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Cell kinetic changes in human squamous cell carcinomas during radiotherapy studied using the *in vivo* administration of two halogenated pyrimidines

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Abstract

The aim of this study was to investigate cell cycle changes during radiation treatment and establish whether treatment intervention could be considered if these changes helped to predict outcome. 33 patients with head and neck cancer were administered iododeoxyuridine (IdUrd) prior to treatment and a second administration of bromodeoxyuridine (BrdUrd) prior to the fifth fraction of 2 Gy. Biopsies were taken several hours after each injection and flow cytometry was used to calculate changes in the cellular kinetics and cell cycle delay *in vivo*. The kinetic response of the tumour cells was variable; some showed an increase in proliferation during the first week of treatment, whilst the majority showed an inhibition of proliferation. Reduction in the labelling index (LI) and the pretreatment DNA ploidy status and not delays in G2 were the only parameters to correlate with clinical outcome. A lack of reduction in the LI after 1 week of radiotherapy and DNA aneuploidy predicted a group of patients where radiotherapy failed. This information could be helpful in planning future treatment interventions.

Keywords: Cell cycle delay; Radiotherapy; Proliferation; Head and neck cancer; Prediction of response

1. Introduction

The treatment of solid tumours with radiation initiates a complex series of processes in which proliferation and cell cycle changes play an important role. It is well known that radiation sensitivity varies throughout the cell cycle [1] and that radiation can induce cell cycle blocks [2] that can lead to a redistribution or reassortment of cells within their cell cycle producing variation in the sensitivity of surviving cells. The short-term response of a tumour to radiation might reveal information that can predict whether the treatment is likely to be successful in achieving local control with a particular schedule of radiotherapy.

Several studies have attempted to relate pre- and posttreatment growth and cellular kinetic parameters and changes to radiosensitivity. Some papers have demonstrated a relationship between the sensitivity of human tumours and their doubling time [3] and between their histological types and their labelling index (LI) [4]. Since it is considered that there is a correlation between the histological type of human tumours and their response to radiotherapy, then this would suggest a possible influence of the LI on radiosensitivity. In addition, radioresistance of experimental tumours has been shown to be a result of repopulation during and after treatment [5–7]. This would suggest that monitoring the LI during treatment might give some indication of whether the tumour is responding, i.e. a reduction in the LI or progressing (an increase in the LI). These studies have been undertaken, in the past, using tritiated thymidine ([^{3}H]TdR) incorporation into biopsy explants *in vitro* [8,9]. The data have suggested that the LI of human tumours generally decreases after irradiation and it continues to diminish during a fractionated course of radiotherapy. Increases in the LI are relatively rare and transient and are often associated with a low pretreatment LI. The relationship between changes in

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the LI and prognosis during treatment has not been strong; although one study [9] did suggest that more pronounced decreases in the LI after 10 Gy were associated with a more favourable outcome.

Within the past decade, the importance of cell cycle regulation mechanisms in the response of mammalian cells to radiation has become a focus of increasing importance and research [10–12]. A series of tightly regulated cell cycle checkpoints ensure the fidelity of cells progressing through the cell cycle in both unperturbed and perturbed cell populations. The existence of cell cycle delays in G1, S and G2, following ionising irradiation, is believed to contribute to the ability of the cell to survive the radiation insult. The integrity of cell cycle checkpoint function in tumours might give an indication as to the ability of the tumour to respond to DNA damaging agents such as radiation. It may also be possible to exploit functioning checkpoints, such as G2 arrest, by administering agents that force cells prematurely through the block. Malfunctioning of radiation-induced cell cycle blocks might also indicate a progressing tumour, dividing in spite of DNA damage, and accumulating chromosomal abnormalities.

In this study, we investigate cell cycle changes during radiation treatment of head and neck cancer patients.

2. Patients and methods

2.1. Patients

A total of 33 patients were entered into this study, 16 females and 17 males, with squamous cell cancer of the head and neck region. There were three T1, 15 T2, 3 T3 and 12 T4 tumours, four tumours were grade 1, 23 grade 2 and six grade 3. The tumour sites were oral cavity (22 patients) and oropharynx (11 patients). It was planned that all patients would receive conventional fractionation (64–70 Gy, 2 Gy per fraction, five fractions per week) and 1 patient also received carbogen and nicotinamide; the medan total dose was 66 Gy. The Ethical Committee of Umeå University sanctioned the protocol and all patients entering the study gave their informed consent.

2.2. Surgical procedures

The first biopsies, for most patients, were removed under general anaesthesia during the diagnostic work-up. The second biopsy was removed under local anaesthesia.

2.3. Administration of halogenated pyrimidines

The formulations of both iododeoxyuridine (IdUrd) and bromodeoxyuridine (BrdUrd) were prepared in the Pharmacy Department of Umeå University. Both drugs were obtained from Apoteksbolagets produktionsenhet (Umeå, Sweden) and were dissolved in normal saline, the dose administered to the patients was 100 mg in 100 ml over a period of <5 min. Biopsies were removed, as described above, 4–8.2 h after the first injection of IdUrd and 3.7–9.3 h after the second injection of BrdUrd. There were no adverse effects associated with the administration of the DNA markers. The biopsies were macroscopically examined and similar pieces were fixed in 10% formal saline for histology and 70% ethanol for flow cytometric (FCM) analysis.

2.4. Treatment response and follow-up

Patients were assessed for treatment response 6–8 weeks after the completion of radiotherapy. If the tumour persisted, surgery was performed whenever possible. In addition, the Department policy was to routinely excise the site of tumours in the oral cavity and positive neck nodes after radiotherapy. 21 of the patents had such surgery. In these cases, any remaining tumour cells in the surgical specimen was considered to represent a failure to obtain local control with radiotherapy. After the initial evaluation of the treatment, patients were followed every 3 months for 2 years and then every 4–6 months until 5 years after treatment. After this time, patients have been followed at least once a year. The follow-up period ranges between 55 and 90 months.

2.5. IdUrd and BrdUrd staining

The scheduling of IdUrd as a pretreatment labelling procedure and BrdUrd as the posttreatment label was governed by the availability of monoclonal antibodies specific for the bromine-substituted DNA precursor. Several combinations of antibody were tested, e.g. IU4 and Br3 (Caltag Ltd.), the former shows a higher specificity for IdUrd. However, the most convenient and successful combination was the combination of a pan-halogen specific mouse monoclonal (Dako Ltd.) to detect the pretreatment IdUrd and a rat anti-BrdUrd monoclonal antibody (Sera-Lab), which has a high affinity for BrdUrd, but a very low affinity for IdUrd. Prior to antibody staining, the ethanol-fixed material was digested with 0.4 mg/ml in 0.1M HCI for 40–60 min at 37 °C to produce a nuclear suspension. DNA was partially denatured, to allow antibody access to the binding sites, using 2M HCI for 12 min at room temperature. After two washes in phosphate-buffered solution (PBS), the monoclonal antibodies were added to the appropriate sample at a dilution of 1:20 in 200 μl of phosphate buffered saline (PBS) containing 0.5% Tween-20 and 0.5% normal goat serum (PNT (PBS with normal goat serum and Tween-20 buffer) for 1 h at room

temperature. After washing in PBS, appropriate secondary antibodies were added (goat anti-mouse or anti-rat), conjugated to fluorescein isothiocyanate (FITC), at a dilution of 200 μl of PNT for 30 min at room temperature. After a further wash in PBS, the suspensions were stained with 20 μl of PNT for 30 min at room temperature. After a further wash in PBS, the suspensions were stained with 20 μg/ml propidium iodide (PI) in 1 ml of PBS.

2.6. Flow cytometry and data analysis

All samples were analysed on a fluorescent activated cell sorter (FACS)can (Becton Dickinson) using Lysys II software. Pairs of samples from each patient were always processed in the same staining run. At least 10 000 events were recorded for each sample and doublet discrimination was used to separate cell doublets and aggregates.

Several different parameters were calculated from the flow cytometry (FCM) profiles and from the comparison of the pre- and posttreatment biopsies. The LI was calculated as the percentage of IdUrd or BrdUrd labelled cells, taking into account cell division between injection and biopsy [13] for either the total cell population or the aneuploid compartment where appropriate. The duration of S-phase was measured according to the method of Begg and colleagues [14], and the potential doubling time (T_{pot}) was calculated from these two parameters. In addition, the percentage of total G1, S and G2 cells and the DNA index were calculated from the DNA profile using CellFit software (Becton Dickinson). Division delay was estimated by calculating the proportion of divided IdUrd or BrdUrd labelled cells as a function of the total BrdUrd or IdUrd population, this was then corrected for the time between injection and biopsy to derive the proportion of divided labelled G1 cells per unit time. From these parameters, the absolute change and percentage change in each parameter, pre- and posttreatment was calculated.

2.7. Statistical analysis

Correlations between the cell kinetic parameters were performed using the Spearman's Rank correlation test; changes during treatment were assessed using a paired '*t*'-test. Univariate survival and local control curves were assessed using a Kaplan–Meier analysis.

3. Results

3.1. Cell kinetic parameters pre- and posttreatment

Fig. 1 shows the comparison between pre- and posttreatment cell kinetic parameters generated by flow cytometry. DNA index showed little change after 1 week of radiotherapy, only five of 33 tumours showed an alteration in their ploidy value. Three tumours apparently became diploid after treatment, whilst two tumours became aneuploid from a pretreatment diploid phenotype. Although there was little change in ploidy, *per se*, there were large differences in the proportion of aneuploid cells in those tumours with abnormal DNA (data not shown).

The LI showed considerable variation after treatment. Two tumours remained exactly the same, 11 increased their LI but the majority, 20, showed a decreased LI after five fractions of radiation. The median LI fell from 6.6 to 4.9% after treatment. The percentage change in the LI, revealed that 5 of the 33 tumours showed less than a 5% reduction or an increase in the LI and can be classified as no significant change. Of those that showed a decrease in the LI, the median percent change in value was 33%. Of the 10 tumours that displayed a significant increase in the LI after treatment, three showed a change of ploidy from diploid to aneuploid. The increase in the LI after treatment was associated with tumours in the group with a lower than median (6.65%) pretreatment LI. Within this group, 10 of 16 tumours showed a less than 5% reduction or an increase in the LI compared with the five tumours in the group with a LI above the median, although this did not reach statistical significance ($P = 0.087$).

The T_s generally increased posttreatment with the median value increasing from 8.8 to 9.6 h. Only eight tumours had a faster pretreatment T_s, 1 showed no change and 23 showed an increase in T_s (it was not possible to estimate the T_s in 1 patient).

The T_{pot} values also generally increased after radiotherapy, due to the combination of lower LIs and longer T_s values. The median pretreatment T_{pot} was 4.5 days, whilst after 1 week of radiotherapy it increased to 6.4 days. There were eight tumours which showed a faster T_{pot} postradiotherapy and two of these were associated with a ploidy change from diploid to aneuploid.

3.2. Cell cycle delays during treatment

Cell cycle delay was examined in the G2 phase of the cell cycle by two different analyses. Firstly, the proportion of cells in G2 + M was estimated from the DNA profile. The data in Fig. 2a shows the percentage change in the posttreatment G2 + M cells. Only nine tumours failed to show an increase in the proportion of G2 + M cells after the first week of treatment, whilst 24 showed a significant accumulation of G2 + M cells with a median increase of 54% in this phase.

The accumulation of the cells in G2 + M does not necessarily indicate a G2 + M delay as the persistence of cells in this phase after treatment might be due to aborted mitosis due to chromosomal aberrations. We utilised the dynamic nature of the IdUrd and BrdUrd analyses versus the DNA profiles to look for evidence of

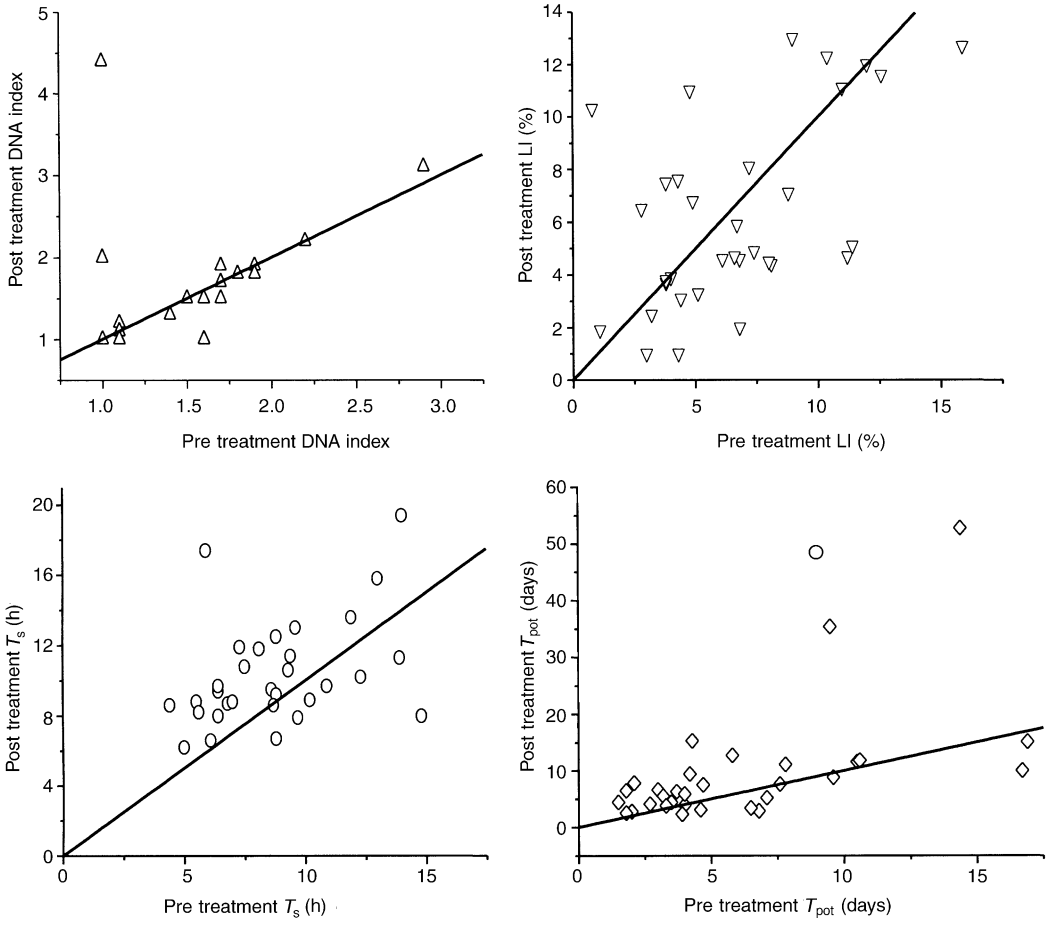

Fig. 1. Changes in cell kinetic parameters during treatment. The line of unity is drawn on each graph. LI, labelling index; T_{pot}, potential doubling time; T_s, duration of S phase.

cell division by studying the presence of labelled cells in G1. This analysis shows two aspects of interest, firstly evidence of cell division during treatment and quantitation of the G2 delay. It can be seen from Fig. 2b that the inhibition of labelled cells progressing into G1 showed a different pattern of delay to that seen when measuring G2 accumulation. The majority of tumour cells showed little change in the proportion of halogenated pyrimidine labelled cells that divide although, for most, any changes seen were positive changes, which is indicative of a G2 delay. Nine tumours showed evidence of greater cell division per unit time during treatment, although these changes were small.

3.3. Correlation between cell kinetic parameters and division delay

None of the pretreatment parameters correlated with the division delay during treatment. In particular, there was no evidence that the tumours that failed to show a LI reduction were those that did not show evidence of a G2 delay.

3.4. Correlation between cell kinetics and clinico-pathological parameters

There was no correlation between any of the pretreatment cell kinetic parameters and the clinico-pathological characteristics in this small cohort of patients. In addition, there was nothing remarkable, in terms of clinico-pathological features, about the group of patients whose tumours showed an increase in the LI during the first week of radiotherapy.

3.5. Cell kinetic changes and treatment outcome

The only pretreatment parameter that had a significant influence on disease-free survival was the DNA index (Fig. 3a). 45% of the tumours were diploid and these were associated with a favourable outcome ($P=0.05$). This finding was not observed when local control was used as endpoint. Of the parameters assessed during treatment, a reduction of the LI of 10% or greater predicted ($P=0.045$) a cohort of patients with a better overall survival (Fig. 3b). The value of 10% had

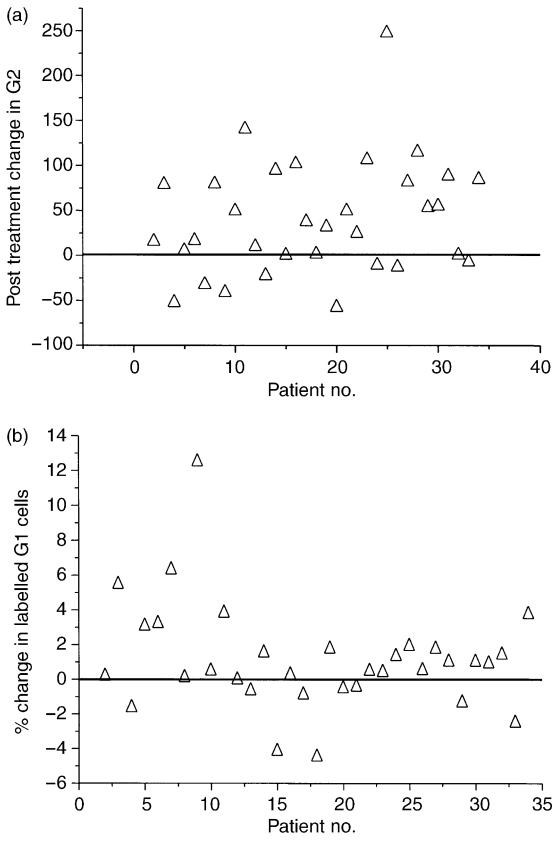

Fig. 2. Changes in cell cycle delays during treatment. (a) shows the change in the G2 + M population measured from the DNA profile and (b) shows the dynamic changes in the progress of labelled S phase cells through G2, mitosis and entry into G1 measured from the halogenated pyrimidine analysis.

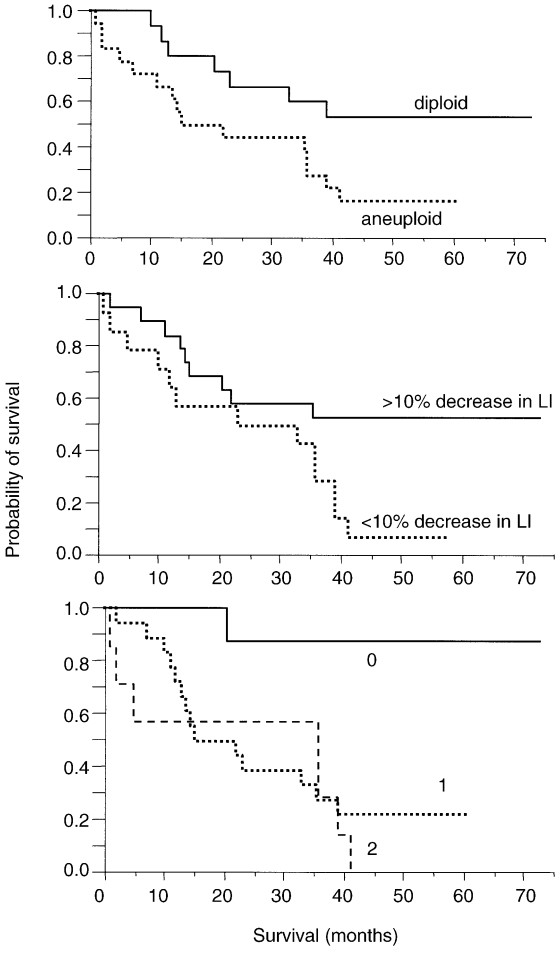

Fig. 3. The influence of the pretreatment DNA index on clinical outcome (a). The effect of a reduction in LI (>10%) on disease-free survival (b). The combination of the DNA index and reduction of LI on disease-free survival (c). In (c), 0 refers to tumours which were diploid and showed a reduction in LI, 2 refers to aneuploid tumours which did not reduce their LI and 1 represent tumours which showed only one of the adverse parameters.

been used in previous studies [9] and was also the median cut-off for this group of patients. Using a cut-off that delineated those patients that either showed a reduction compared with those which did not change or increased their LI further improved the discrimination ($P = 0.026$). Neither of the division delay parameters was able to discriminate for patient survival. In this small sample of patients, the combination of the pretreatment DNA index and a reduction in the LI was able to delineate a group of patients, 8/33 (24%) with diploid DNA and a reduction in the LI in which only 1 patient has failed treatment (Fig. 3c). In addition, all of the patients with aneuploid tumours that did not show a reduction in the LI failed on the treatment (7/33).

4. Discussion

This study has shown the feasibility of using two halogenated pyrimidines as DNA markers to measure cell kinetic parameters *in vivo* during radiotherapy. The technique we have reported has enabled the simultaneous study of more parameters than was previously possible with either [^{3}H]TdR or DNA profile analysis. We have studied changes in the DNA index, LI, T_s, T_{pot} and division delay in a clinical setting. We were able to demonstrate that all of the tumours were proliferating after the first week of treatment and that there were significant changes in the cell kinetic parameters during the first week of conventional radiotherapy. Reductions in the LI, but not cell cycle delays, during the first week of treatment were associated with a more favourable outcome.

Like other studies [8,9], we have demonstrated a reduction in the LI, in the majority of the tumours, as a result of treatment. The change we observed (30%) was similar to that reported by Courdi and colleagues [8], but less than the reduction observed by Silvestrini and colleagues [9]. In addition, we observed a lengthening of T_s which, combined with the lower LI, produced longer T_{pot} values for most of the tumours after the first week of treatment (4.5 versus 6.4 days). However, some caution must be applied with regard to the accuracy of the

T_s results due to the method of calculating T_s that involves measuring the mean DNA content of the IdUrd or BrdUrd labelled cells as a function of the difference in DNA content between G1 and G2. Any delay in the movement of the labelled cells through the G2 window will influence the mean DNA value of the population. Interestingly, this would normally result in shorter T_s values as the mean value is biased towards G2; we have found a lengthening of T_s that might suggest a true biological effect of radiation.

Neither of the two techniques to study G2 delay showed any relationship with the alterations of the LI, T_s or T_{pot} nor with clinical outcome. This suggests that the modulation of proliferation characteristics and the integrity of cell cycle regulation are independent mechanisms. Indeed, there was evidence of a division delay in the vast majority of specimens suggesting that abrogation of G2 delay is not a common event in head and neck tumours. The technology we have used did not allow us to study G1 delay with any great certainty as this checkpoint is clearly associated with mutation of the *TP53* gene and may have given insight into the biological consequences of gene malfunction during treatment.

The data presented in this study would certainly suggest that tumours show considerable proliferation, even after 10 Gy of X-rays *in vivo*. The data do not, however, show any evidence of increased proliferation (repopulation) at this early time point. In the study of Courdi and colleagues [8], the LI was still depressed after 30 Gy, again providing no evidence of an increased proliferation. The interpretation of this type of study does have its limitations. First, clonogenic cells cannot be distinguished from doomed cells. Second, there may be sampling artifacts between the two biopsies produced by intrinsic tumour heterogeneity. These are valid criticisms, but often in experimental tumours the time–course of the LI changes mirrors that of the clonogenic repopulation, albeit with at least an order of magnitude difference, in absolute cell terms, than the observed clonogenic surviving population. The problem of heterogeneity cannot be answered from the FCM data, but the consistent effects seen in the reduction in the LI in most tumours and the increase in the LI associated with the tumours of low pretreatment proliferative activity, suggest that these are true differences due to radiation.

The reduction in the LI after 1 week of radiotherapy appears to be able to discriminate patients who do better and this is in accord with a previously reported study [9]. When this parameter was combined with DNA ploidy information, it was able to identify a cohort of patients with a particularly good outcome. This observation might be a surrogate for a successful reduction in clonogenic cells in a genetic background without gross chromosomal abnormalities resulting in a radiosensitive phenotype. In contrast, the presence of aneuploidy and a failure to reduce the LI was associated with a total failure of treatment. This information could be helpful in planning treatment intervention in the latter group of patients where conventional radiotherapy has failed and an alternative therapy may have a better chance of success. However, the numbers are small and more patients will be required to make definitive statements. In a parallel investigation on this material, we are studying the histology of these tumours and the expression of a range of biological markers including p53, Ki-67, bcl-2, CD31 and the cyclins and the amount of apoptosis to establish whether any changes in the expression of these parameters during radiotherapy are associated with a poor prognosis. Using this combined approach, and accruing more patients, we hope to be able to apply a multifactorial analysis of relevant biological processes to pre- and posttreatment specimens that will allow us to better define the likely response of individual patients to radiotherapy.

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