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ORIGINAL ARTICLE

Oxidative damage to guanine nucleosides following combination chemotherapy with 5-fluorouracil and oxaliplatin

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Abstract
Purpose Recent in vitro and animal studies have suggested that the cytotoxicity of 5-fluorouracil and oxaliplatin is linked to increased formation of reactive oxygen species (ROS). This prospective study was undertaken to examine the generation of oxidative stress, in 106 colorectal cancer patients, by 5-fluorouracil and oxaliplatin combination (FOLFOX) therapy as measured by urinary excretion of 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydro-guanosine (8-oxoGuo).
Methods The amounts of 8-oxoGuo and 8-oxodG were measured in 3 spot urine samples from 106 patients by using ultra performance liquid chromatography and tandem mass spectrometry. Furthermore, we collected information on other clinical and demographic variables hypothesized to be associated with oxidative stress. Repeated measures linear mixed models were used to model the relationship between urinary concentrations of 8-oxoGuo and 8-oxodG and the treatment effect and the other variables.
Results The analysis showed that chemotherapy increased the excretion of 8-oxoGuo and 8-oxodG around 15% ($P < 0.0001$ and $P = 0.02$, respectively) though there was a significant interaction with CRP levels. Additionally, we found that sex, smoking status, age, and c-reactive protein were related to urinary excretion of 8-oxoGuo and 8-oxodG in colorectal cancer patients.
Conclusion These results indicate that FOLFOX induces ROS in patients and that ROS-generating mechanisms interact.

Keywords Fluorouracil · Oxaliplatin · Colorectal neoplasms · 8-oxo-7,8-dihydro-2-deoxyguanosine · 8-oxo-7,8-dihydro-guanosine

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Introduction

5-Fluorouracil (5-FU) and oxaliplatin have been shown to be effective in the treatment of colorectal neoplasms and is widely used for this purpose and the treatment of other solid cancers. The main action mechanisms of 5-FU are inhibition of thymidylate synthetase and RNA function, whereas oxaliplatin forms oxaliplatin–DNA adducts inhibiting DNA synthesis. Other minor mechanisms of action have also been suggested for both drugs such as production of reactive oxygen species.

Several anticancer drugs, including 5-FU and oxaliplatin, have been shown to increase the intracellular concentration of ROS, and the inhibition of the drug-induced increase in ROS concentrations partly reverses the cytotoxicity of these agents [1–11]. Furthermore, in vitro models indicate that oxaliplatin cytotoxicity depends on ROS production but is not necessarily related to oxidatively modified DNA [9].

ROS, especially H_2O_2, have a dual role in cellular homeostasis; stimulation of cells with low concentration of ROS leads to stimulation of cell growth, while high concentrations of ROS induce cell senescence and apoptosis [6, 12–19]. Higher production of ROS and higher levels of oxidative stress is observed in tumor cells than in normal cells [6, 20, 21]; correspondingly, increases in ROS have been shown to be toxic to cancer cells, whereas smaller decreases in intracellular ROS concentration have induced cell growth in cancer cells [6].

The consequence for normal cells will depend on the level of ROS induced by 5-FU and oxaliplatin, and larger increases could induce cell dysfunction or even cell death and hence systemic toxicity [22].

ROS can induce 8-hydroxylation of guanine bases in DNA and RNA, and the resulting products are 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydro-guanosine (8-oxoGuo). These products can be measured in urine and are estimates of the combined DNA and RNA oxidation of guanine in an intact organism [23]; in contrast to many other measures of oxidative stress, they represent intracellular processes.

No studies have been conducted to analyze whether 5-FU and oxaliplatin treatment increases oxidative stress in cancer patients. Based on the experimental evidence, we hypothesize that 5-FU/oxaliplatin (FOLFOX) combination therapy should increase oxidative stress in cancer patients. To this end, we have conducted a prospective study to detect the formation of 8-hydroxylated guanine nucleosides during adjuvant FOLFOX therapy in colorectal cancer patients.

Materials and methods

Study population

A total of 161 consecutive patients with stage II-III colorectal cancer were prospectively recruited into this study from August 2005 to September 2008 at the Department of Oncology Rigshospitalet. Among these patients, 106 completed the study, whereas 55 were excluded because of retraction of consent, refusal to receive treatment, or disease recurrence. All patients were treated with complete resection of their tumors followed by adjuvant FOLFOX-4 chemotherapy. The FOLFOX-4 regimen consisted of 12 cycles of oxaliplatin 2-h infusion (85 mg m^{-2}) and 5-FU bolus injection (400 mg m^{-2}) followed by flat continuous infusion of 5-FU for 46 h (2,400 mg m^{-2}) every 2 weeks.

We evaluated several clinical variables as possible independent predictors of urinary excretion of 8-oxodG and 8-oxoGuo. Hypertension was assessed either by self-reported physician diagnosis, previous or current antihypertensive medication, or blood pressure >140/90 after repeated measurements. Diabetes was assessed by self-reported physician diagnosis, use of anti-diabetic diet or medication, and an incidental plasma glucose ≥11.1 mmol/l or HgbA1c >6.5%. Hypercholesterolemia was defined by the use of cholesterol-lowering drugs or total cholesterol >6.5 mmol/l or LDL-cholesterol >4.0 mmol/l based on 3 measurements 3 months apart. Body mass index (BMI) was calculated as usual and categorized according to the WHO classification into 20–24.9 kg m^{-2} (normal), 25–29.9 kg m^{-2} (overweight), and ≥30 kg m^{-2} (obese). Smoking habits were assessed by self-reported consumption of cigarette pack years and categorized as never smokers, previous smokers (stopped smoking before inclusion), and current smokers.

Patients were accrued after informed consent. The local Research Ethics Committee approved this study (KF 01-267812).

Sampling procedure and analyses

Spot urine samples were assayed for the oxidatively modified guanine nucleosides 8-oxodG and 8-oxoGuo using ultra performance liquid chromatography and tandem mass spectrometry. 8-oxodG and 8-oxoGuo were normalized against urinary creatinine concentration. Chromatographic separation was performed on an Acquity UPLC system (Waters, Milford, MA, USA). The column used was an Acquity UPLC BEH Shield RP18 column (1.7 μm, 2.1 × 100 mm) protected with in-line filter (4 × 2 mm, 0.2 μm) both obtained from Waters. The MS detection was performed on an API 3000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ESI ion source (Turbospray) operated in the positive ionization mode. Details of the analysis are described elsewhere [24].

Three spot urines were sampled before chemotherapy, immediately after one of the courses 5–7, and at follow-up at least 2 weeks after end of chemotherapy. The middle time point was chosen at courses 5–7 to imitate a chronic exposure state.

Statistics

The 8-oxoGuo or 8-oxodG to creatinine ratio was ln transformed before statistical analysis to obtain normally distributed variables.

The relations between demographic and clinical factors and oxidatively modified guanine nucleosides were analyzed using repeated measures linear mixed models to estimate fixed effects across patients and time/treatment effects on individual patients. The optimal co-variance structure of the linear mixed model was selected using the Akaike information criterion. Furthermore, least square

means were estimated from the model to quantify the differences between the different patient groups. The optimal co-variance structure was identified as autoregressive heterogeneous for 8-oxoGuo and heterogeneous compound symmetry for 8-oxodG. The assumptions for using the linear mixed model were fulfilled. Outlier detection was based on model residuals with a deviation of 4 standard deviations being used as the exclusion criterion.

The ratios between different periods of measurements could not be transformed into a normally distributed variable, so we did a non-parametric two sample median test instead.

All statistical analyses were performed using the SAS software version 9.2 (SAS Institute Inc. Cary, NC, USA). Statistical significance was defined as $P < 0.05$. All statistical tests were two-sided.

Results

Study population

The study population consisted of 106 patients yielding a total of 302 measurements of oxidatively modified guanine nucleosides with 16 missing measurements. The measured raw values normalized against creatinine showed an increase in 8-oxoGuo during chemotherapy (Table 1). Patients receiving full dose had higher increases from treatment than patients receiving reduced doses of chemotherapy The median dose reduction was 25% (range: 10–50%). 8-oxodG seemed unchanged by FOLFOX-4 treatment. The dose reductions were due to toxicities prohibiting optimal treatment. The demographic and clinical variables for the patient population are listed in Table 2.

CRP had a median value of 3 (range: <1–50) and was measured at baseline. For statistical analyses, the top tertile of CRP was compared to remaining patients (cut-off CRP = 5).

8-oxoGuo and 8-oxodG was not associated with diabetes, hypercholesterolemia, or hypertension (data not shown).

Repeated measures analysis

Based on the repeated measures linear mixed models (RMLMM), the variables associated with 8-oxoGuo and 8-oxodG excretion differed. For both RMLMMs, we found the variables that were significantly associated at the $P < 0.05$ level and the least square means (LS-Means) for each variable (Tables 3, 4). The treatment or time variable was a within-patient test (repeated measure), whereas the other variables modeled the between-patient, or baseline, differences.

The variables associated with increased baseline 8-oxoGuo concentration in urine were age >60, CRP > 5, sex = Female and smoking. Treatment was strongly associated with increased 8-oxoGuo excretion during chemotherapy ($P < 0.0001$), and none of the baseline characteristics interacted with this effect. The results demonstrated that treatment was associated with an average increase of 15% in excretion of 8-oxoGuo.

Only two patient variables were associated with baseline 8-oxodG excretion, namely sex and smoking. To supplement our analyses, we carried out interaction analyses. The model for 8-oxodG revealed a significant interaction between CRP and the time/treatment effect ($P = 0.0005$). The model showed that in patients with CRP > 5, there was a significant sustained drop in 8-oxodG excretion, whereas there was a sustained increase in 8-oxodG excretion in patients with CRP ≤ 5 (Table 4). No other interactions were detected in any of the models. There was an estimated increase of 10% in 8-oxodG in patients with low CRP, while patients with higher CRP experience a drop of 25% in urinary excretion of 8-oxodG.

Table 1 The distribution of 8-oxoGuo and 8-oxodG across the studied time periods

	Before chemotherapy $N = 105$ Median (90% IQR[a])	During chemotherapy $N = 104$ Median (90% IQR)	After chemotherapy $N = 93$ Median (90% IQR)
8-oxoGuo[†]	2.8 (1.6–6.1)	3.5 (2.0–5.4)	2.8 (1.7–4.8)
8-oxodG[†]	1.8 (0.81–5.2)	1.7 (1.1–4.0)	1.8 (0.95–4.0)
Dose dependency			
100% of planned dose			
8-oxoGuo	Baseline	126% (65–221%)**	111% (46–261%)
<100% planned dose			
8-oxoGuo	Baseline	109% (53–201%)**	93% (40–202%)

** Significantly different from each other $P = 0.0227$

[†] *IQR* Interquantile range

[a] Measured in nmol/mmol creatinine

Table 2 Patient demographic and clinical characteristics

$N = 106$	No.	%
Gender		
Male	47	44
Female	59	56
Age		
60>	71	67
60≤	35	33
Primary tumor		
Colon	74	70
Rectum	32	30
Stage		
II	22	21
III	63	59
IV	21	20
CRP		
5≥	71	71
5<	35	35
Cardiovascular disease	8	8
Hypercholesterolemia	31	29
Hypertension	26	25
Diabetes	16	15
BMI		
Normal	56	53
Overweight	33	31
Obese	17	16
Smoking		
Never/previously	82	77
Currently	24	23
Dose reduction		
100% of planned dose	60	57
<100% of planned dose	46	43

Analysis revealed 4 outliers in the 8-oxoGuo model and 3 outliers in the 8-oxodG model; excluding them did not change the models or estimates significantly.

Discussion

Our results demonstrated a transient increase in RNA oxidation (8-oxoGuo) and a more sustained increase in DNA oxidation (8-oxodG) associated with FOLFOX-4 treatment. The increase in 8-oxodG depended on the presence of systemic inflammation; patients with CRP > 5 experienced a drop in 8-oxodG excretion associated with FOLFOX-4 treatment, whereas patients with CRP ≤ 5 had a small increase in 8-oxodG excretion. Patients with CRP > 5 had higher levels of 8-oxoGuo and 8-oxodG excretion before treatment compared to patients with low CRP. Furthermore, this interaction was restricted to inflammation, as the other variables did not interact with the treatment effect. Inflammation in patients have been shown to increase ROS formation and oxidative stress [25–29]; chemotherapy is immunosuppressive, so the decrease in 8-oxodG in patients with inflammation might be a result of immunosuppression as the degree of oxidative insult in inflammation has been shown to be very high [29]. The source of inflammation in cancer patients may be co-morbidity or the cancer itself. Another explanation could be that the mechanisms by which oxaliplatin or 5-fluorouracil induced ROS overlapped with inflammatory processes. The ROS-inducing capacity may be saturated by inflammation, which blunted the effects of the chemotherapeutics, or the processes may have been mutually inhibitory. Remarkably, the concentration of 8-oxodG seemed to converge toward the same level in two groups, which was higher than the initial concentration in cancer patients with low CRP. Moreover, it was interesting to note that some of these effects would have been missed or misinterpreted if proper multiple variable analysis using RMLMM models had not been carried out, i.e., if analysis had been restricted to univariate analysis.

In cancer patients, smoking was associated with an increase in both 8-oxoGuo and 8-oxodG excretion when compared to non-smokers and previous smokers; this was in accordance with previous studies on healthy subjects [30]. Our results further demonstrated that female cancer patients seem to excrete higher levels of oxidatively modified guanine nucleosides than male patients. Age was only associated with the excretion of 8-oxoGuo in patients older than 60 years, who had a greater concentration in their urine than younger patients, which is in accordance with previous studies [31, 32]. The association of demographic and clinical variables with 8-oxoGuo or 8-oxodG should be interpreted with care as our population represents selected cancer patients and not the general population.

The relative importance of the oxidation products with regard to cytotoxicity has not been determined. Experimental studies have indicated that DNA oxidation is not necessarily the most important target in ROS-induced cytotoxicity [9, 33]. Oxidative damage to RNA has also been shown to disrupt cell function through decreased translation, defective protein products, and altered function of non-coding RNA [34]. Urinary markers represent whole-body guanine nucleoside oxidation and not only the tumor, so the increased oxidative stress may also induce side effects [22]. However, our study is in accordance with previous studies showing an increase in oxidatively modified guanosine nucleosides during chemotherapy [35, 36].

One can speculate as to the source of increased 8-oxoGuo and 8-oxodG excretion due to treatment. Three possible sources come to mind: increased excretion due to

Table 3 Repeated measure linear mixed model results showing the variables included in the final model and the time effect on 8-oxoGuo excretion

Effect	Parameter estimates (95% CI)	LS-Means[a] (95% CI)	P_{Model}	$P_{LS\text{-}Means}$
Time			<0.0001	
Before chemotherapy (baseline)	0	2.93 (2.72–3.17)		Ref
During chemotherapy	0.14 (0.08–0.20)	3.38 (3.17–3.60)		<0.0001
After chemotherapy	−0.04 (−0.13–0.04)	2.81 (2.62–3.01)		0.3132
Sex			<0.0001	
Male (baseline)	0	2.75 (2.56–2.96)		Ref
Female	0.19 (0.11–0.28)	3.34 (3.12–3.58)		<0.0001
Smoking			0.003	
Never/previously (baseline)	0	2.81 (2.66–2.96)		Ref
Current	0.15 (0.05–0.26)	3.27 (2.98–3.59)		0.003
Age			0.0002	
60≤ (baseline)	0	2.78 (2.56–3.01)		Ref
60>	0.18 (0.09–0.27)	3.31 (3.11–3.52)		0.0002
CRP			0.007	
5≥ (baseline)	0	2.85 (2.68–3.02)		Ref
5<	0.13 (0.04–0.22)	3.23 (2.97–3.51)		0.007

[a] LS-means converted back to original scale, the unit is nmol 8-oxoGuo/mmol creatinine

Table 4 Repeated measure linear mixed model results showing the variables included in the final model, the time effect and interaction effects on excretion of 8-oxodG

Effect	LS-means[a] (95% CI)	P_{Model}	$P_{LS\text{-}Means}$
Time		0.2371	
Before chemotherapy (baseline)	2.04 (1.82–2.28)		Ref
During chemotherapy	1.86 (1.70–2.03)		0.1046
After chemotherapy	1.88 (0.53–2.08)		0.1858
Sex		0.0051	
Male (baseline)	1.76 (1.58–1.95)		Ref
Female	2.10 (1.91–2.32)		0.0051
Smoking		0.0493	
Never/previously (baseline)	1.78 (1.66–1.92)		Ref
Current	2.07 (1.81–2.37)		0.0493
CRP		0.0392	
5≥ (baseline)	1.79 (1.64–1.95)		Ref
5<	2.07 (1.83–2.33)		0.0392
CRP*time interaction		0.0005	
5≥ CRP			
Before chemotherapy (baseline)	1.65 (1.45–1.87)		Ref
During chemotherapy	1.82 (1.64–2.01)		0.1313
After chemotherapy	1.92 (1.72–2.14)		0.0228
5< CRP			
Before chemotherapy (baseline)	2.52 (2.11–3.02)		Ref
During chemotherapy	1.90 (1.65–2.18)		0.0028
After chemotherapy	1.84 (1.57–2.15)		0.0020

[a] LS-means converted back to original scale, the unit is nmol 8-oxoGuo/mmol creatinine

cell death, increased repair of oxidatively modified guanine nucleotides, or oxidative modification of guanine nucleotides from the nucleotide pool. Important studies have demonstrated that increased excretion of oxidatively modified guanine nucleotides was not due to cell death [36, 37]. The in vivo concentration of ribonucleotides is many times higher than the deoxyribonucleotide pool, and if the excretion was due to oxidation of the nucleotide pool, one would expect a much higher 8-oxoGuo/8-oxodG ratio. In summary, the source of the oxidized nucleosides is

difficult to decipher; the low ratio 8-oxoGuo/8-oxodG makes the nucleotide pool an improbable source, but 8-oxoGuo has never been shown to be the product of a repair process; therefore, firm conclusions regarding the source of the oxidized nucleotides cannot be reached.

Limitations of this study were that we could not account for other oxidation products than guanine nucleosides, e.g., lipid peroxidation, which also affects cell homeostasis, and we could not determine oxidation levels in specific organs.

In conclusion, our study provides evidence that FOLFOX-4 treatment induces increased excretion of oxidatively modified guanine nucleosides in cancer patients that can be measured in urine and that different ROS-generating processes may interact in patients. In future studies, the significance of oxidative stress induced by FOLFOX-4 should be studied as a biomarker for treatment efficacy and toxicity.

Conflicts of interest No conflicts of interest to disclose for any author.

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