTGGCC	ACGTTGGATGAGGAA	GTTCCGGGGCA
rs2279017 [C/A]	AAATGCTGACTTG	GAGGTACACATTCCC	CCTGTG
XPC	ACGTTGGATGAGCCAT	ACGTTGGATGTCGCT	AAGAGCTTGAG
rs2228000 [C/T]	CGTAAGGACCCAAG	GCACATTTTCTTGCC	GATGCC
NQO1	ACGTTGGATGGCAT	ACGTTGGATGTCCA	CAATGCTATA
rs1800566 [C/T]	TTCTGTGGCTTCCAAG	GGATTTGAATTCGGGC	TGTCAGTTGAG
XRCC4	ACGTTGGATGCTTTTAC	ACGTTGGATGCAGGT	GTGAAGAATC
rs1805377[G/A]	TCTATAACAGAAG	AGTGAAGAATCAGGC	AGGCCTAGAA
NAT1	ACGTTGGATGACATAACC	ACGTTGGATGTTCCAA	CACAGGCCTCT
rs15561 [C/A]	ACAAACCTTTTC	GATAACCACAGGCC	TTAAAA
NAT1	ACGTTGGATGACATAACC	ACGTTGGATGTTCCAA	GCCATCTTTAA
rs1057126 [C/A]	ACAAACCTTTTC	GATAACCACAGGCC	AAGACATTT
NAT1	ACGTTGGATGCAATTGT	ACGTTGGATGCTGAT	CCTAGAAGACA
rs5030839 [C/T]	TCGAGGCTTAAGAG	CTCCTAGAAGACAGC	GCAAATAC
NAT1	ACGTTGGATGCAATTGT	ACGTTGGATGCTGAT	CTAGAAGACA
rs4986782 [A/G]	TCGAGGCTTAAGAG	CTCCTAGAAGACAGC	GCAAATACC

[a]Accession number for polymorphisms in the SNP database of National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp.

PCR assays, positive controls for each genotype as well as negative control (no template) were included. To ensure that the observed polymorphisms, analyzed with MALDI-TOF mass spectrometry, were specific and not the result of experimental variation, negative controls were included and all polymorphisms were analyzed in a well-characterized material of 10 families. Furthermore, for all assays at least 5% of the samples were reanalyzed and the concordance between these analyses was 100%.

Telomere length assessment

Telomere length quantification was performed on a subset of the samples, i.e. 63 cases and 93 controls with a method described by Cawthon (24). There was little difference in the patient and control groups for telomere analysis with regard to distributions of the sex (17 and 19% women in the different groups) and age [medians 69 (range 35–85) and 68 (21–90) years, respectively]. In brief, relative telomere length was determined by PCR through two steps of relative quantification. In the first step, the ratio of telomere repeats product to single copy gene product to (*36B4*, encoding acidic ribosomal phosphoprotein PO) was established for each sample by using standard curves. This ratio is proportional to the average telomere length. In the second step, the ratio for each sample was normalized to a calibrator DNA in order to standardize between different runs. Telomere PCRs and *36B4* PCRs were always performed in separate 96 wells. A standard curve of a diluted reference DNA (the same DNA sample for all runs), as well as the calibrator DNA were included in each run and relative quantification of the samples were allowed.

Two master mixes were prepared, one with telomere primers [1× PCR Buffer (Invitrogen; Carlsbad, CA), 0.8 mM dNTPs, 1.5 mM $MgCl_2$, forward and reverse primers (for the concentration, see Table I), 0.3 μM SybrGreen I (Invitrogen), 1× Rox (Invitrogen), 0.5 U *Taq* Platina (Invitrogen)], and one with *36B4* primers [1× SybrGreen Universal mix (Applied Biosystems; Foster City, CA), forward primer and reverse primers]. An aliquot of 4 ng template DNA was added to each reaction (end volume 25 μl). Each sample was run in triplicates. For each standard curve one reference DNA sample was diluted serially by ~1.68-fold per dilution to produce six concentrations of 0.076–1 ng/μl. In each run three negative controls as well as two different positive controls (run in duplicates) were included. The positive controls were derived from two different tumor cell lines, one with very short (mean telomere length 0.13, SD = 0.067), and the other with long telomeres (mean = 2.3, SD = 0.065).

The PCR was performed on a real-time PCR machine (ABI 7000, Applied Biosystems). The thermal cycling profile for the telomere amplification was 95°C for 10 min, followed by 30 cycles of 95°C for 15 s and 54°C for 2 min, and for the *36B4* amplification: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves were generated with the ABI Prism 7000 SDS software 1.1. R^2 for each standard curve was $\geq$0.998. Standard deviations (for C_t values) were accepted at <0.2.

Statistical analysis

Telomere length was analyzed as a continuous variable and as a categorical variable. The Wilcoxon rank sum test was used to compare the differences in telomere length between case patients and control subjects as a continuous variable. Association between age and telomere length was assessed with Spearman rank correlation coefficient. As a categorical variable, the quartile values of telomere length, according to its distribution in control subjects, were used to compare the differences in telomere length between case patients and control subjects, according to Wu *et al*. (25). In addition, telomere length was dichotomized at the 75% value in control subjects. The cases and controls were categorized into three age groups <60, 60–75 and >75 years. A never smoker was defined as an individual who had never smoked or had smoked <100 cigarettes before the year of enrolment in the study (i.e. the year of bladder cancer operation for the case studies and the year of sampling from the Regional Population Registry for the control studies). A former smoker was one who had a history of smoking but had stopped at least one year before year of enrolment. A current smoker was a smoker at the time of enrolment or one who had stopped smoking less than a one year before. Categorized telomere length, smoking status and genotype frequencies in cases and controls were compared with the χ^2-tests. The strength of the associations was estimated as odd ratios (ORs) together with 95% confidence intervals (CIs) by unconditional logistic regression with adjustments for age and sex. Statistical significance refers to $P \leq 0.05$ (two-tailed) or, equivalently, 95% CI for the OR that excludes 1.0. For smoking and genotypes that showed significant associations with bladder cancer risk, we also evaluated (synergistic) interaction

with telomere length. Interaction is here defined as departure from the additivity of effects (26). As a measure of interaction, Relative Excess Risk due to Interactions (RERI), was estimated with the formula:

$$\mathrm{RERI} = \mathrm{OR}_{11} - \mathrm{OR}_{10} - \mathrm{OR}_{01} + 1,$$

where OR_{11} denotes the odds ratio associated with the presence of two risk factors A and B under investigation (e.g. A = smoking and B = short telomere length), OR_{10} denotes the odds ratio associated with the presence of A but not B (smokers without short telomere length) and OR_{01} denotes the odds ratio associated with the presence of B but not A (non-smokers with short telomere length). If effects are additive, RERI equal to zero should be expected.

Results

There was a strong association between telomere length and bladder cancer. We found that the telomere length was significantly shorter in case patients than in control subjects. The median telomere length ratio was 0.95 (range 0.53–3.2) for cases and 1.1 (0.51–2.4) for controls ($P = 0.001$). We next categorized the subjects into quartiles of telomere length determined from the telomere length distribution in control subjects: the quartile of the longest telomere length (fourth quartile) was the referent (Table III). Increased risk for bladder cancer and degree of telomere shortening was most pronounced in the first quartile OR = 4.0 (95% CI 1.1–11). After adjusting for age, sex and smoking status, the adjusted ORs for bladder cancer associated with decreasing quartiles of telomere length were: 3rd = 2.0 (95% CI = 0.63–6.1), 2nd = 1.5 (0.48–4.6) and 1st = 4.5 (1.7–12). We also analyzed the association between telomere length and age. An inverse correlation between age and telomere length was found that was statistically significant in case patients ($r = -0.29$, $P = 0.023$), but not in control subjects ($r = -0.06$, $P = 0.59$) (Figure 1).

Smoking is a known risk factor for bladder cancer and implies exposure to a mixture of carcinogenic agents that causes DNA damage, which could be suspected to enhance telomere attrition. Therefore, we wanted to analyze whether a joint effect of smoking and telomere length exists for bladder cancer risk. There was a significant association between risk for bladder cancer and smoking in the year of enrolment ($P = 0.006$). An increasing trend in risk could be observed from non-smokers, former smokers (OR = 2.2, 95% CI 1.0–4.6), to current smokers (OR = 4.0, 95% CI 1.7–9.8).

Table III. Quartile distribution of telomere length in control subjects and corresponding distribution in case patients

Telomere length	No. of controls (%)	No. of cases (%)	OR (95% CI)
1st quartile	23 (25)	32 (51)	4.0 (1.5–11)
2nd quartile	24 (26)	11 (17)	1.3 (0.45–3.9)
3rd quartile	23 (25)	12 (19)	1.5 (0.52–4.4)
4th quartile	23 (25)	8 (13)	1.0[a]

[a]Reference category.

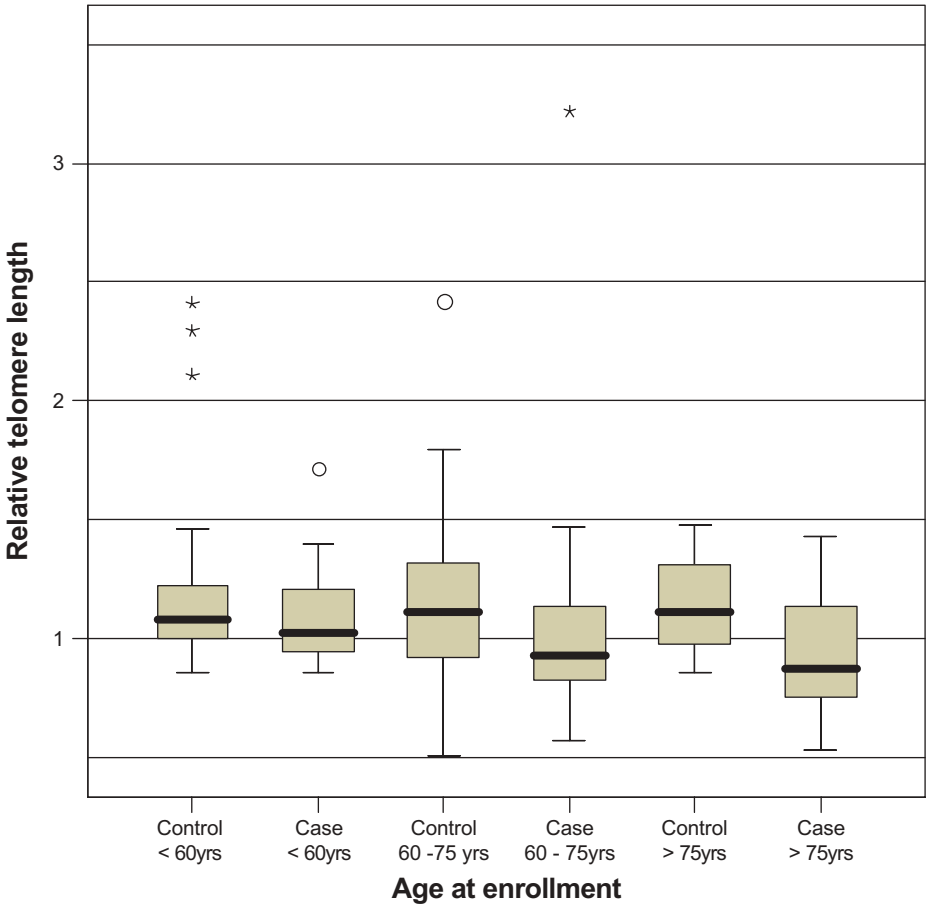

Fig 1. Relative telomere length in cases and controls categorized in three age groups. There were 21 individuals among controls <60 years, 14 cases <60 years, 50 controls 60–75 years, 35 cases 60–75 years, 22 controls >75 years and 14 cases >75 years. Extreme values and outliers are presented separately: asterisks indicate extreme value, defined as subjects with values >3 box lengths from the upper or lower edge of the box; closed circles indicate outliers, defined as values between 1.5 and 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range.

Table IV. Bladder cancer risk—joint effect of telomere length and smoking[a]

Smoking/telomere length[b]	No. of cases	No. of controls	OR	95% CI	*P*-value
Non-smokers + former smokers/long	21	59	1.0[c]	—	—
Current smokers/long	10	11	2.6	0.92–7.2	0.072
Non-smokers + former smokers/short	23	19	3.4	1.5–7.5	0.003
Current smokers/short	9	4	6.3	1.7–23	0.005

[a]Logistic regression with adjustment for sex and age.
[b]Categorized by the 75th percentile value of telomere length in the control subjects.
[c]Reference category.

In order to analyze the interaction of smoking and telomere length, we used the 75th percentile value of telomere length in control subjects as the cutoff between long and short telomeres. Smoking here, was categorized in two groups: non-smokers/former smokers and current smokers (Table IV). A clear effect of both smoking and telomere length could be observed. Current smokers with short telomeres had more than six times as high a risk as non-smokers/former smokers with long telomeres. However, no interaction between telomere length and smoking could be proved (RERI = 1.3, 95% CI −6.7 to 9.3; $P = 0.75$).

We also analyzed other genetic markers for susceptibility to bladder cancer, i.e. different polymorphisms in enzymes involved in biotransformation of carcinogens as well as DNA repair (Table V). All the single nucleotide polymorphisms were in Hardy–Weinberg equilibrium. Of the biotransformation genes, only lack of *GSTM1* was significantly associated with risk for bladder cancer (OR = 2.2, 95% CI 1.2–4.2). Among the DNA-repair genes, *XPD* rs1799793 (also referred to as Asp312Asn) was associated with increased risk of cancer (rare genotype displayed OR = 3.6, 95% CI 1.3–9.5). An elevated risk was also indicated, but did not reach statistical significance, for the variant homozygous for *XPC* rs2228000 (also known as Ala497Val) with a risk of OR 3.1 (95% CI 0.94–11).

Finally, we analyzed whether there was an interaction on the risk for bladder cancer between the telomere length and two genotypes that showed significant associations with bladder cancer risk, namely, *GSTM1* and *XPD* rs1799793. Thus, logistic regression analysis was performed with adjustment for sex, age and smoking status. Subjects that had short telomeres and homozygous deletion of *GSTM1* had more than six times (OR = 6.5, 95% CI 2.4–18) the risk of cancer relative individuals with long telomeres and presence of *GSTM1* (RERI = 3.4, 95% CI −2.2 to 8.9, $P = 0.23$; Table VI). Subjects with short telomeres and homozygous for *XPD* rs1799793 displayed OR = 17 (95% CI 1.9–150) (RERI = 13, 95% CI = −24 to 50, $P = 0.49$). Thus, no significant interaction between the investigated genotypes and telomere length could be proven.

Discussion

The results of our study demonstrated that telomere length was significantly shorter in buccal cells from patients with bladder cancer than in control subjects. When dividing the telomeres length into the four quartiles after the telomere length among the control subjects, only half of the expected number of cases was in to the longest quartile of telomere length. Moreover, when stratifying for smoking, more than a 6-fold risk was observed among current smokers in the shortest quartile of telomere length. The data suggest an additive effect of smoking and telomere length.

These results are in agreement with a recently published study on telomere length and risk for different smoking-related epithelial cancer forms (cancers of the bladder, lung, kidney, and head and neck) by Wu *et al*. (25). In this study, individuals with constitutionally short telomeres were at a considerably higher risk for developing cancer and, when stratified for smoking, a greater than additive association between telomere status and smoking was suggested. However, no statistically significant evidence for interaction between smoking and telomere length could be shown [RERI, calculated for adjusted OR of 25 (their Table V), $P = 0.12$]. Nevertheless, individuals with short telomeres were more likely to exhibit genetic instability analyzed with the comet assay. Other studies on telomere length and carcinogenesis indicate that telomere dysfunction, reduced function caused by telomere shortening in particular, is a very early and prevalent genetic alteration acquired in the multi-step process of malignant transformation (27), and that telomere shortening leads to increased frequencies of telomeric associations and chromosome instability (10,28).

We also analyzed the impact of polymorphisms of genes, involved in metabolism of genotoxic agents and DNA repair, on bladder risk, and furthermore, whether they could modulate the association between telomere length and bladder cancer. Our result showed that amongst the genes involved in metabolism, homozygous deletion of *GSTM1* was significantly associated with bladder cancer. The increased risk was >2-fold. These results are in agreement with previous reports on *GSTM1* deficiency and bladder cancer (19,29), although our risk estimates are higher than what has been reported from other analyses of larger materials (OR = 1.7 in ref. 19; and OR = 1.4 in ref. 29). This discrepancy could be due to the rather small number of individuals in ours as well as in the referred studies, which also is reflected in the large confidence interval for all ORs obtained.

Two variants of the DNA repair genes were associated with bladder cancer risk. In *XPD*, the variant rs1799793 in exon 10 (also referred to as Asp312Asn) was associated with bladder cancer. The homozygous rare variant 312Asn of *XPD* had more than a 3-fold increased risk. To our knowledge, no other study on bladder cancer has analyzed this variant of *XPD* in relation to cancer risk. Nevertheless, studies on breast, lung and prostate cancer have shown increased risk for this genotype (30–34). The *XPD* gene codes for a DNA helicase involved in transcription and nucleotide excision repair, and its function is critical for repair of genetic damage from tobacco and other carcinogens (35). The variant 312Asn results in a non-conservative replacement in an evolutionary conservative helicase region of the enzyme, implying an effect on the enzyme activity. Indeed, in studies based on the host cell reactivation assay, reduced repair efficiency of lesions from UV light and the tobacco carcinogen BPDE, has been shown for the 312Asn variant (36,37).

The other DNA repair genotype with some effect on bladder cancer risk was the homozygous variant of *XPC* rs2228000 (also referred to as Ala497Val). There is very limited data on the impact of this sequence variant and the effect of cancer

Table V. Bladder cancer risk estimates for genotypes of genes involved in biotransformation of carcinogens and DNA repair[a]

Gene polymorphism alternative titles	Genotype	No. of cases[b]	No. of controls	OR	95% CI	*P*-value
EPHX1	TT	27	81	1.0	—[c]	
rs1051740 [d]	TC	27	57	1.7	0.84–3.3	0.15
Tyr113His	CC	7	16	1.1	0.40–3.1	0.83
EPHX1	AA	41	84	1.0	—	
rs2234922	AG	19	65	0.64	0.33–1.2	0.18
His139Arg	GG	1	6	0.27	0.03–2.4	0.24
EPHX1[e]	Slow	26	45	1.0	—	
	Intermediate	22	73	0.56	0.28–1.1	0.11
	Fast	12	36	0.55	0.24–1.3	0.17
GSTA1	GG	24	45	1.0	—	
rs3957356	AG	28	75	0.68	0.34–1.3	0.26
A/B	AA	9	35	0.61	0.24–1.5	0.29
GSTM1	Present	19	78	1.0	—	
	Absent	41	74	2.2	1.2–4.2	0.016
GSTP1	AA	24	71	1.0	—	
rs947894	AG	27	69	1.1	0.58–2.2	0.75
Ile105Val	GG	10	15	2.3	0.87–6.1	0.092
GSTT1	Present	54	132	1.0	—	
	Absent	7	22	0.85	0.33–2.2	0.59
NAT1[f]	Slow	36	104	1.0	—	
	Fast	21	41	1.7	0.88–3.4	0.11
NAT2[g]	Fast	19	67	1.0	—	
	Slow	41	88	1.7	0.86–3.2	0.13
NQO1	CC	43	107	1.0	—	
rs1800566	CT	13	46	0.72	0.34–1.5	0.38
Pro187Ser	TT	5	3	3.9	0.85–18	0.080
APE1	TT	9	37	1.0	—	
rs3136820	TG	35	79	1.8	0.77–4.2	0.18
Asp148Glu	GG	17	39	1.7	0.64–4.3	0.30
NBS1	GG	21	63	1.0	—	
rs1805794	GC	36	67	1.5	0.79–3.0	0.21
Gln185Glu	CC	4	24	0.53	0.16–1.8	0.30
XPC	CC	35	92	1.0	—	
rs2228000	CT	20	55	1.1	0.56–2.2	0.78
Ala497Val	TT	6	8	3.1	0.94–11	0.063
XPC	CC	29	63	1.0	—	
rs2279017	CA	23	70	0.65	0.33–1.3	0.22
PAT+/−	AA	9	22	0.72	0.28–1.9	0.50
XPD	GG	16	61	1.0	—	
rs1799793	AG	29	71	1.7	0.81–3.5	0.17
Asp312Asn	AA	12	13	3.6	1.3–9.5	0.012
XPD	AA	23	59	1.0	—	
rs1052559	AC	24	76	0.79	0.40–1.6	0.51
Lys751Gln	CC	14	19	1.8	0.77–4.4	0.17
XRCC1	CC	26	80	1.0	—	
rs25487	CT	31	62	1.4	0.74–2.7	0.29
Arg399Gln	TT	4	13	0.95	0.27–3.3	0.94
XRCC3	CC	23	60	1.0	—	
rs861539	CT	33	72	1.3	0.68–2.5	0.42
Thr241Met	TT	5	21	0.62	0.20–1.9	0.41
XRCC3	AA	25	57	1.0	—	
rs1799796	AG	30	74	1.0	0.53–2.0	0.97
IVS5-14	GG	3	21	0.30	0.08–1.1	0.076
XRCC4	GG	44	103	1.0	—	
rs1805377	AG	9	23	1.1	0.44–2.6	0.90
Asn298Ser	AA	1	1	2.5	0.11–58	0.56

[a]Logistic regression, adjusted for sex, age and smoking.
[b]Due to variations in success rate of the genotype analyses, the number of analyzed individuals differs to some extent between the different polymorphisms.
[c]Reference category.
[d]Accession number for polymorphisms in the SNP database of National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/entrez/query. fcgi?db=snp
[e]The combined *EPHX1* genotypes were categorized as predicted enzyme activity categories based on classification established by Benhamou *et al*. (20).
[f]The *NAT1* genotypes are categorized as fast or slow according to Badawi *et al*. (17) and Taylor *et al*. (18).
[g]The combined *NAT2**4, *5, *6 and *7 genotypes were characterized as rapid and slow acetylators according to (19).

risk. Blankenburg *et al*. (38) did not find any association of rs2228000 with malignant melanoma risk. The polymorphism encodes a non-synonymous amino acid change, with unknown impact on the protein function, just outside a protein domain of low complexity. Our finding of increased risk for bladder cancer among carriers with the *GSTM1* deletion agrees with prior knowledge. Moreover, an elevated risk for *XPD*312Asn homozygotes has previously been reported for other epithelial

Table VI. Interaction between genetic markers of susceptibility for bladder cancer[a]

Telomere length[b]/ *GSTM1* genotype	No. of cases	No. of controls	OR	95% CI	*P*-value
Long/present	11	38	1.0[c]	—	—
Long/absent	18	30	1.9	0.77–4.8	0.16
Short/present	8	11	2.2	0.69–7.2	0.18
Short/absent	23	12	6.5	2.4–18	<0.0001
Telomere length/XPD rs1799793 genotype					
Long/GG or AG	22	59	1.0	—	—
Long/AA	6	6	2.2	0.62–8.0	0.22
Short/GG or AG	23	22	2.6	1.2–5.7	0.018
Short/AA	6	1	17	1.9–150	0.012

[a]Logistic regression with adjustment for sex, age, as well as smoking.
[b]Categorized by the 75th percentile value of telomere length in the control subjects.
[c]Reference category.

cancer types than bladder cancer. It is, however, important to note the fact that we only found significant effects of gene-specific variants in two out of nineteen polymorphisms analyzed, and we can therefore not rule out the possibility that the significant findings concerning gene polymorphisms are the results of multiple testing.

Many other studies have reported associations of bladder cancer risk and polymorphisms in genes involved in DNA repair or metabolism of genotoxic agents. However, several of these associations have not been observed in our material, stressing the difficulties in finding conclusive genetic markers for risk. For example, recently it has been reported a significant association between the null homozygote of *GSTT1* and increased risk of bladder cancer (17,39), whereas no such trend could be observed in our study. Two recent meta-analysis of *NAT2* have shown a modestly increased risk of bladder cancer among subjects with slow acetylators alleles (40,41). We did find an elevated cancer risk for slow acetylators of *NAT2* in our material, but it was not significant.

Our hypothesis was that since oxidative stress and alkylation enhances telomere shortening, there would be a joint effect between telomere length and certain susceptibility genotypes for DNA damage on bladder cancer risk. However, although individuals with shorter telomeres and certain genotypes showed increased risk, we could not demonstrate significant interaction between telomere length and genotype of susceptibility genes. It should be noted that the limited number of enrolled subjects and the low prevalence of certain risk genotypes, implied that only very substantial departure from additive effects could be detected in this study. Surprisingly, we found a correlation between age and telomere length among the cases, but not among the controls. A possible explanation could be that telomere shortening is most pronounced in childhood with a gradual attrition later in life (14,42). We have analyzed telomere length in a limited age span (50–80 years). Moreover, the study subjects of the same age generally display a large variation in telomere length. Thus, a larger age interval might be necessary for a positive correlation of telomere length and age for both controls and cases. On the other hand, the same phenomenon was reported by Wu *et al*. (25). Thus, a more speculative reason for this finding could be a selection of individuals with shorter telomeres in the cases group.

A limitation with the present study was the low participation rate of cases (64%) and controls (52%). A probable consequence of the reduction of study subjects is that the most rapid progressors of the disease are less likely to be in the study. This could yield biased effect estimates if telomere length is a predictor of aggressive forms of bladder cancer and/or survival. Smoking was not presented as a main issue in the written information given before the interviews. However, selection bias could be a concern with regard to smoking, since higher smoking prevalences among non-participants than among participants in health studies have been reported by others (43). Thus, a possible outcome of the falling off in our study could be a tendency toward overestimation of the effect of smoking on bladder cancer risk. The analyses regarding effects of smoking were based on smoking status in the year of enrolment (i.e. the year of bladder cancer operation for the cases and the year of sampling from the Regional Population Registry for the controls). The number of years between enrolment and interview could therefore be a limiting factor as well. However, using smoking status in the year of interview would tend to yield biased effect estimates if the patients quit smoking as a consequence of the disease. This notwithstanding, since data on life-long smoking habits were obtained in the interviews, we could compare the association between smoking and bladder cancer risk using smoking status either in the year of enrolment or in the year of interview. However, the effect of smoking on bladder cancer risk was still evident when smoking status in the year of interview was used. As an example, the OR for bladder cancer, comparing smokers and non-smokers in the year of enrolment was 4.0 (95% CI 1.7–9.8; see Results), whereas the OR for bladder cancer, comparing smokers and non-smokers in the year of interview was 3.7 (95% CI 1.4–9.6). Moreover, we analyzed effects of telomere length and smoking when it was categorized in the year of enrolment, as well as the year of interview. The OR for bladder cancer, contrasting current smokers with short telomere length and non-smokers/former smokers with long telomere length, was 6.3 based on smoking status in the year of enrolment (95% CI 1.7–23; adjusted for sex and age, see Table IV). The corresponding effect estimate based on smoking status at the year of interview was similar (OR = 5.2, 95% CI 1.2–23).

This study is based on telomere length from buccal cells as a proxy for telomere length in bladder cells. The use of a surrogate tissue is reasonable, since the majority of the inter-individual variation of telomere length seems, from studies among monozygotic and dizygotic twins, to be genetic (44). Further support for this observation comes from studies on cells with different origins, like fibroblasts and lymphocytes from peripheral blood, which have different microenvironment and replicative history, but show intraindividual correlation for telomere length (45). Moreover, our results are in concordance with those published by Wu *et al*. (25), which was based on telomere length assessments on peripheral blood lymphocytes. The possibility to use buccal cells instead of blood lymphocytes could be an advantage, since many subjects are reluctant to provide a blood sample. On the other hand, buccal cells are directly exposed to cigarette smoke that could give biased telomere length results among smokers. However, we did not find any difference within the groups of controls and cases, in telomere length between current smokers, former smokers and non-smokers (data not shown). Nevertheless, further studies on the relation between telomere length in lymphocytes and buccal cells, among smokers/non-smokers, needs to be conducted.

In this study, we have used a real-time PCR method for quantification of telomeric length. This method has several

advantages relative to the two more common analyses for assessment of telomere length, i.e. Southern blot analysis and Q-FISH^{LSC} (Quantitative Fluorescent In Situ Hybridization Laser Scanning Cytometry). For Southern blot analysis with selected restriction enzymes, a large amount of DNA is required, and contributions of individual subtelomeric DNA fragments limit the ability of this method to provide accurate telomeric lengths. The Q-FISH^{LSC} approach is not suitable for analysis of a larger number of samples. In contrast, the real-time protocol we have utilized is feasible for high throughput processing of samples and requires only small amounts of DNA.

In conclusion, we have shown strong association between telomere length and bladder cancer risk. Moreover, we found increased risk for bladder cancer for individuals carrying deletions of *GSTM1*, and the rare variant of *XPD* rs1799793 (312Asn). These genetic susceptibility markers, as well as smoking, seem to be independent risk factors for bladder cancer.

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