SHORT COMMUNICATION

Cisplatin-DNA adduct formation in patients treated with cisplatin-based chemoradiation: lack of correlation between normal tissues and primary tumor

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Abstract

Purpose In this study, the formation of cisplatin-DNA adducts after concurrent cisplatin-radiation and the relationship between adduct-formation in primary tumor tissue and normal tissue were investigated.

Methods Three intravenous cisplatin-regimens, given concurrently with radiation, were studied: daily low-dose (6 mg/m^2) cisplatin, weekly 40 mg/m^2, three-weekly 100 mg/m^2. A ^{32}P-postlabeling technique was used to quantify adducts in normal tissue [white blood cells (WBC) and buccal cells] and tumor.

Results Normal tissue samples for adduct determination were obtained from 63 patients and tumor biopsies from 23 of these patients. Linear relationships and high correlations were observed between the levels of two guanosine- and adenosine–guanosine-adducts in normal and tumor tissue. Adduct levels in tumors were two to five times higher than those in WBC ($P < 0.001$). No significant correlations were found between adduct levels in normal tissues and primary tumor biopsies, nor between WBC and buccal cells.

Conclusions In concurrent chemoradiotherapy schedules, cisplatin adduct levels in tumors were significantly higher than in normal tissues (WBC). No evidence of a correlation was found between adduct levels in normal tissues and primary tumor biopsies. This lack of correlation may, to some extent, explain the inconsistencies in the literature regarding whether or not cisplatin-DNA adducts can be used as a predictive test in anticancer platinum therapy.

Keywords Chemoradiation · Cisplatin · DNA adducts · Head and neck cancer · Cervical cancer

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Introduction

Concurrent chemoradiotherapy is more effective than radiotherapy (RT) alone, both in in vitro studies [1, 2] as well as in clinical studies in many different tumor types, including advanced head and neck squamous cell carcinoma (HNSCC) and cervical cancer, leading to improvements in locoregional control and/or survival [11, 13, 21, 28]. In a metaanalysis on concurrent chemoradiation in HNSCC, the addition of concurrent single agent cisplatin to RT was the most effective treatment regime with the largest improvement on overall survival [5]. Concurrent cisplatin-based chemoradiation is now considered standard care in advanced-stage HNSCC and cervical cancer.

In addition to the increased efficacy of the combined treatment, it was shown that the concurrent regimens are accompanied by higher acute toxicity rates compared to radiation alone [11, 21], with more severe mucositis and gastrointestinal toxicity.

Since a substantial number of patients treated with concurrent chemoradiation still fail to respond to this toxic treatment, there is a need for an accurate predictive assay, based on which patients that are likely to respond to the therapy can be selected. This strategy may also provide a tool to individualize and tailor treatment, based on evaluation of the predictive assay, early during therapy.

One potential predictive marker is the formation of cisplatin-DNA adducts, which are formed when cisplatin reacts with the cellular DNA by binding to nucleotides. The majority of adducts are either intrastrand adducts with cisplatin bound between two guanosine (GG) nucleotides or adenosine–guanosine (AG) nucleotides [8]. Cisplatin-DNA adducts can be measured in tumor and normal tissue. The level of adducts has been shown to correlate with cytotoxicity in vitro [34], and with response to therapy in patients [3, 17, 32, 35]. In most of these studies [3, 32, 35], adduct measurements were performed in the normal tissue, with the assumption that normal tissue can be used as surrogate marker for tumor.

In our institute, study protocols with cisplatin-DNA adduct measurements are ongoing in patients with HNSCC and cervical cancer, who are all treated with concurrent cisplatin-based chemoradiation. The objectives of the current study are (1) to investigate the two major forms of cisplatin-DNA adducts (GG and AG adducts) after different schedules of cisplatin given concurrently with radiation and (2) to explore relationships between adducts in primary tumor and normal tissue. We specifically wanted to investigate whether the level of adducts in tumors are reflected by those in normal tissues. In studies focused on the predictive value of cisplatin-DNA adduct levels, this would then justify the use of more easily obtained normal tissues as a surrogate for tumor samples.

Patients and methods

Concurrent chemoradiation protocols

This study on adduct formation was approved by the medical ethical committee of the participating hospitals. The main eligibility criteria were: patients scheduled for cisplatin chemoradiation, no previous treatment with cisplatin, and informed consent. Eligible patients were informed about the nature of the protocol and after written informed consent they were entered in the study. Patients were recruited from one of the following regimens.

In advanced-stage HNSCC patients, two different cisplatin-based concurrent chemoradiation protocols (RADPLAT) were used. The *RADPLAT 100* schedule, which is the most commonly administered schedule in HNSCC [11], consisted of cisplatin given intravenously (IV) at a dose of 100 mg/m^2, as a 30 min infusion, 1–2 h before RT at days 1, 22, and 43 of treatment. This treatment was part of a randomized trial on IV vs. intra-arterial chemoradiation. In *RADPLAT daily LD*, low-dose (LD) cisplatin was given as a 1–2 min IV infusion at a dose of 6 mg/m^2 daily, for a total number of 20 doses, 1–2 h prior to RT. This treatment was shown to be an effective alternative in HNSCC [6, 19]. Patients ineligible for or refusing the randomized trial on intra-arterial chemoradiation were treated with RADPLAT daily LD, since this treatment could be given on an outpatient basis. The RT target volumes for all schedules included the primary tumor and the bilateral neck at a dose of 46 Gy in 23 fractions. A boost was given to the macroscopic tumor extensions at the primary tumor site and lymph node metastases at a dose of 24 Gy in 12 fractions, resulting in a total dose of 70 Gy in 35 fractions.

In patients with advanced-stage squamous cell cervical cancer, concurrent chemoradiation (*CERVIX 40*) consisted of weekly administration of cisplatin IV as a 4-h infusion at a dose of 40 mg/m^2, followed by RT within 1–2 h. The total number of doses was 5–6, depending on external beam RT schedule and the number of intracavitary brachytherapy applications. The total radiation dose was usually 46 Gy to the cervical tumor, uterus, and pelvic lymph nodes, with a boost to the cervix tumor and other involved regions, to a total dose of 60–74 Gy, depending on treated volume and whether or not intracavitary brachytherapy was given.

Cisplatin-DNA adducts

Before and after chemotherapy, normal tissue samples [white blood cells (WBC) and buccal cells] were collected. In patients with an accessible primary tumor, a biopsy of the tumor was also taken. To avoid harvesting necrotic tissue, the biopsy was taken at the viable peripheral rim of the tumor. Samples were obtained at different times, due to

logistic reasons: for the patients in the RADPLAT 100 study, this was done 23 h after the end of administration of the first cisplatin infusion (given on day 1 of treatment). In the patients in the CERVIX 40 study, samples were taken 20 h after the end of administration of the first weekly cisplatin infusion (given on day 1 of treatment). For the patients in the RADPLAT daily LD group, samples were taken 1 h after the 5th dose on day 5 of treatment. WBC were isolated from whole blood samples according to a previously published protocol [24]. Buccal cells were collected in phosphate-buffered saline by scraping the bilateral buccal mucosa using a cotton swab. In HNSCC patients, the buccal mucosa could be located within the RT treatment fields, depending on the tumor site. The harvested cells were centrifuged (5 min at 4°C, 1,000 rpm) and resuspended in a Tris–EDTA buffer and stored at −80°C until analysis. Tumor biopsies were taken and immediately frozen at −80°C until analysis. Quantification of GG- and AG-intrastrand adducts was performed by a ^{32}P-postlabeling technique as previously described [29]. Internal standardization was incorporated in the present analysis method, by adding 300 fmol of TT nucleotides to each sample. From previous work, the reproducibility of the assay is known by analysis of duplicate specimens within the same experiment (within-run reproducibility) and by analysis of duplicate specimens in separate experiments (between-run reproducibility) [29]. The reproducibility was described for WBC and tumor samples and was within 10% for the within-run precision and between 2–20% for the between-run precision. It was also determined for buccal cells in the same way and similar reproducibility was obtained. The concentration of DNA present in the samples was measured spectrophotometrically at 260 nm with the Nanodrop ND-1000 (Nanodrop Technologies Inc, Wilmington, DE, USA). The cisplatin-DNA adduct levels were expressed as fmol/μg DNA. The lower limit of quantification for the Pt-GG and Pt-AG adducts was 0.087 and 0.053 fmol/μg DNA, respectively.

Statistical analysis

Analysis of data was performed in SPSS software (version 11.5, SPSS, Inc.). For quantitative comparison of numerical data between groups, the Student's t-test was applied. The Pearson correlation coefficient and Spearman's rank correlation coefficient were calculated for analysis of correlations between different samples (WBC, buccal cells, and primary tumor) on an intra-patient level.

Results

Samples for cisplatin-DNA adduct determination were obtained from 63 patients: 27 from RADPLAT daily LD,

15 from CERVIX 40, and 21 from RADPLAT 100. WBC samples were taken from 61 patients, buccal cells from 25, and tumor biopsies from 23 of these patients. The reasons for the missing data for the normal tissue samples were: no collection of samples due to logistics or (in minority of cases) not sufficient volume for analysis. The reason for missing primary tumor biopsy data were: tumor not accessible for direct outpatient-based biopsy (in HNSCC patients) or refusal (in cervix cancer patients).

In WBC, all but three of the 60 available baseline samples were below the LLQ for the GG adducts and all but two below the LLQ for the AG adducts. This was probably due to some background signal inherent in the postlabeling method, since all patients had not been treated before with platinum chemotherapy. The yield of DNA, obtained from the buccal cell samples was rather low, ranging from 1–10 μg. Baseline samples of buccal cells were available from 16 of 25 patients, of whom posttreatment samples were also available. The baseline values of GG adducts in buccal cells ranged from 0.067 to 0.745 fmol/μg DNA (mean 0.282, SD 0.19) and baseline values of AG adducts in buccal the cells ranged from 0.087 to 1.538 fmol/μg DNA (mean 0.398, SD 0.38). All but two of the baseline GG-adduct values were above the LLQ and all the baseline AG-adduct values were above the LLQ. This was probably due to the low DNA quantities obtained from the buccal cell samples. The difference in adduct levels from baseline to post-infusion values was significant for the GG adducts, but not for the AG adducts. This implies that for measuring low quantities of AG adducts in the low amounts of buccal cell DNA available, we reached the limits of quantification with this postlabeling method.

In Table 1, the results are presented for the GG- and AG-adduct levels in normal tissue and primary tumor for the three different cisplatin-chemoradiation regimes. Adduct levels in primary tumor were two to five times higher than those in WBC for all three treatment regimes for both GG- and AG-adduct formation (Student t-test, $P < 0.001$ for both adduct types). For the comparison of the adduct levels from the three different treatment protocols, the data from the RADPLAT daily LD were omitted, since the daily administration schedule and sampling time (1 h after the infusion) were different from the other two regimens. The adduct levels in the RADPLAT 100 schedule were statistically significantly higher than those after the CERVIX 40 schedule for both tumors (Student t-test, $P = 0.01$) and normal tissues (Student t-test, $P < 0.01$).

A highly significant linear correlation (Pearson correlation, $r = 0.93$, $P < 0.001$, $n = 61$) was observed between the level of GG and AG adducts in WBC (see Fig. 1a), with a mean ratio of GG/AG adducts of 7.6 ± 2.1 SD. Similar linear relationships and ratios were found for GG and AG

Table 1 Cisplatin-DNA adducts (in fmol/μg DNA) in normal tissue and primary tumor after different schedules of cisplatin-based chemo-radiation

Treatment schedule	WBC		Buccal cells		Tumor	
	GG	AG	GG	AG	GG	AG
RADPLAT daily LD						
N	26	26	11	11	6	6
Mean	0.34	0.05	0.84	0.21	0.66	0.10
SD	0.10	0.19	0.39	0.14	0.37	0.05
CERVIX 40						
N	14	14	7	7	10	10
Mean	0.44	0.06	0.87	0.22	1.94	0.26
SD	0.17	0.03	0.26	0.06	1.47	0.26
RADPLAT 100						
N	21	21	7	7	7	7
Mean	1.047	0.124	1.563	0.340	3.866	0.413
SD	0.377	0.049	0.434	0.090	1.101	0.089

See text for explanation of treatment schedules

N number of patients, *SD* standard deviation, *WBC* white blood cells, *GG* GG-adducts, *AG* AG adducts

adducts in primary tumor ($r = 0.89$, $P < 0.001$, $n = 23$, and ratio 8.5 ± 2.8; Fig. 1b) and buccal cells ($r = 0.85$, $P < 0.001$, $n = 23$, and ratio 3.9 ± 1.3; Fig. 1c).

A trend was observed between GG-adduct levels in WBC and buccal cells, although not significant ($r = 0.38$, $P = 0.07$, $n = 24$). No significant correlations were found between tumor and normal tissue: tumor vs. WBC ($r = 0.35$, $P = 0.13$, $n = 21$) and tumor vs. buccal cells ($r = -0.003$, $P = 0.99$, $n = 9$). See Fig. 2 for scatter plots. Similar results were found for the AG adducts: no significant correlations were found between adducts in tumor vs. WBC ($r = 0.14$, $P = 0.55$, $n = 21$), tumor vs. buccal cells ($r = 0.25$, $P = 0.58$, $n = 7$) or WBC vs. buccal cells ($r = 0.26$, $P = 0.24$, $n = 22$).

Discussion

There are two main conclusions from the 63 patients included in these analyses. First, intra-tumoral adduct levels were substantially higher than those in normal tissue (WBC) at all cisplatin-dose levels examined. Second, no positive correlations were evident between adducts in tumors and normal tissues. It should be noted that the various schedules, the cisplatin doses, and the duration of infusions differed, as well as the sampling times. However, all analyses on adducts were performed on paired samples, within the same patient. This eliminates variance of these factors since the normal tissue and tumor samples all were obtained at similar time points after the cisplatin infusion.
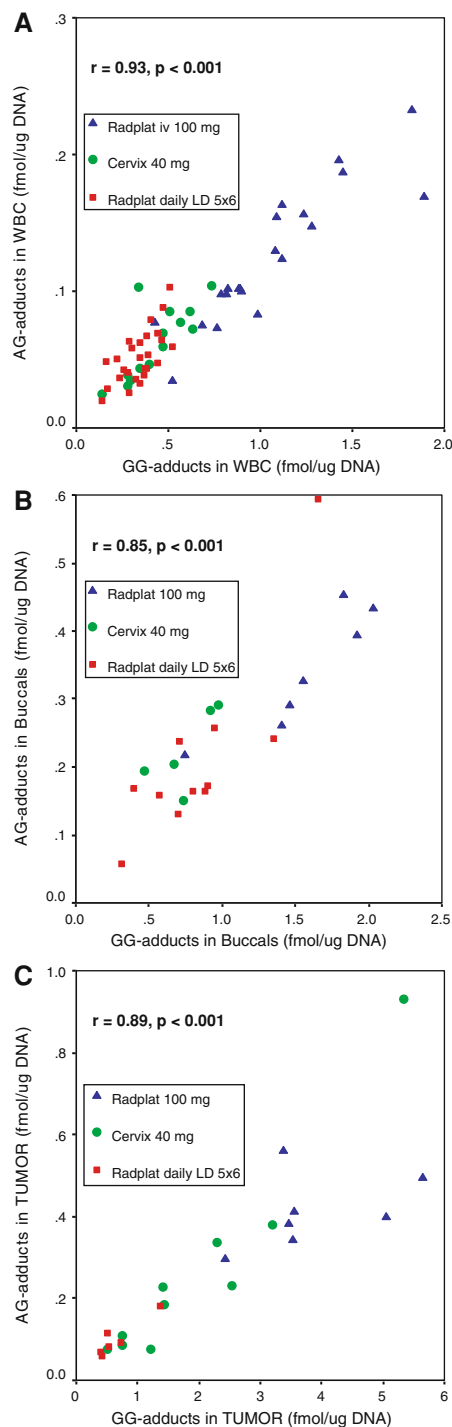
Fig. 1 Correlation-plots of GG- and AG-adduct levels in white blood cells (WBC) (panel **a**), primary tumor biopsy (panel **b**), buccal cells (panel **c**). In each panel, the three different treatment groups are depicted: RADPLAT daily LD 5 × 6 mg (*red squares*), CERVIX 40 mg (*green circles*), and RADPLAT 100 mg (*blue triangles*)

In the RADPLAT daily LD, some accumulation from the previous four daily 6 mg cisplatin infusions would have occurred and affected the day 5 measurement after the 5th
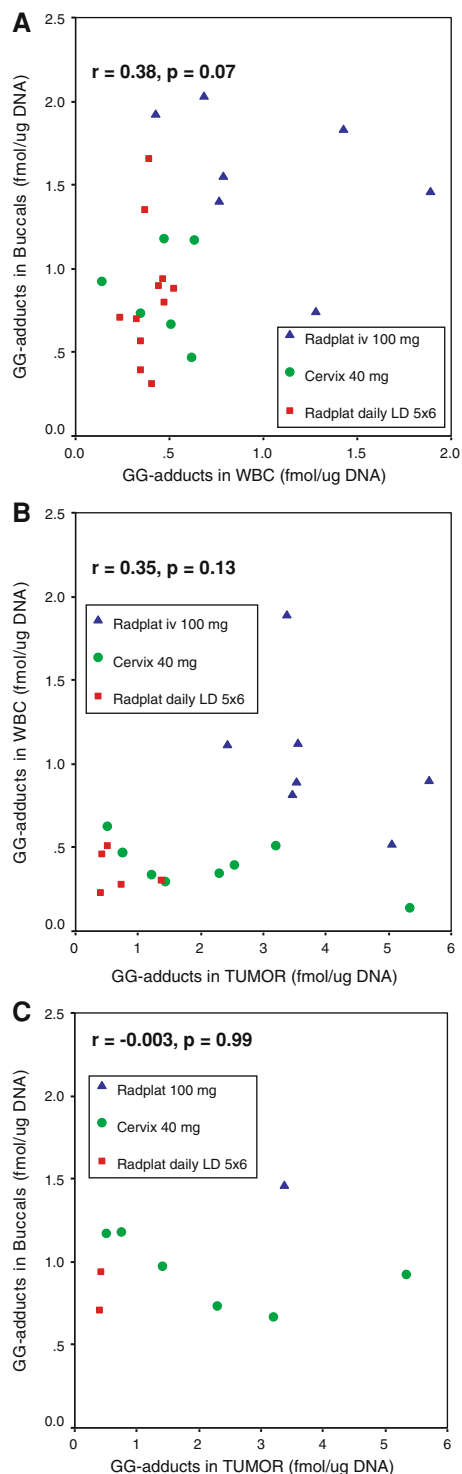
Fig. 2 Correlation plots of GG adducts in white blood cells (WBC) vs. buccal cells (panel **a**), and normal tissue vs. tumor (panels **b** and **c**). In each panel, the three different treatment groups are depicted: RAD-PLAT daily LD 5 × 6 mg (*red squares*), CERVIX 40 mg (*green circles*), and RADPLAT 100 mg (*blue triangles*)

infusion. In the RADPLAT 100 and CERVIX 40 patients, no such accumulation would have occurred, since the sampling was done 20–23 h after the first infusion of cisplatin.

Relatively little information is available regarding the in vivo formation of intra-tumoral cisplatin-DNA adducts in clinical series [17, 23]. Most studies focused on intra-tumoral platinum concentrations, both in HNSCC [12, 33] and cervical cancer patients [15, 22]. These studies are mostly characterized by relatively low numbers of patients, probably due to the invasive nature of the procedure. The data on correlations between adducts and platinum content are contradictory: In an experimental study [37], no relationship could be established between the intra-tumoral adduct levels and platinum content, although in one clinical study, a significant correlation was found [23].

Adduct levels in primary tumors were consistently two- to fivefold higher than in WBC. This was true for both GG and AG adducts. This finding was previously described in anecdotal clinical cases [9, 30]. Similar observations were made in platinum content studies in an experimental tumor model [18] and in HNSCC patients [33]. Adducts in tumor were also higher than in buccal cells in the CERVIX 40 and RADPLAT 100 group, but not in the RADPLAT daily LD group. We observed a linear relationship between the two major adduct forms (AG and GG) for both normal tissues and tumor (Fig. 1a–c), although GG-adduct formation was 5–12 times increased relative to the AG adducts, as reported earlier [8, 36]. The linear relationships between GG and AG adducts serve as a validation of the assay. Apparently within a sample, both types of adducts are present in an equal proportion, although the absolute amounts differ greatly. In previous studies, the type of adduct responsible for the cytotoxic effect of platinum compounds has been investigated. From two of these studies [7, 36] it was concluded that the AG adduct was responsible for the platinum cytotoxicity. From our study, such a conclusion cannot be made, since both types of adducts were present in equal proportions.

Adduct formation in different tissue samples showed a lack of correlation between tumor and normal tissue. One might have expected that higher adduct levels in normal tissue would be accompanied by more adducts in tumor, although this was not the case. Similar results were found in an animal study [25] and in a clinical series of uterine cervix cancer patients [22], both demonstrating lack of correlation between tumor platinum and serum platinum concentrations. If adduct formation were merely a matter of cisplatin exposure, then a positive correlation would be expected. The reasons for the higher levels of adducts in tumor vs. normal tissue and the lack of correlation between them may be explained by differences between tumor and normal tissue in one of the following factors: Tumors are heterogeneous in terms of blood supply and perfusion, resulting in differences in cisplatin uptake and diffusion, drug-pumps may diminish intra-cellular cisplatin concentrations by active transmembrane transport of cisplatin,

prohibiting adducts to be formed, and tumor cells may have less effective capacities to repair damage from cytotoxic agents.

Adducts are formed rapidly and in a dose-dependent fashion within 1–2 h after cisplatin exposure, with a gradual decrease (repair) within the next 20–24 h [20, 32, 36]. The persistence of cisplatin-DNA adducts may therefore be regarded as a measure of repair and possibly be used as predictive assay. We therefore chose to measure adducts 20–23 h after the end of chemotherapy infusion. Ideally, more frequent measurements would have generated more information on the rate of adduct formation, repair, and total exposure to adducts (like an AUC analysis) [24]. However, obtaining repeated biopsies is not feasible in clinical practice.

The results from the buccal cell samples need to be interpreted with caution, especially the AG-adduct levels, since uncertainties remain. With the low quantities of AG-adducts in the low amounts of buccal cell DNA we could extract, we reached the limits of quantification of the post-labeling method.

The rationale for measuring cisplatin-DNA adducts is that it could be used as a predictive assay: higher levels of adducts would predict favorable treatment outcome. Many studies have been performed for this purpose, investigating adducts in normal tissue (WBC and buccal cells) [3, 4, 10, 14, 26, 31, 32, 35]. These study designs are based on the assumption that normal tissue can be used as a surrogate marker for tumor tissue with respect to cisplatin-DNA adduct formation. In our present study, however, we did not find such a correlation. This might explain why the results on the role of adduct formation to predictive outcome are heterogeneous and contradictory, as illustrated below.

Several studies on adduct formation in WBC showed that the level of adducts was positively correlated with response to chemotherapy in patients with advanced disease in a variety of tumor sites [31, 32], while others showed no correlation [4, 26]. One study showed a positive correlation in one tumor site (ovarian cancer), but not in the other (breast cancer) [14], and in another study the level of adduct formation showed a negative association with survival for day-5 adducts, while there was no difference for day-1 adducts [10]. In studies on adduct levels in buccal cells, a positive correlation was found between adducts and either disease response [3] or better survival in non-small cell lung cancer (NSCLC) [35]. We recently showed that adduct formation in primary tumor appeared to be associated with better progression-free survival in HNSCC [17].

Differences were observed between the levels of intratumoral adducts for the three chemoradiation schedules, with lower adducts after lower dosages of cisplatin. Based on this, however, one cannot predict that the schedules with less adducts will result in less cytotoxicity, since not only

the cisplatin dose, but also timing and schedule of cisplatin administration are crucial determinants of efficacy [1]. These factors may contribute to differences in the formation and rate of repair of adducts, resulting in different exposures to cisplatin-DNA adducts. These differences make it difficult to extrapolate from the observed adduct values in tumor and normal tissues to a prediction of superiority of one schedule over another.

In future studies, immunohistochemistry on repair proteins like ERCC1 [27] or gene expression profiling studies on platinum resistance [16] could be used to improve the prediction of cisplatin sensitivity and prediction of therapy response.

In conclusion, we have demonstrated that in concurrent chemoradiotherapy schedules, cisplatin adduct levels in tumors were significantly higher than in normal tissues (WBC). No evidence of a correlation was found between adduct levels in normal tissues and primary tumor biopsies. This lack of correlation may, to some extent, explain the inconsistencies in the literature regarding whether or not cisplatin-DNA adducts can be used as predictive test in anticancer therapy.

Conflict of interest: none declared.

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