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ARTICLE

Predictive Value of p53, Bcl2 and Bax in the Radiotherapy of Head and Neck Cancer

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Radiation is known to induce DNA damage resulting in the onset of apoptosis. The apoptosis is modulated by p53, Bcl2 and Bax proteins. High level of wild type p53 is required for radiation induced apoptosis. The p53 status, therefore, may be a crucial determinant of radiosensitivity of tumor cells. Overexpression of Bcl2, however, inhibits apoptosis via hetero- and homodimeric interaction. Bax might function as a cell death effector molecule that is neutralized by Bcl2. The aim of the present study is to investigate the correlation between p53, Bcl2, Bax and c-myc levels and the clinical response of head and neck cancer patients to radiation. The base line and 30 GY gamma radiation induced values of p53, Bcl2, Bax and c-myc were estimated by Western blot in 40 biopsies of head and neck cancers. We found that the radiosensitivity of head and neck cancer patients depends on the ratio of p53, Bcl2 and Bax protein levels. High Bcl2 levels resulted in radioresistance of cancer patients. Overexpression of Bax and c-myc may ensure the radiosensitivity of head and neck cancer patients. Our studies indicate that prediction of radiation sensitivity of tumors could be based on the simultaneous evaluation of p53, Bax and Bcl2 levels. (Pathology Oncology Research Vol 3, No 3, 204–210, 1997)

Key words: radiosensitivity, head and neck cancer, p53, Bcl2, Bax

Introduction

Fractionated radiotherapy plays an integral role in the management of head and neck cancers. Most squamous cell carcinomas of the head and neck (SCCHN) respond to radiotherapy, but not all. Tumor growth might continue during radiotherapy or metastasis may develop in the neck during treatment.

There have been some attempts to identify factors predicting response to radiotherapy. Among others, measurement of the tumor doubling time[1] and the micronucleus assay[32] have been applied. It was shown recently that ionizing radiation induces DNA damage followed by apoptosis.[14] An increasing body of evidence suggests that apoptosis is an integral part of the cytotoxicity of various anticancer therapeutic agents.[20] Therefore, several attempts have been made to establish a correlation between apoptosis and drug sensitivity of the tumors. Positive correlation between apoptosis and chemosensitivity of cancer cells has been reported.[16] Regarding the molecular mechanism of apoptosis, a central role of p53 in the regulation of programmed cell death has been proposed. While most reports lend support for the idea that wild type p53 is indispensable for apoptosis,[13] others have provided evidence for the existence of a p53 independent apoptotic pathway.[5] It has become clear that several genes are involved in the regulation of apoptosis, including p53 and various Bcl2 related genes (Bax, $BclX_{L/S}$, Bad). The mechanism of p53-mediated apoptosis was linked to p53-dependent upregulation of Bax and downregulation of Bcl2.[22] p53 is known to bind to DNA in a sequence specific manner and to regulate the transcription of several genes,[8,11,15] involved in cell proliferation (p21), DNA repair (GADD45), and apoptosis (Bax, Bcl2).

Wild type p53 maintains genomic stability by arresting cells at the G_1/S interface, thus providing opportunities for repairing damaged DNA.[7] If the repair fails, p53 may trigger cell suicide by apoptosis. Intracellular levels of p53

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increase dramatically in response to a variety of DNA damaging agents.[14] The chemoresistance of the tumors partly may be attributed to a decreased cellular susceptibility to apoptosis. Therefore, the p53 status of the tumor may be an important determinant of the efficacy of many treatment protocols. p53 mutation is very common in SSCHN.[38] It has been reported that only wild type p53 can mediate the radiation induced apoptotic pathway.[21] On the other hand, increased therapeutic sensitivity of tumor cells containing mutated p53 has also been reported.[2,18] Some studies have demonstrated that not all forms of apoptosis require p53.[5] p53 independent cytotoxicity was shown to be more pronounced at relatively high doses of radiation.[15] Factors that modulate p53 function could also influence cellular resistance to anticancer therapy. These factors include human papilloma virus E6 protein[35] and overexpression of Bcl2 oncogene.[4] Upregulation of the c-myc oncogene also sensitizes cells to apoptosis.[9] The aim of this study was to determine whether p53, Bcl2, Bax and c-myc levels have any influence on the outcome of curative radiotherapy in head and neck cancer patients.

Our results have shown that the predictive value of the constitutive p53 level is very low. Upregulation of Bcl2 induced the radioresistant phenotype. Whereas upregulation of Bax and overexpression of c-myc were associated with the radiosensitivity of head and neck cancer patients.

Materials and Methods

Patients

Twenty patients (3 females and 17 males) with stage T_1 N_0-T_3N_2 head and neck tumors were included in this study. The median age at the time of diagnosis was 55,6 years (range 44–75 years). All tumors were histologically verified squamous cell carcinoma. Eight tumors were well differentiated, six tumors were moderately differentiated and six tumors proved to be undifferentiated. All tumors were localised in the mesopharynx. Fourty biopsies have been collected before and after radiotherapy form 20 patients for Westwrn blot analysis and histological studies. The patients received no chemotherapy before the radiation. All patients received 60 GY in 2 GY fractions, five fractions per week. The efficacy of radiotherapy was evaluated by the palpation of the regional lymph nodes, and measurements of tumor volume were recorded after 6 weeks of radiotherapy. Complete remission of the tumor (CR) means that no residual tumor could be observed at the original site of the primary tumor after 6 weeks of the 60 GY irradiation. In the case of partial remission (PR), the tumor volume decreased by 50%. The volume of the resistant tumor (R) declined by less than 50%. The levels of apoptotic proteins have been evaluated before the irradiation and after 30 GY. The second biopsies were collected after 4 hours of 30 GY irradiation.

Western blot

Tissue were homogenized in RIPA buffer containing 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 1.0 mM PMSF, 50 µg /ml leupeptin in PBS and disrupted using a polytron homogenizer. Debris was removed by centrifugation at 12.000g for 30 min. The resulting supernatants were size-fractionated by 7.5-12% SDS-PAGE and transferred to Immobilon P membrane (Sigma), blocked in PBS containing 10% FCS and 0.1% Tween. Membranes were incubated overnight at 4°C in primary antibodies dissolved in blocking buffer, then washed three times in blocking buffer (15 min each) and incubated for 45 min in secondary antibody. (Anti-rabbit and anti-mouse IgG-alkaline phosphatase conjugates.) Following the secondary antibody, blots were washed three times in PBS/0.1% Tween, then developed using NBT/BCIP. Primary antibodies were as follows: p53-specific monoclonal antibody, PAb 1801 (Biogenex) Bcl2, and c-myc MAb (Dako).

Results

Response to radiotherapy

Among the 20 untreated patients who received 60 GY fractionated radiotherapy, 8 patients (40%) achieved complete remission (CR). 8 patients (40%) showed partial remission (PR). Four patients (20%) had minimal or no response, showing that these cancers were radioresistant (R).

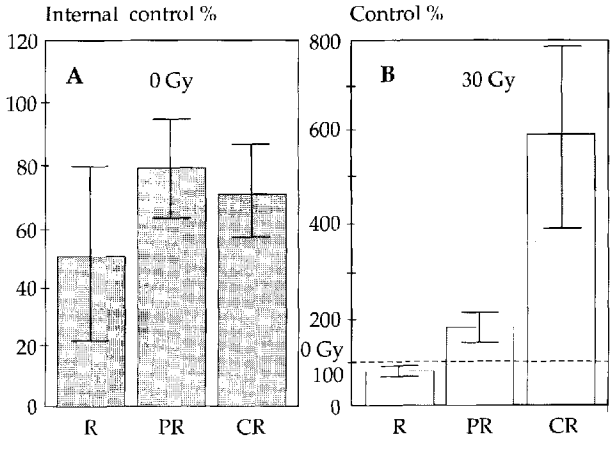

Figure 1. *P53 levels of head and neck cancers*

A. The baseline levels of p53 were estimated prior to the radiotherapy, by densitomery of Western blots. All data were normalized to 20 µg protein of Hep2 laryngeal carcinoma cells. R= radioresistant patient, PR= partial remission, CR= complete remission. Differences in the constitutive p53 levels of patients were not statistically significant.

B. Radiation induced p53 values of head and neck cancer. 30 GY irradiation induced a six-fold increase in the p53 levels of CR patients ($p<0.05$).

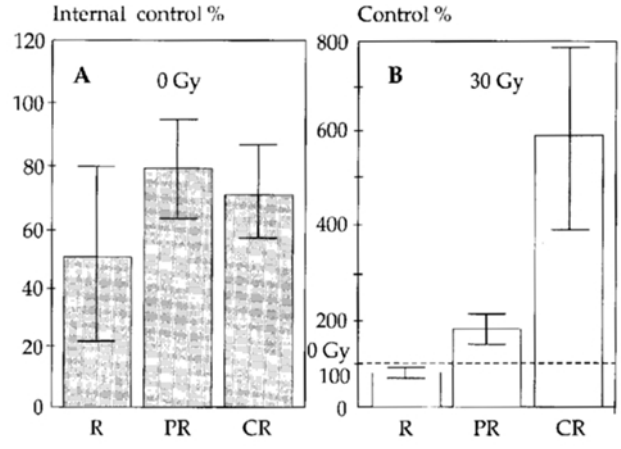

Figure 2. *Bcl2 levels of head and neck cancers*

A. Constitutive Bcl2 levels of R, PR, CR head and neck cancer patients do not show statistically significant differences (p<0.05).

B. Bcl2 level is upregulated in R patients and downregulated in CR patients (p<0.05).

Constitutive level of p53

The p53 status of the patients was determined prior to the radiotherapy using 1801 MAb, which recognizes wild-type as well as mutant forms of p53 proteins. The constitutive levels of p53 were measured by densitometric analysis of Western blot in 20 biopsies of head and neck carcinomas. The major goal of this investigation was to decide whether the radiosensitivity of squamous carcinomas of head and neck (SSCHN) could be predicted on the basis of p53 levels prior to the therapy. The baseline p53 levels (0 GY) of R, PR and CR patients are shown in *Fig. 1A*. Hep2 laryngeal carcinoma cells have been applied as an internal control. The densitometric values of each blot were normalized to the O.D. value of 20 µg protein of HEP cells. Our results have shown that there is no significant correlation between the baseline p53 value (0 GY) and the clinical response of the patients. We did not observe any p53 protein overexpression in radioresistant patients prior to the radiotherapy *(Fig. 1A)*.

Although the highest constitutive p53 level could be detected in PR and CR patients, its statistical significance could not be proved (p=0,0797 R/PR). Similarly, although the p53 level is the lowest in; the radioresistant patients, this finding is statistically not significant.

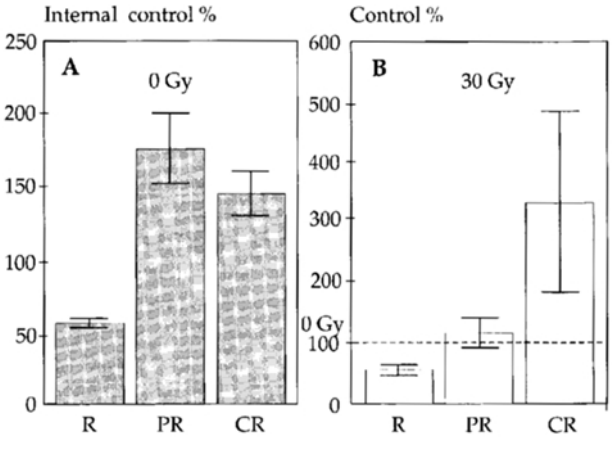

Figure 3. *Bax levels of head and neck cancers*

A. The Bax level is the lowest in the radioresistant patients (p<0.05).

B. 30 GY irradiation significantly (p<0.05) increased the Bax levels of radiosensitive patients (CR).

Radiation induced p53 levels in SSCHN patients

Since our studies have shown that the baseline levels of p53 have no effect on the clinical response, the radiation-induced p53 expression was also been studied in SSCHN patients. We found no increase in the p53 levels of the radioresistant patient (4/20) after 30 GY irradiation. At the same times, a six-fold increase in the p53 level could be detected in the patients with complete remission *(Fig. 1B)*. The radiation induced increase in p53 levels of the CR patients proved to be statistically significant (p<0,05) compared to the radioresistant patients.

Bcl2 status of SSCHN patients

The baseline value (0 GY) of Bcl2 is almost identical in the PR and CR patients *(Fig. 2A)*. The Bcl2 level in radioresistant patients is lower than that of PR and CR patients. The most important finding of our studies is that Bcl2 level is elevated after 30 GY radiotherapy upregulates Bcl2 in the radioresistant (R) patients *(Fig. 2B)* and downregulates Bcl2 in the radiosensitive patients (CR). The radiation induced changes in Bcl2 levels proved to be statistically significant (p=0,0078; R/CR).

Bax status of SSCHN patients

The baseline Bax levels of SSCHN patients are shown on *Fig. 3A*. The constitutive levels of Bax were found to be lowest in the radioresistant patients. The Bax levels of CR and PR patients were significantly higher than that of the R patients (p<0,05). The expression of Bax has also been evaluated after irradiation with 30 GY. Densitometric analysis revealed no evidence for post-irradiated increase of Bax in radioresistant patients *(Fig.3B)*. In contrast, a three-fold increase in Bax levels was detected by immunoblot analysis in tumor samples of SSCHN patients with complete remission *(Fig. 3B)*. The post-irradiated increase in Bax expression was found to be statistically significant (p<0,05).

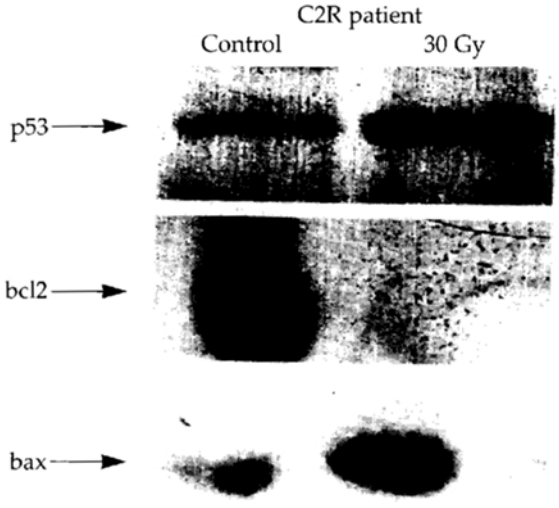

Figure 4. Western blot of p53, Bcl2, and Bax proteins of radiation sensitive patients (CR). Radiation induced upregulation of p53 and Bax and downregulation of Bcl2 could be observed.

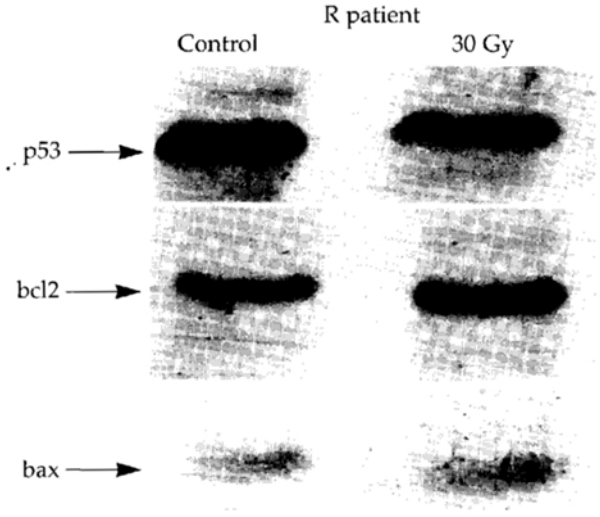

Figure 5. Western blot of p53, Bcl2, and Bax levels of radioresistant head and neck tumors. Radiation induced no increase in p53 or Bax level. Bcl2 level is increased after irradiation.

Fig. 4 shows a Western blot on p53, Bcl2 and Bax levels of radiation sensitive patients. Radiation induced p53 protein stabilization, upregulation of Bax and downregulation of Bcl2. We found that radioresistant tumors failed to exhibit p53 accumulation in response to ionizing radiation (*Fig. 5.*). The Bcl2 levels are elevated while the Bax changes remained unchanged in the radioresistant patients after 30 GY irradiation.

c-Myc status of SSCHN patient

The c-myc levels have been evaluated by densitometry of Western blot. Our results have shown that the c-myc levels in tumors of CR patients are significantly ($p<0.05$) higher compared to the radiation resistant patients (*Fig. 6*).

Discussion

The finding that p53 function is lost in many human tumors, including head and neck cancers, has led to the assumption that mutation of p53 is causally associated with sensitivity/resistance to anticancer therapy.[19] If tumors lack wild-type p53, their capacity for DNA repair is also lost.[14,35] This model predicts that tumors with wild-type p53 have enhanced DNA repair leading to increased survival (decreased therapeutic sensitivity) compared to tumors with mutated p53.

Some previous studies have indeed shown that p53 mutation resulted in enhanced sensitivity to ionizing radiation.[2,27] At the same time, others have found no correlation between p53 status and radiosensitivity.[3,34] More recently, a positive correlation between wild-type p53 and radiosensitivity has been reported.[33] Because of the conflicting data in the literature, in this study we have addressed the importance of p53 status with respect to the sensitivity of head and neck tumors to irradiation. The p53 protein has been detected by Western blot analysis using 1801 MAb, which is able to detect both the wild and mutant forms of p53.

It is presumed that p53 overexpression recognized by antibodies (immunohistochemistry, Western blot) might indicate the presence of mutant p53. Mutation can increase the half life of p53 and induce protein accumulation.[30] Our results have shown that the constitutive level of p53 is higher in patients with partial or complete remission, than in the radioresistant patients. This finding might favour the idea that p53 mutation is correlated with the radiosensitiv-

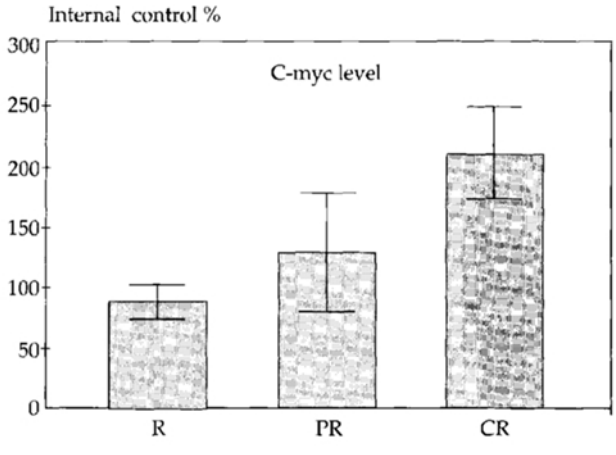

Figure 6. The c-myc values of head and neck cancers. The radiosensitive tumors (CR) contain higher amounts of c-myc than the radioresistant head and neck cancers ($p<0.05$).

ity of head and neck cancer patients. However, it turned out recently, that no precise correlation was found between p53 gene alteration and increased level of protein.[30] Missense mutations of p53 were associated with protein accumulation, and it is logical that no such mutation was found among the immunonegative tumors.[6] The molecular basis of the p53 protein stabilization is not clear and could be due to a variety of mechanisms including phosphorylation/dephosphorylation and binding to stabilizing proteins (mdm2, E6). All these mechanisms may function to prevent entry of p53 into ubiquitin-dependent proteolysis.[7]

The mutated p53 protein may favour a proliferation-permissive conformation. It has been suggested that wild-type p53 can adopt a mutant conformation (protein stabilization) under conditions of active cellular proliferation. Our data have shown that there is no significant correlation between the baseline p53 levels and the clinical response of head and neck cancer patients. Our results agree with the results of previous investigations of Wilson et al 1995 and Tan 1997,[37,38] who also found no correlation between the baseline p53 status and response to radiotherapy of the head and neck cancer patients. Therefore we can conclude that the constitutive levels of p53 have a very low predictive value in the case of radiotherapy. Since the p53 accumulation did not indicate either the p53 mutation or the transcriptional activity of p53, we studied the radiation induced p53, Bax and Bcl2 expression after 30 GY irradiation. We found that the p53 level is elevated in patients with complete remission but no p53 increase could be detected in the radioresistant patients. This difference is found to be statistically significant ($p<0.05$). To our knowledge this study is the first one which analyze the p53 levels during radiotherapy.

Head and neck cancer offers an excellent model for the monitoring of p53 status because the continuos collection of biopsies during the therapy is feasible. Our data indicated that the post-irradiation p53 status has a higher predictive value than that of the baseline value. Radiation has been shown to increase not only the p53 protein level but also to enhance its transcriptional activity.[17] It should be noted that the transcriptional targets of the wild type and mutated p53 are not the same. Transcriptional activation of basic fibroblast growth factor gene by mutant p53 has also been demonstrated.[38] Mutant p53 regulation of MPRI gene promoter activity has also been documented.[40] The wild type p53 is known to increase the transcription of p21, GADD 45, Bax[17,25] and downregulates the expression of Bcl2.[23] Therefore the evaluation of GADD 45, Bax gene expression after irradiation might contribute to the identification of wt or mutated p53.

Bcl2 is known to play a role in promoting cell survival by inhibiting apoptosis.[12] Recently, various Bcl2 related genes (Bax, $BclX_{L/S}$, Bak, Bag) have been identified.[29] Overproduction of Bcl2 resulted in blockage of apoptosis.[24] The anti-apoptotic activity of Bcl2 is modulated by Bax. Bax homodimerizes or heterodimerizes with Bcl2. When Bax is in excess and Bax homodimers dominate cells are susceptible to apoptosis. Bax-Bcl2 heterodimers prevent apoptosis.[26] High levels of Bcl2 expression occur frequently in acute myeloid leukemia resulting in chemoresistance.[4] p53 may induce apoptosis by altering the ratio of Bcl2 and Bax . Radiation has been shown to increase the level of Bax mRNA in tumors containing wild-type p53, but failed to induce such an increase in tumors with mutant p53.[39] Our studies have shown that the constitutive level of Bax protein is significantly higher ($p<0,05$) in the CR patients than in the radioresistant patients (R). We also found that the Bax protein level is elevated after 30 GY irradiation in CR patients. At the same time, radiation failed to induce a detectable increase in the Bax level of R patients. Our results suggest that the differential sensitivity of tumors to radiation-induced apoptosis correlates strongly with whether or not Bax expression is induced. It has been reported previously that mutation in p53 is accompanied by a marked reduction in the expression of Bax gene.[28] Therefore, the upregulation of Bax in CR patients demonstrated by us lends support for the involvement of wild-type p53 in radiation sensitivity. Our data suggest that both baseline values and radiation-induced high Bax levels have an outstanding predictive value indicating the therapeutic sensitivity of the tumors.

The outcome of apoptosis induction depends on the ratio of Bax: Bcl2. Thus, Bcl2 protein at levels which are sufficiently high to compete for the p53 induced Bax, may protect cells from p53-induced apoptosis. We found no significant correlation between the constitutive levels of Bcl2 and the clinical response of head and neck cancer patients. However, a significant downregulation of Bcl2 ($p<0,05$) was observed after 30 GY irradiation in CR patients. The Bcl2 levels are elevated after irradiation of the radioresistant patients. The promoter region of Bcl2 gene is known to have a wild-type p53 responsive element therefore, its downregulation by therapeutic treatment is well documented.[22] The increased Bcl2 levels in the irradiated R patients could be explained by the elevation of free Bcl2 rather than any changes in Bcl2 expression. Recently, a new Bcl2-related gene BclX was identified. Alternative splicing results in two BclX species called $BclX_L$ and $BclX_S$. $BclX_L$ appears to have functions similar to Bcl2 in preventing apoptosis.[31] Therefore, it could be suggested that the estimation of the apoptotic threshold of tumors has to be based on the levels of Bcl2 and $BclX_L$, as well.

It has been reported that enhanced expression of the $BclX_S$ gene can promote apoptosis in tumors with either wild-type or mutant p53.[36] This finding suggests that a

p53 independent apoptotic pathway also exists, such as mediation of apoptosis by $BclX_S$ or c-myc genes. C-myc has been shown to induce apoptosis under growth-restrictive states such a hypoxia, lack of nutrients.[9] We found that the c-myc level in the CR patients is significantly higher ($p<0.05$) compared to the radioresistant patients. Our finding lends further support to the idea that high c-myc levels promote apoptosis.[10] Our data presented here support the role of wild-type p53 in radiation induced cytotoxicity. Our results also indicated that the estimation of the apoptotic threshold of tumor cells could be based on the simultaneous determination of p53, Bcl2 and Bax levels of tumor cells. The low baseline value of Bax and Bcl2 is predictive of the radiosensitivity of patients. Further considerations of the predictive and prognostic use of these markers is warranted.

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