Free Radical Research, May 2010; 44(5): 497–504

Lipid peroxidation product acrolein as a predictive biomarker of prostate carcinoma relapse after radical surgery

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(Received date: 8 December 2009; In revised form date: 13 January 2010)

Abstract
Cancer recurrence after radical surgery might happen even in the case of patients with localized prostate carcinoma treated by radical prostatectomy. Therefore, identifying predictive markers of tumour recurrence is very important, so this study evaluated the presence of lipid peroxidation product acrolein in primary prostate carcinomas, assuming that acrolein could be involved in prostate carcinogenesis as was recently shown for colon cancer. Samples obtained by radical prostatectomy of 70 patients were analysed, out of which 27 patients suffered afterwards from tumour recurrence, while 43 patients were disease free. Immunohistochemistry using genuine monoclonal antibodies against acrolein-protein adducts revealed the association of acrolein with progression of carcinoma. The logistic regression combining clinical parameters together with the biochemical markers of disease and acrolein immunohistochemistry has shown that the relapse might be predicted with 90% accuracy if tumour-positive surgical margins, stage of disease and the intensity of acrolein presence in tumour stroma were taken together.

Keywords: *Lipid peroxidation, reactive aldehydes, acrolein, 4-hydroxynonenal, oxidative stress, prostate carcinogenesis, immunohistochemistry*

Introduction

While benign prostatic hyperplasia (BPH) and prostate cancer are the most common conditions of prostate in ageing men, prostate cancer represents the leading cause of mortality and morbidity in men due to cancer [1]. Prostate cancer is a multi-focal neoplasm which forms solid tumours of glandular origin. The majority of prostate cancers are androgen-dependent, due to the role of androgens in development and normal function of the prostate [2]. Therefore, traditional therapy of prostate cancer was based on hormone deprivation. Still, in advanced stage of the cancer, cells become androgen-independent, with aggressive phenotypes associated with the inefficiency of conventional therapy [3]. The most widely used indicator to detect prostate cancer in the general population is a serum measurement of prostate-specific antigen (PSA), a serine protease produced by the prostate epithelium [4]. Other possible indicators of prostate cancer are citrate, myo-inositol, and spermine [5]. Although the majority of the diagnosed prostate cancers remain localized and never produce clinical symptoms during the lifetime of the patient, a sub-set of these cancers will progress to a more malignant state requiring therapeutic intervention [6]. Progressive changes in cellular metabolism, either inherited or acquired, occurring over the years may play a very important role in the development of this disease. Dietary factors, environmental carcinogens, and inflammatory diseases have been linked with increased risk

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd. (Informa Healthcare, Taylor & Francis AS)
DOI: 10.3109/10715761003636831

of prostate cancer and are therefore considered as factors that influence the promotion phase of prostate carcinogenesis [3,7,8].

Another factor with a major role in the initiation of prostate carcinogens is imbalance in prooxidant–antioxidant status in prostatic tissue [9]. Hence, significant impairment of the oxidation/reduction balance, infections and inflammation, as well as ageing itself, are considered as risk factors of prostate cancer. Chronic symptomatic and asymptomatic prostate inflammatory processes generate significantly elevated levels of reactive nitrogen and oxygen species, which cause higher lipid peroxidation and lower antioxidant levels [10]. One of major lipid peroxidation products is acrolein, the strongest electrophile among α,β-unsaturated aldehydes, which shows the highest reactivity with nucleophiles such as proteins [11]. Einhorn [12] linked acrolein as one of the major toxic by-products of smoke developed during pyrolysis or combustion of plastic materials with severe pulmonary damage and as one of the main reasons of death following smoke inhalation. Because acrolein is found in all types of smoke (cigarette smoke, the exhaust from internal combustion engines, the vapours of overheated cooking oil), it might be assumed that it can act as cytotoxic, mutagenic and carcinogenic factors. Hence, acrolein could also be an important pathological factor related to the historical findings of Percival Pott, who described in 1775 an association between exposure to soot and high incidence of scrotal cancer in chimney sweeps, thus pointing for the first time to the harmful environmental carcinogens present in soot that act not only due to direct contact but also on a systemic level. Environmentally, acrolein exists naturally in foods and is formed during the combustion of organic materials. Industrially, acrolein is used as an herbicide and slimicide as well as a starting material for acrylate polymers and in the production of acrylic acid. Because it was identified as one of the harmful components of tobacco smoke [13], a number of reports have appeared describing the damaging effects of acrolein [14]. *In vivo*, acrolein is also known as a metabolic product of the anti-cancer drug cyclophosphamide and has been found to be formed from threonine by neutrophil myeloperoxidase at sites of inflammation [15,16].

In particular, importance of acrolein for prostate carcinogenesis might be stressed by findings of this particular aldehyde as one of the oxidative products of polyamines spermine and spermidine, ubiquitous aliphatic, cationic amines that are abundant in prostate and are involved in the regulation of cellular proliferation and differentiation, even inducing cell death by oncosis [17,18]. Therefore, we assume that acrolein could be especially important in prostate carcinogenesis acting as a toxic, environmental pollutant and as a toxic oxidation product of spermine and spermidine.

As the role of acrolein in prostatic carcinogenesis has not been studied to date, in the present study we give evidence that acrolein is differentially distributed in prostatic tissue in men who suffered from recurrent cancer after radical prostatectomy in comparison with a control group which remained free of recurrence. Hence, the level and localization of acrolein expression were evaluated in respect to clinical stage and pathological grade of the disease, showing that acrolein could be important for prediction of recurrence of prostate cancer.

Materials and methods

Patients

This study included 70 men who had a radical prostatectomy for clinically localized prostate cancer. Patient age at the time of surgery, clinical stage (2002 TNM classification) and serum PSA (prostate-specific antigen) level before and after surgery, pathological stage including capsular penetration, surgical margins and perineural invasion were included.

TNM classification outlines three parameters: T (0–4) primary tumour size; N (0–3) degree of spread to regional lymph nodes and M (0/1) presence of metastasis. According to TNM classification criteria, the following analyses were used to analyae clinical stage; digital rectal examination (DRE), abdominal computerized tomography (CT) recording (Schimadzu Model Intellect; Shimadzu, Kyoto, Japan), scintigraphic bone scan, serum PSA measurement and transrectal ultrasonography (TRUS, Siemens Sonoline Prima, Multiplanar Probe Siemens P II, transducer 5/7,5 mHz; Munich, Germany). The final selection criteria were: PSA level $\leq$20 ng/mL and Gleason score (GS) $\leq$7, the absence of DRE, scintigraphic, TRUS or CT evidence of an extracapsular prostate cancer invasion. At the time of surgery none of the patients had clinical or radiological evidence of lymph node metastases and did not receive hormonal or radiation therapy, as well as they did not receive any drug during the time from diagnosis up to the time of surgery. Standard lymphadenectomy done during radical prostatectomy showed no evidence of the lymph node metastases in any of the cases.

At the follow-up period, serum PSA level was determined every third month in the first year, semi-annually for another 2 years and annually thereafter, when the serum PSA remained undetectable.

Biochemical relapse was defined as persistent or rising serum PSA level $\geq$0.20 ng/mL after radical prostatectomy.

Tissue processing

Prostatectomy specimens were paraffin fixed and whole-mount step sections were cut transversely at 5 mm intervals from the apex of the prostate to the tips of seminal vesicles. The sections were carefully examined for traces of cancer location, capsular penetration, surgical margins involvement and seminal vesicle invasion. One slide of each tumour was stained with hematoxylin and eosin (Kemika, Croatia) and

the other prepared for immunohistochemistry on acrolein-protein adducts. Two registered pathologists diagnosed each specimen independently.

Immunohistochemistry

Immunohistochemistry specific for acrolein-protein adducts was carried out as described recently for colon tissue and carcinoma samples [19]. For immunohistochemical staining, one slice of each tumour was cut in 5 μm sections, mounted on the slide coated with 3-aminopropyl-triethoxy silane, deparaffinated in xylene and rehydrated through a series of ethanol. Immunohistochemistry was done in a three step procedure using a LSAB kit (Dako, Denmark) where the first step was incubation with monoclonal antibody (mAb5F6, 2 μg/ml) during 2 h in a humid chamber at room temperature. This particular anti-acrolein-modified protein monoclonal antibody was generated as previously described by Uchida et al. [20]. The specificity study has shown that, among the aldehydes tested, acrolein was the only source of antigenic materials generated in the protein. The second step was incubation with biotinylated secondary goat anti-mouse and anti-rabbit immunoglobulins during 30 min. The third step was incubation with streptavidin peroxidase (HRP) during 30 min. Finally, the reaction was visualized by a DAB stain (3,3,-diaminobenzidine tetrahydrochloride in organic solvent) giving a brown colour after 5 min, using haematoxylin contrast staining (blue). Negative control was done on one histological slice of the same tissue without application of acrolein-lysine specific monoclonal antibodies. Intensity and distribution of acrolein in the tumour were evaluated semi-quantitatively. The absence of immunopositivity was marked with a negative sign (−), while the ± was assigned to the samples showing occasional positivity in some cells. Weak immunopositivity manifested as a yellow to light brown coloured immunochemical reaction in stroma and epithelium was marked numerically with one cross (+) numerically assigned as one (1). Medium immunopositivity was manifested by a brown colour and was marked with two crosses (++) numerically assigned as two (2), while strong immunopositivity was manifested by an intense, dark brown colour and was marked with three crosses (+++) numerically assigned as three (3) (Figure 1).

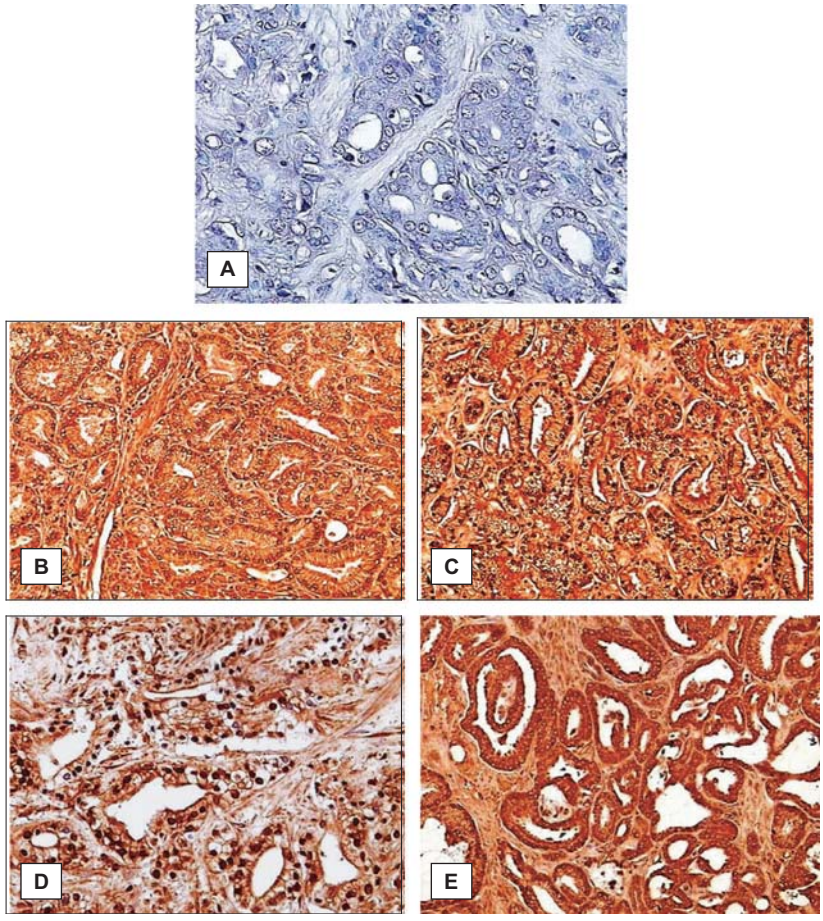

Figure 1. Immunohistochemical staining for acrolein-protein tissue in prostate cancer. (A) negative control—without primary antibody (400×); (B) organ-confined prostate cancer—moderate positivity in stroma, tumour cell cytoplasm and nuclei (200×); (C) extra-capsular prostate cancer—moderate positivity in stroma and cytoplasm, strong positivity in the nuclei of cancer cells (200×); (D) prostate cancer with extra glandular progression—weak positivity in stroma, moderate positivity in cytoplasm, strong positivity in nuclei (400×); (E) prostate cancer spread to urethra with positive surgical margins, moderate positivity in stroma, intensive positivity in cytoplasm and in nuclei of atypical glandular formations (200×).

Statistical analysis

For statistical analysis, the intensity of acrolein positivity was considered as a non-continuous quantitative variable. To compare group differences with respect to the expression of acrolein, the Kruskal-Wallis test and chi-square test were incorporated. Possible differences in intensity of staining were done by Mann-Whitney test, using a numerical description of positivity corresponding to respective standard grading of positivity as described above. A multivariate logistic regression analysis was used to determine the predictive factors of prostate cancer progression after radical prostatectomy. Calculations were carried out using statistical software SAS for Windows, ver. 9.1. (Sas Institute Inc. Cary, NC).

Results

There were 70 patients included in this study diagnosed with clinically localized prostate cancer with no extension of cancer to lymph nodes and no metastases. The subsequent pathological findings revealed an organ-confined disease in 42 patients (pT2N0M0, 60.0%) and extraprostatic tumour in 28 patients (pT3N0M0, 40%).

All extraprostatic carcinoma from T3 group, which comprised 28 patients, were positive to acrolein, showing usually medium positivity of total acrolein immunostaining in the stroma, cytoplasm and nucleus (Figure 2). The organ confined group, T2, comprised 42 carcinomas which were also positive to acrolein, showing mostly medium positivity of total acrolein immunostaining (Figure 2).

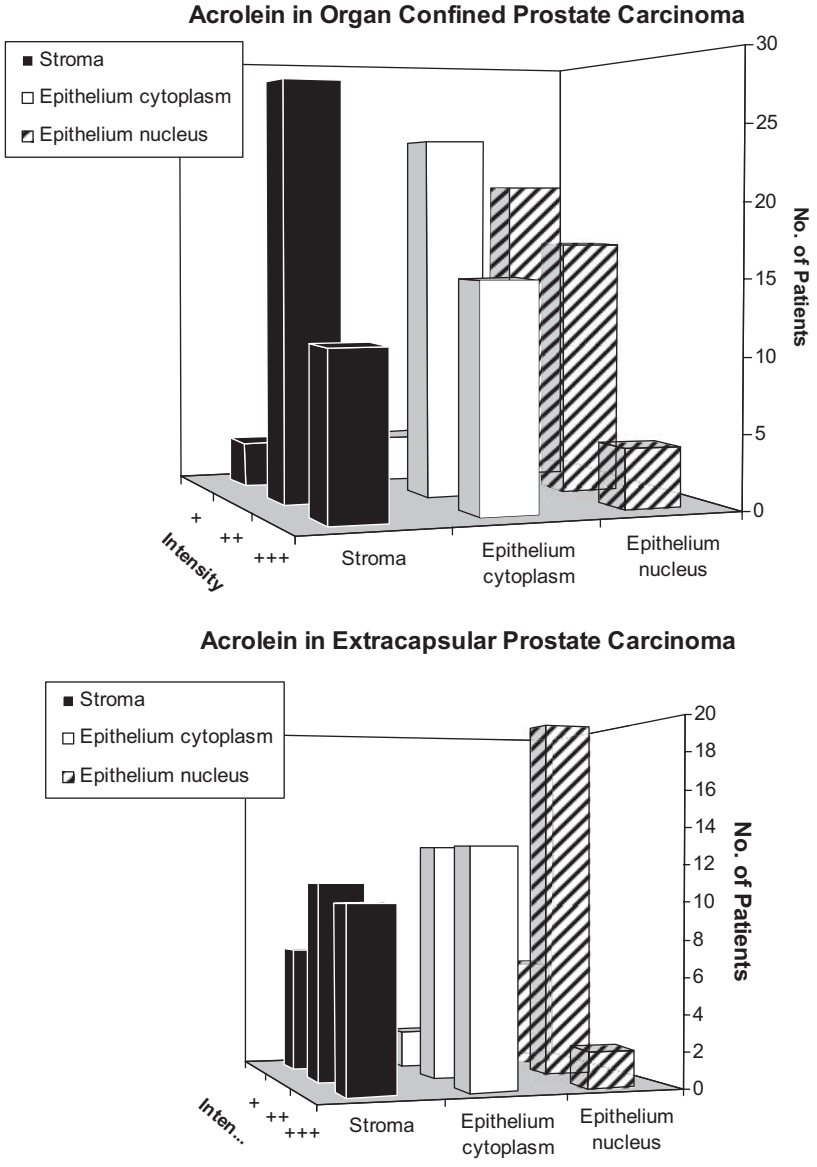

Figure 2. Acrolein presence in cancer cells (epithelium) and in tumour stroma in respect to the tumour stage.

Table I. Distribution of acrolein in organ-confined and extraprostatic prostate carcinomas.

Acrolein immunopositivity		Pathological stage		
Location	Intensity	Extraprostatic	Organ-confined	Significance (p)
Tumour stroma	+	7*	3	
	++	11	28	0.732
	+++	10	11	
Cytoplasm (cancer epithelium)	+	2	3	
	++	13	24	0.430
	+++	13	15	
Nuclear region (cancer epithelium)	+	6	21	
	++	20	17	0.03
	+++	2	4	
Number of patients	70	28	42	

*Numbers given represent the number of patients.

Due to the obvious immunohistochemical presence for acrolein in all specimens, the immunohistochemical analysis was further carried out for acrolein presence in tumour stroma, in epithelial nuclei and in cytoplasm of cancer cells. Thus, obtained results are given in Table I. The results showed that the intensity of acrolein positivity associated with nuclei was stronger in extraprostatic tumours than in organ-confined tumours (difference between groups was significant $p=0.03$). Also, in extraprostatic tumours, medium acrolein immunostaining in the nucleus of cancer cells was stronger than in organ-confined disease (difference between groups was significant; $p=0.009$). Acrolein immunostaining in the cytoplasm and in stroma were equally distributed in extraprostatic and in organ-confined tumours ($p=0.732$, $p=0.430$).

Furthermore, out of 70 patients, 21 (30%) had tumour positive surgical margins (PSM), while 49 (70%) had negative surgical margins (Table II). In patients with positive surgical margins, the intensity of acrolein positivity in the cytoplasm and the total acrolein positivity were stronger than in patients with negative surgical margins (difference between groups was significant with respective $p=0.032$, $p=0.008$) (Table 2).

Table II. Distribution of acrolein in the patients with positive surgical margins (PSM).

Acrolein immunopositivity		PSM		
Location	Intensity	Positive	Negative	Significance (p)
Stroma	+	3*	7	
	++	9	30	0.245
	+++	9	12	
Cytoplasm (cancer cell)	+	0	5	
	++	9	28	0.032
	+++	12	16	
Nuclear region (cancer cell)	+	5	22	
	++	13	24	0.072
	+++	3	3	
Number of patients	70	21	49	

*Numbers given represent the number of patients.

Table III summarizes fundamental characteristics of our patients that were monitored for biochemical relapse of the disease during the period of follow-up after radical prostatectomy. Out of 70 patients, 27 developed biochemical relapse of disease and the other 43 patients had no biochemical relapse of disease after radical prostatectomy. All patients had undetectable PSA levels at the time of analysis. The median interval between radical prostatectomy and the occurrence of biochemical relapse was 56.4 months (95% CI 48.92–63.94).

In order to analyse prevalence of lipid peroxidation in biochemical relapse, the presence of the acrolein-protein adducts was evaluated in prostate tumour tissue in respect to the biochemical relapse (Table IV). In patients with biochemical relapse with extraprostatic disease dominating the intensity of acrolein, positivity in the nucleus was medium (++, $p=0.003$), while patients with extraprostatic disease who developed biochemical relapse had higher acrolein positivity in the nucleus and cytoplasm than patients with

Table III. Characteristics of the patients before radical prostatectomy in respect to the biochemical relapse (recurrence) of the disease.

Parameter	With Biochemical Relapse	Without Biochemical Relapse	Significance (p)
Age	67.17±4.95	64.67±5.48	0.08
Preoperative PSA, ng/mL	12.48±4.91	8.96±4.43	<0.001
Gleason score			
5	1*	7	
6	7	25	<0.001
7	19	11	
Pathological stage:			
Organ confined	5	37	<0.001
Extracapsular carcinoma	22	6	
PSM	17	4	<0.001
PNI	15	6	0.07
Number of patients	27	43	

*Numbers given represent the number of patients.

Table IV. Distribution of acrolein in the prostate carcinoma tissue in respect to the biochemical relapse.

Acrolein immunopositivity		Biochemical relapse		
Location	Intensity	Positive	Negative	Significance (p)
Stroma	+	3*	7	
	++	14	25	0.088
	+++	10	11	
Cytoplasm (cancer cell)	+	1	4	
	++	16	21	0.010
	+++	10	18	
Nuclear region (cancer cell)	+	8	19	
	++	18	19	0.003
	+++	1	5	
Number of patients		70	27	43

*Numbers given represent the number of patients.

organ-confined disease with biochemical relapse (difference between groups was significant (respective $p=0.033$, $p=0.010$).

Finally, to determine a possible predictive value of acrolein for prediction of the biochemical relapse of prostate carcinoma, the obtained results of acrolein intensity of immunopositivity and distribution were compared with the established combination of total PSA, age, Gleason score, pathological stage of cancer, stroma, malignant epithelium cytoplasm, malignant epithelium nucleus, perineural invasion and surgical margins. The total reliability of the logistic regression (backward stepwise in seven steps) model was 90.0%. The variables such as PSA, age, Gleason, epithelium cytoplasm, perineural invasion and malignant epithelium acrolein presence in the nucleus were excluded from further analysis due to their limited predictive value. According to the logistic regression model, equally valid predictors for biochemical relapse of disease were pathological stage of cancer, positive surgical margins and acrolein presence in stroma (Table V). Using the logistic regression model (forward stepwise) we got similar results. The logistic regression model was statistically significant (-2 Log likelihood $=53.6$, $\chi^2=33.5$, df $=1$, $p < 0.001$).

Therefore, the final conclusion of the logistic regression has shown that the combination of pathological stage, positive surgical margins and stromal acrolein were the most reliable prediction markers with 90.0% prediction accuracy.

Discussion

One of the priorities in cancer research is to define novel prognostic markers of the outcome. Therefore, our aim was to see if acrolein could be associated with progression of prostate cancer and accordingly used as a novel prognostic marker.

Previous studies on the involvement of oxidative stress in prostate carcinogenesis were mainly based on the measurements of antioxidative capacity of serum/plasma [21,22]. These studies correlated low antioxidant serum capacity with higher risk of prostate cancer development [10]. The research on animal models showed that acrolein can inactivate GSH-reductase in prostate cells and seminal vesicles of epididymis, thereby making them primed to harmful oxidative stress [23]. Association of oxidative stress with prostate carcinogenesis was also shown by Yilmaz et al. [21], who found increased serum levels of lipid peroxidation products in patients with prostate cancer, in comparison to patients with benign prostatic hyperplasia. Kumar et al. [24] have shown also that ROS generation might be essential for aggressive phenotypes of prostate cancer cells, including deregulated growth, colony formation, cell migration and cancer invasion. Another link between oxidative stress and prostate cancer is glutathione peroxidase 3, the enzyme secreted and present in plasma which plays a critical role in the detoxification of ROS and reactive aldehydes produced by lipid peroxidation. This enzyme was found to be frequently deleted in prostate cancer samples, while opposite to that enhanced expression of glutathione peroxidase 3 in prostate cancer cell lines may cause suppression of the tumour growth and metastases [25].

Therefore, the assumption that oxidative stress and lipid peroxidation products may play important roles in prostate cancer development seems feasible. This possibility is further supported in the case of acrolein by the fact that prostate is rich in spermine and spermidine, the polyamines subjected to oxidation by spermine oxidase

Table V. Logistic regression analysis for prediction of biochemical relapse (tumour recurrence) after radical prostatectomy.

						95% C.I. for EXP(B)		
Variable	B	S.E.	Wald	df	Exp (B)	lower	upper	Sig. (p)
Pathological stage	3,812	0,995	14,675	1	45,251	6,435	318,21	0.001
Acrolein in stroma	−2,204	1,199	3,382	1	0,11	0,011	1,156	0.042
Positive surgical margin	1,995	0,901	4,907	1	7,353	1,258	42,957	0.023

C.I.; confidence interval.
Pathological stage includes extracapsular extension and seminal vesicle invasion.
PSM-positive surgical margin is defined as cancer presence observed at the inked margin of the specimen obtained by surgical removal of the tumor.

(SMO) generating N,*N*-bis(3-propionaldehyde)-1,4-butanediamin or *N*-(4-aminobutyl)-aminopropionaldehyde, respectively, that generate eventually acrolein [26]. It was shown that individuals with elevated SMO activity have higher risk for prostate cancer than those with lower SMO activity, thus further supporting lipid peroxidation and acrolein production as mechanisms by which prolonged induction of SMO activity due to prostatitis may contribute to prostate carcinogenesis [27]. Induction of SMO was recently demonstrated in human gastric epithelial cells by *Helicobacter pylori* infection and the pro-inflammatory cytokine tumour necrosis factor-*α* (TNF-*α*), respectively, which result in oxidative stress causing oxidative DNA damage [28,29]. Although acrolein was not studied under such circumstances, these findings further stress the relevance of reactive aldehydes as lipid peroxidation products involved in carcinogenesis. Complementary to that, in the recent study *Helicobacter pylori* infection was found to be associated with development of another reactive aldehyde, 4-hydroxynonenal (HNE), which is considered as a major bioactive marker of lipid peroxidation, although it seems to be less reactive than acrolein [30–32]. HNE is a well known growth regulating factor and cell signalling molecule, which achieves its growth factor-like activities interacting with the bioactivities of various cytokines [32–34]. Although such growth regulating activities were not yet described for acrolein, it seems probable that acrolein could have similar roles in carcinogenesis as HNE, in particular because for both aldehydes similar immunohistochemical appearances were described in colon carcinogenesis related to the cancer progression [19,35,36].

In current study we have shown that acrolein-protein adducts are present in prostate cancer tissue and could be related with biochemical relapse, i.e. the prostate cancer recurrence. Acrolein was differentially distributed in prostatic tissue in men with biochemical relapse after radical prostatectomy than in carcinoma specimens of patients which remained free of cancer recurrence. The presence of the aldehyde was more intense, as evidenced by immunohistochemistry in the cytoplasm and in the nuclei of cancer cells. Together with the acrolein content in stroma this resulted in the overall higher immunopositivity of acrolein in patients with extraprostatic disease than in those with organ-confined disease, suggesting that acrolein presence correlates with progression of prostate carcinoma. This indicates that prostate cancer cells are subjected to oxidative stress manifested by lipid peroxidation and acrolein formation. The environmental origin of acrolein seems probable in carcinogenesis in general, while the fact that spermine content in these patients also increases, making pre-conditions for further acrolein production, allows the assumption that prostate carcinogenesis is associated with spermine and lipid peroxidation as well as with lowered antioxidative protection allowing persistent oxidative stress.

Accordingly, the cells in the organ-confined tumour might probably stand the acrolein-caused damage in the cytoplasm, while with progression of the tumour production of acrolein could overwhelm the cellular capacity to tolerate and localize the aldehyde in the cytoplasm, which is therefore accumulated in the nuclear region, as was observed in the current study. Thus, the observed intracellular distribution of acrolein resembles the recent findings on HNE nuclear accumulation in the case of liver carcinogenesis [37]. This might in both cases (HNE in the liver and acrolein in prostate) result in further deterioration of the cell growth control and more aggressive tumour phenotype with increasing malignancy. The results of the follow-up study for our patients support this hypothesis as patients with positive surgical margins develop biochemical relapse of the disease.

In conclusion, the results obtained indicate that biochemical relapse of prostate carcinoma after radical surgery might be predicted with 90% accuracy if positive surgical margins, pathological stage of disease and the intensity of acrolein presence in tumour stroma are taken together. These findings indicate novel possibilities to study prostate carcinogenesis and for the complementary therapy with antioxidants, such as lycopene [38], in prevention of prostate cancer relapse.

Declaration of interest: This study was supported by the Croatian Ministry of Science, Education and Sports research grants and by the research grant from the of Education, Culture, Sports, Science and Technology, by COST B35 Action and by the COEProgram in the 21st Century in Japan. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 19 February 2010.