

Checkpoint Effectors CDKN1A and Gadd45 correlate with Oxidative DNA Damage in Human Prostate Carcinoma

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Abstract. *Background:* Although cellular oxidative stress is a major cause of DNA damage, it is still not clear to what degree it affects genetic instability and malignant progression in established prostate carcinoma (PCa). *Materials and Methods:* We examined the expression of CDKN1A and Gadd45 proteins acting on cell cycle checkpoints and DNA repair in PCa relative to the presence of oxidative DNA damage, as measured by the detection of the DNA adduct 8-hydroxy-2-deoxyguanosine (8-OHdG.). Sixteen PCa and 28 benign prostate hyperplasias (BPH) were analyzed. RT-PCR was used to evaluate WAF1 and Gadd45 transcripts. Western blot and ELISA were used to analyze proteins and 8-OHdG adducts. Proliferation was studied by Ki67 image cytometry; telomerase activity was detected by TRAP- ELISA. *Results:* Multivariate factor analysis provided evidence that, in PCa, DNA checkpoint proteins were associated with 8-OHdG and did not prevent neoplastic cells proliferation. Conversely, in BPH, oxidative DNA damage was inversely correlated with DNA checkpoint proteins and proliferation, suggesting the presence of energy-depleted senescent cells. *Conclusion:* Although in non-malignant tissue extensive oxidative DNA damage drives cells to a metabolic blockage, in PCa neoplastic cells it activates repair mechanisms favoring the escape from senescence and the expansion of DNA-damaged clones.

Epidemiological evidence indicates that prostate cancer (PCa) has become one of the five most common causes of cancer death (1).

A mechanism under investigation to explain increased prostate cancer aggressiveness with time and acquired

resistance to hormone therapy is genetic instability (GI) (2). GI is widely observed in human tumors, where it is always associated with DNA damage and genomic imbalance. Endogenous oxidative damage to prostate cell DNA, which can be modulated by several anti-oxidant compounds present in the diet (3), has been proposed as a mechanism that could sustain GI and promote prostate carcinoma (4). Indeed, when DNA repair mechanisms are no longer able to cope with the DNA damage caused by reactive oxygen intermediates, oxidative stress becomes associated with increasing DNA damage (5). Thus, biomarkers related to DNA damage and repair may be of added value to identify aggressive prostate carcinomas. For example, an elevated expression of checkpoint protein cyclin-dependent kinase-inhibitor 1 (CDKN1A), formerly known as p21^{waf1/cip1/sdi1}, has been related to prostate cancer progression and recurrence (6).

In this study, we analyzed *ex vivo* fragments of human PCa to determine whether the expression of major proteins acting on cell cycle checkpoints and DNA repair is related to the extent of oxidative DNA damage, as measured by the detection of the DNA adduct 8-OHdG. To evaluate the engagement in DNA repair activity, we analyzed the expression of CDKN1A, a cyclin-dependent kinase inhibitor implicated in the G1/S and G2/M cell-cycle checkpoints (7) and the product of the growth-arrested and DNA damage-inducible gene Gadd45, implicated in the G2/M growth arrest (8). These two proteins are rarely mutated in human tumors (9, 10) and their expression may be induced through the activation of transcription factors other than p53 (11, 12).

Materials and Methods

Selection of tissue samples. Surgical samples from 40 patients, ranging in age from 57 to 88 years (median age 67), were collected from 1998 to 2001 and frozen in liquid nitrogen. Frozen sections examination of the samples confirmed the presence of more than 90% of cancerous tissue in 16 of the 28 samples obtained from the patients submitted to radical prostatectomy. Benign epithelial cells

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Key Words: Checkpoints, CDKN1A, Gadd45, human, oxidative stress, prostate cancer, DNA repair.

Table I. Primer sequences for RT-PCR analysis.

Gene	Primer sequence (5'-3')	Starting Position	Annealing Temperature (°C)	Cycles number	Amplified product (bp)
β-Actin*	Fw 5'-GAAATGGTGCCTGACATTAAG-3'	2284	55°	25	509
	Rv 5'-CTAGAAGCATTTGCGGTGGA-3'	3000			
CDKN1A 1§	Fw 5'-AAGACCATGTGGACCTGTCA-3'	417	55°	30	168
	Rv 5'-GGCTTCCTCTTGGAGAAGAT-3'	585			
Gadd45#	Fw 5'-TGACTTTGGAGGAATTCTCGGC-3'	2553	58°	31	395
	Rw 5'-ATGAATGTGGATTCGTCACCAGCACGCAGT-3'	4730			

Abbreviations: Fw, forward; Rv, reverse; bp, base pairs.
Genbank accession numbers: *M10277; §NM000389; #L24498

were found in a sufficient amount for analysis in all samples, taken as non-neoplastic tissue adjacent to PCa (n=16) or as benign prostate hyperplasia (BPH) from adenomectomy (n=12). Informed consent was obtained from each patient and the present work was performed according to the ethical guidelines of the Declaration of Helsinki in its latest version.

Protein extraction and Western blot analysis. CDKN1A and PCNA protein levels were analyzed in soluble and in detergent insoluble proteins fraction, respectively, according to a previously detailed protocol (13). Digital images of autoradiographies were acquired with Fluor-S MultiImager (BioRad, Hercules, CA, USA), and band signals were quantified in the linear range of the scanner using specific densitometric software (Quantity-one, BioRad) in integrated optical densities (IOD) above the background.

Enzyme-linked immunosorbent assay (ELISA). *Gadd45*: Plastic ELISA plates (Nalge Nunc, Roskilde, Denmark) coated in triplicates with 150 mg of sample proteins, were incubated for 2 h at room temperature. Wells were filled with 5% nonfat milk and then incubated at 4°C overnight with the anti-Gadd45 monoclonal antibody (clone 4T-27, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100. Bound antibody was revealed with the EnVision dextran polymer visualization system (DAKO, Denmark) using 3,3',5,5'-tetramethyl benzidine (Roche, Basel, Switzerland) as chromogenic substrate for HRP. Final readings are expressed in absorbance units (A_{450}) using a microtiter plate reader (Labsystem, Helsinki, Finland). *8-OHdG*: To minimize artifactual 8-OHdG generation during sample preparation, DNA was isolated according to the DNAzol protocol (Invitrogen, Paisley, Scotland), which does not include a phenol/chloroform precipitation step (14). ELISA plates were coated in triplicates with 35 mg/ml of DNA at room temperature for 4 h. Bound DNA was treated with 10mg/ml of RNase A and 0.7% of fish gelatine was then added to block non specific sites. A monoclonal antibody anti 8-OHdG (clone 15A3; QED Bioscience Inc, CA, USA) diluted 1:100 was used and the development and measuring procedures were those described for Gadd45. To rule out false-positive results, all samples were re-

evaluated after DNase I digestion and the overall assay was repeated. Final readings were all included between 0.12 A_{450} – 1.3 A_{450} . Considering that the background amount of 8-OHdG residues in the steady state is still considered uncertain at best (14), and that the 15A3 clone has shown a low affinity cross reactivity with deoxyguanosine (15), the 0.12 A_{450} lowest reading found in the present set of samples was taken as the reference basal value. Therefore, 8-OHdG measurements were calculated as relative units (RU) using the ratio of the average A_{450} reading of each sample to the reference value. We adopted this strategy because the multivariate data analysis approached in this study (see below) transforms data in standardized z-units and does not require real units. **Telomerase:** Telomerase activity was determined using a polymerase chain reaction (PCR) ELISA detection kit (Roche Diagnostics, Mannheim, Germany) as previously detailed (16). The final reading is expressed in absorbance units at 450 nm (A_{450}) and is given by the absorbance of the test sample minus the absorbance of a tissue sample devoid of telomerase activity.

Immunohistochemistry and image cytometry: Cell proliferation was analyzed by immunohistochemistry in formalin- fixed, paraffin-embedded tissue using rabbit antiserum against proliferating cells (polyclonal Ki-67, DAKO) as previously described (13).

Proliferating Ki-67-positive and resting cells were counted by image cytometry using an unbiased counting frame on random selected fields (16). The final labelling index (LI) measurement is given as the percentage of Ki-67-positive epithelial or neoplastic cells over the total of, respectively, epithelial or neoplastic cells.

Semi-quantitative RT-PCR. RT-PCR was performed in 30 µl of reaction mixture including 1X RT buffer, 0.4 µM dNTP, 5 µM dithiothreitol, 0.5 µM oligodT, 3 µM random primers, 240U Superscript II and 4 µl of RNA, isolated using RNAzol and treated with DNase I (all reagents from Invitrogen). The cDNA was normalized for the equivalent template amount by competitive PCR, for β-actin, carried out by the addition of 1 µl of a scalar competitor to a fixed aliquot (1 µl) of cDNA. The annealing

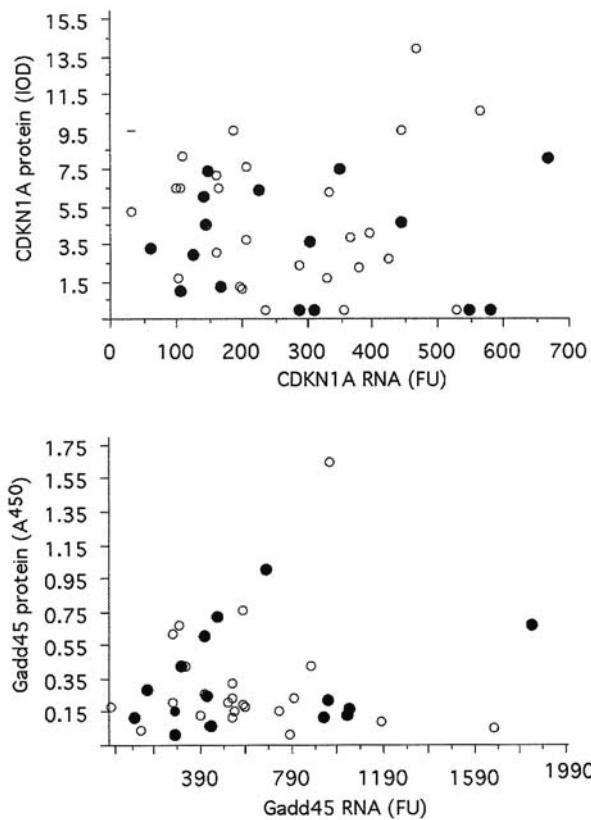


Figure 1. The two scattergrams show the lack of relationship between the extent of mRNA (x-axis) and protein expression (y-axis) for CDKN1A and Gadd45 in matched samples. The Pearson correlation was $r=0.034$ ($z=0.22$, $p=ns$) for CDKN1A and $r=0.13$ ($z=0.77$, $p=ns$) for Gadd45. Open circle=BPT, closed circle=PCa.

temperatures and the number of cycles for the different sets of primers are listed in Table I. PCR products were resolved on 2% agarose gels stained with ethidium bromide and the fluorescence was quantified using the Fluor-S Multimager analyzer and Quantity One software (BioRad) set to 10 sec of light integration. The fluorescence units (FU) were normalized against a positive internal sample present in each each gel to give relative emission measurements.

Statistical analysis. The aim of the statistical analysis was to explore the relationships between the presence of DNA oxidative damage, the expression of DNA repair activity and cell proliferation. Factor analysis (FA) using the principal component extraction method and Varimax rotation was adopted in the present study (17, 18). FA is a powerful multivariate analysis that highlights the strength of association among correlated variables and allows the graphical mapping of variable associations (18).

For univariate and bivariate data analyses, we followed procedures and principles described in Snedecor and Cochran (19). Analyses were performed using StatView 5.0 statistical software (SAS Institute, Cary, NC, USA).

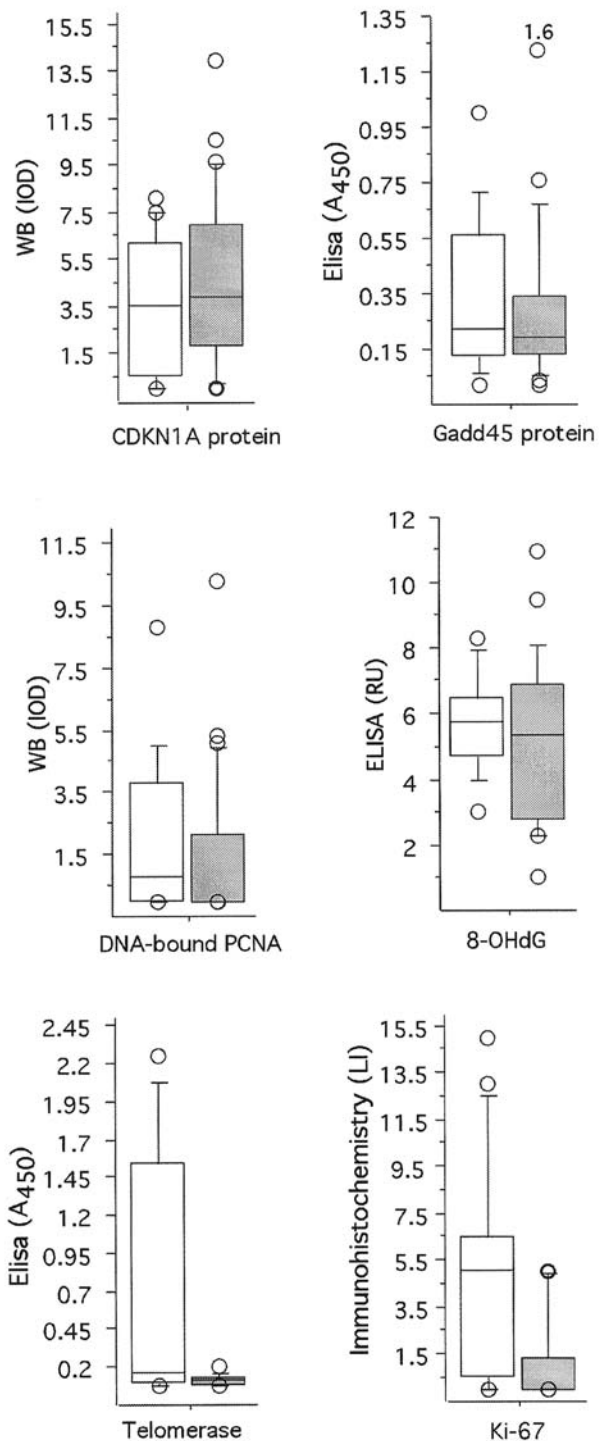


Figure 2. Box-plot graphic display of CDKN1A, Gadd45, DNA-bound PCNA, 8-OHdG, Telomerase and Ki-67 distribution in neoplastic (left white boxes) and non-neoplastic (right filled boxes) samples. The three horizontal bars in the box from top to bottom are the 75th, 50th (median) and 25th percentiles, respectively. The upper line ends at the 90th percentile and the lower line at the 10th percentile. Outliers are represented by circles.

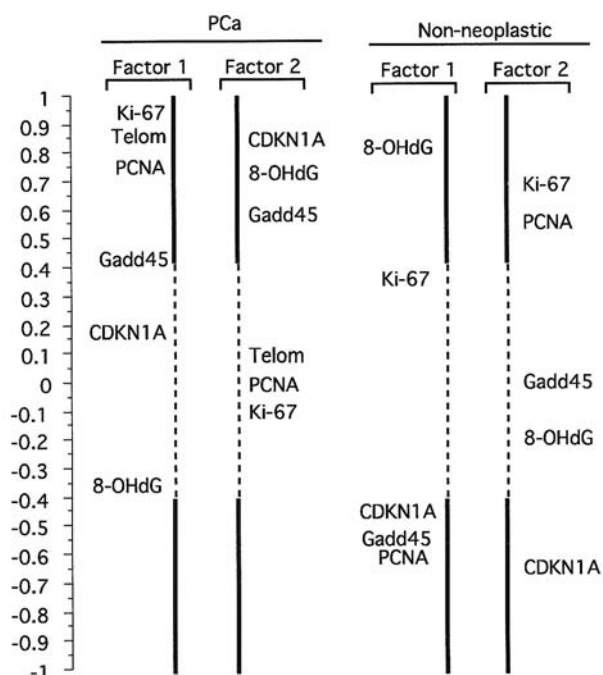


Figure 3. FA analyses for PCa (left) non-neoplastic (right) samples. In PCa, the first two eigenvalues were 2.59 and 1.61, summarizing 43% and 27% of variance, respectively. In non-neoplastic samples, the first two eigenvalues were 1.7 and 1.25, summarizing 34% and 25% of variance, respectively. The variables are listed along the axis of factors 1 and 2 and are horizontally lined up with their factor loading value, the scale of which is on the left of the graph. The dotted lines indicate the interval of factor loadings between 0.4 and -0.4. Variables whose loading falls in this interval are not important for the definition of the factor.

Results

mRNA and protein expression. When neoplastic and non-neoplastic tissue fragments were tested for differences in variance (F-test) and in mean value (Student's *t*-test), no significant difference was observed in CDKN1A mRNA, Gadd45 mRNA, CDKN1A protein-WB and Gadd45 protein-ELISA. All sample distributions were approximately normal. To investigate whether the relative transcript abundance levels correlated with cellular protein expression in matched samples, the CDKN1A and Gadd45 mRNAs were contrasted with protein WB and protein-ELISA, respectively. As clearly depicted in Figure 1, no relationships could be observed between mRNA levels and protein expression for either gene.

Association of checkpoint proteins with proliferation and DNA damage. To examine how the expression of DNA repair and proliferation-related variables associate with DNA damage, we adopted FA. Because of the above reported complete

lack of relationship between RNA and protein expression, CDKN1A and Gadd45 were introduced in the FA as WB and ELISA results, respectively. The other variables introduced into the analysis were Ki-67, PCNA telomerase activity and 8-OHdG.

The univariate distribution of all these variables in neoplastic and non-neoplastic samples is reported in Figure 2. Only telomerase and Ki-67 measurements were higher in tumor specimens ($p=0.0454$ and $p=0.0017$, respectively with the Mann-Whitney *U*-test). The mean values of CDKN1A, Gadd45, PCNA and 8-OHdG were not different between neoplastic and non-neoplastic samples when tested with either unpaired or paired Student's *t*-test. The variance of 8-OHdG was higher in non-neoplastic samples (p value=0.053 with the *F*-test). No correlation was found between age and 8-OHdG in non-neoplastic samples.

FA was conducted separately for neoplastic and non-neoplastic samples. The expected pattern would be a bipolar general (unrotated) factor with proliferation-associated variables having a loading of opposite sign with respect to DNA repair-associated variables. The results of FA in PCa and non-neoplastic tissue are graphically summarized in Figure 3. In both subsets, the first two eigenvalues resulted higher than one and, therefore, we extracted only the first two factors. In the PCa subset, the first factor showed a clear and strong association (all loadings higher than 0.7) between the proliferation-associated variables Ki-67, telomerase and PCNA. The two repair proteins, CDKN1A and Gadd45, and 8-OHdG were not important in the definition of this factor. The second factor, however, highlighted a strong association between repair proteins and 8-OHdG. In both factors, the loadings of proliferation-associated variables did not contrast with the loadings of DNA repair-associated proteins, indicating that DNA repair activity was not slowing down proliferation.

In the non-neoplastic data set, telomerase activity was not introduced into FA because it was uniformly absent from the samples. As can be seen in Figure 3, the expected contrast between proliferation and repair is well evident in the second factor where Ki-67 and CDKN1A load on the opposite sites of the factor. 8-OHdG was well represented only in the first factor and, surprisingly, it loaded in the opposite site of repair proteins. In non-neoplastic samples it is evident that PCNA partitions equally with repair proteins, in the first factor, and with proliferation in the second factor.

The above described FA structure in PCa and non-neoplastic samples were strong enough to be confirmed by an orthogonal rotation where variables maintained substantially the same relative position in the factors (data not shown).

Discussion

Unlike non-neoplastic cells, which enter growth arrest in a pre- or proto-differentiation stage, in cancer cells a high proliferation rate parallels a non-terminal, yet aberrant, cell differentiation (20). Because of the close relationship between proliferation and differentiation in neoplastic cells, reactive oxygen species produced by uncontrolled cell oxidative metabolism gain easy access to DNA chains, where they induce damage and mutations. In prostate cancer cells, for instance, abnormal responsiveness to androgen has been shown to increase oxidative stress simply by increasing mitochondrial activity (21).

In our carefully selected series of cases, we used multivariate FA to explore how variables related to DNA repair and proliferation associate in *in vivo* prostate carcinoma. Although not extensively adopted in cancer research, FA is nevertheless a robust multivariate procedure widely used in several medical research fields, that uses firm mathematical foundations to explain covariances and reduce dimensionality within a complex data set (17). The factors resulting from FA have a high dimensionality (sum of data) that decrease the error associated with a single, univariate, measurement.

The main findings resulting from FA in PCa indicate that: *Factor 1*: Bound PCNA and telomerase activity strictly associates with cell proliferation (Ki-67).

Factor 2: The presence of DNA damage was associated with a high level of the repair proteins CDKN1A and Gadd45.

It is important to emphasize that no contrast between repair proteins and either oxidative DNA damage or proliferation appeared in either factor 1 or factor 2. A contrast would have been highlighted by a bipolar factor showing a cluster with high loadings for a first set of variables (*e.g.* proliferation), along with another cluster with high loadings of the opposite sign for a second set of variables (*e.g.* DNA repair). In the first factor, the one with high loadings for proliferative variables, the loadings of Gadd45, CDKN1A and 8-OHdG did not cluster at the opposite site of the axis, but were spread between 0.4 and -0.4, very close to the non-significant zone. Therefore, the results of FA suggest, first, that the cellular response to DNA repair activity parallels cell proliferation, thus implying that oxidative damage does not slow down the expansion of neoplastic cells. Second, that cell cycle checkpoints are well functioning and associate with the presence of DNA damage. This conclusion is in line with studies indicating that, in PCa, CDKN1A overexpression is an unfavorable prognostic factor often associated with a high growth fraction (6).

In non-malignant tissue, FA revealed a different situation: *Factor 1*: A strong contrast was evident between the level of 8-OHdG on the one hand and the expression of reparative proteins on the other.

Factors 1 and 2: Bound PCNA correlated both with repair activity and with proliferation, as revealed by its strong association with CDKN1A and Gadd45 in the first factor and with Ki-67 in the second factor.

Factor 2: The second factor picked out a strong contrast between proliferation and the cell cycle blocking protein CDKN1A, which was not found in carcinomas.

In the non-neoplastic prostate tissue, the definite contrast between 8-OHdG and the expression of repair proteins shown by the first bipolar factor indicated that cells with high DNA oxidative damage do not activate DNA repair. However, the contrast between CDKN1A and Ki-67 shown by the second factor indicates that the homeostasis of the tissue is maintained by a cycling-cells pool that is able to respond to CDKN1A checkpoint activation. The important differences in these patterns with respect to those observed in PCa suggest that, in non-neoplastic prostate tissue, oxidation-damaged cells enter a state of replicative senescence. Indeed, it has recently been shown that oxidative DNA damage, as revealed by 8-OHdG levels, is dramatically high and accumulates in several chronic inflammatory or degenerative diseases and in aged organs (22, 23). No previous study, however, evaluated DNA oxidative damage and DNA repair on the same *ex vivo* tissue fragments.

The persistence of 8-OHdG in chronic inflammation and the results of our FA suggest that, in non-cancerous tissue, an excess of oxidative damage may block DNA repair. Considering that reactive oxidative species, and more precisely •OH radicals, are able to generate single-strand interruptions (24), a rise in poly (ADP-ribose) polymerase (abbreviated in PARP, a family of DNA nick sensors and DNA repair enzymes which open the condensed structure of chromatin nearby broken DNA) can lead to metabolic blockage. It is known that excessive activation of PARP proteins can deplete cellular NAD leading to energy failure (25, 26). A probable scenario is that altered oxidative metabolism, as occurs in inflammation, may induce continuous oxidative damage that is strong enough to stall NAD-depleted cells, impeding their production of repair proteins (26).

Our data indicate that, in PCa, checkpoint proteins are strictly associated with 8-OHdG levels, implying that the metabolic blockage observable in non-neoplastic prostate cells does not occur in PCa. A plausible hypothesis can be advanced to explain this difference. It has long been known that, although *in vivo* cancer cells are metabolically reprogrammed to convey energy from several sources towards replication and related vital functions such as increased purine production (27), they still use a consistent quota of NAD and ATP to fuel the structures and activities of their original phenotypic commitment (28). Since an aberrant differentiation state – as is typically found in early

stage tumors – is easily dispensable, a large amount of NAD could be diverted to PARP without leading to relevant energy depletion for the primary biochemical programs of the neoplastic cells

The evidence from this study underlines the need to better understand the role of DNA repair and the extent of DNA oxidative damage in benign and neoplastic tissues.

Acknowledgements

The authors are grateful to Luca Ronci for clinical information about patients, to Robin M. T. Cooke and Debora Marchiori for the scientific editing. This study was supported by a grant from the Italian Ministry of Instruction, University and Research (MIUR), by Fondazione Cassa di Risparmio in Bologna and by SAMUR (Advanced Studies Urologic Diseases), Italy.

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Received July 13, 2004
 Revised August 31, 2004
 Accepted October 6, 2004