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Cell Cycle Inhibitors and Outcome after Radiotherapy in Bladder Cancer Patients

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The aim of this study was to correlate the expression of cell cycle inhibitors with outcome of patients with muscle-invasive bladder cancer treated with preoperative radiotherapy (46 Gy/4–5 weeks or 20 Gy/1 week) and cystectomy. Patients with pT3b (n = 42) or pT0 (n = 17) were included in the study. Expression of $p16^{INK4a}$ and $p27^{KIP1}$ was assessed immunohistochemically in pre-radiotherapy biopsies and cystectomy specimens. Previously reported results of $p21^{CIP1}$ expression were also included. No difference in pretreatment protein expression was found between patients with pT0 and pT3b. Expression of $p21^{CIP1}$ and $p27^{KIP1}$ was lower in cystectomy specimens than in pretreatment biopsies. None of the proteins showed significant impact on survival when analysed separately. However, patients with tumours showing > 50% expression of $p16^{INK4a}$, $p21^{CIP1}$, or $p27^{KIP1}$ displayed poorer cancer-specific survival rates compared with the remaining patients (p = 0.025). This effect was more pronounced in patients receiving 46 Gy than in those receiving 20 Gy. In conclusion, low expression of cell cycle inhibitors is related to favourable survival after pre-cystectomy radiotherapy.

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Response to radiotherapy varies considerably among patients with muscle-invasive transitional cell carcinoma of the bladder. Local control is obtained in 30–50% of the patients, depending on the radiotherapy regimen and possible use of concomitant chemotherapy (reviewed in (1)). The identification of factors that predict treatment outcome of these patients is warranted.

Radiosensitivity of human tumours is affected by the cells' position within the mitotic cycle (2–5), and thus by the cell population's relative distribution between cell cycle phases. Progression through the cell cycle is regulated by cyclin-dependent kinases (CDKs), whose activity is regulated by phosphorylation and activated by binding of A-, B-, D-, or E-cyclins (reviewed in (6)). At least two families of CDK inhibitors (CDKIs), the INK4 proteins and the CIP/KIP proteins, regulate the cell cycle negatively by binding to and inhibiting CDKs. The members of the INK4 family specifically inhibit cyclin D-associated kinases (7). In addition to cyclin E- and cyclin A-associated kinases, cyclin D-associated kinases control the passage through G1 into the S-phase of the cell cycle (reviewed in (8)). Members of the CIP/KIP family bind and inhibit cyclin E-A/CDK2 complexes, but are also required for the assembly of the active cyclin D/CKD4-6 complexes (reviewed in (9)). Their biological effect seems to depend on the abundance of the proteins, where lower levels promote assembly factor functions and higher levels represent a prerequisite for their inhibitory activity (10).

Through their effect on the cell cycle, CDK inhibitors are involved in senescence, quiescence and differentiation, but details about their role and significance are still unclear. The p16 protein level is upregulated in senescent cells in parallel with an increasing number of population doublings. p16 function is commonly lost in immortalized cells (reviewed in (11)). Reduced p16 expression is predictive of shortened overall survival in T3-T4 TCC tumours (12). p21 expression is induced in response to extracellular stress signals (reviewed in (13)), but is also thought to be involved in senescence and differentiation (reviewed in (14)). p27 expression and activity is highest in quiescent cells and declines as cells re-enter the cell cycle. Many antimitogenic or differentiation signals lead to p27 accumulation, including TGF-β, mitogen/cytokine withdrawal, cell–cell contact, and agents such as cAMP and rapamycin (reviewed in (15)).

Cell cycle inhibitors may influence the outcome after radiotherapy in several ways: In addition to their abovementioned influence on the cell population's relative distri-

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bution between cell cycle phases with different radiosensitivity, CDK inhibitors may modify the cells' ability to recover from radiation damage: ionizing radiation can induce cell cycle arrest or delay in G1, S and G2 (reviewed in (16)), through increased expression of cell cycle inhibitors. This mechanism is thought to be important for repair of damage induced by radiation (17, 18). The various CDK inhibitors may have a different impact on the cell cycle distribution and repair, and the response may be modulated further by the number of radiation fractions and dose per fraction. CDK inhibitors may also have other functions yet to be discovered, which may influence the radiotherapy response.

The aim of this retrospective study was to investigate whether the expression of CDK inhibitors in biopsies from human bladder cancer predicts radioresponsiveness in patients with muscle-invasive transitional cell carcinoma (TCC), and if the expression is associated with survival after planned preoperative radiotherapy followed by cystectomy. In an earlier study, our group investigated the role of immunohistochemically detectable p53, p21 and mdm2 expression in pretreatment biopsies from a highly selected group of preoperatively irradiated bladder cancer patients, for predicting outcome after radiotherapy (19). In the present study, we have extended the results from p21-staining in pretreatment biopsies with staining in cystectomy specimens from those patients carrying residual tumour after radiotherapy, and supplemented with staining of another two CDK inhibitors; p16 and p27, in pre- and post-radiotherapy tumour specimens.

MATERIAL AND METHODS

Treatment principles

From 1980 to 1995, radical cystectomy after preoperative pelvic radiotherapy was the treatment of choice for patients with operable T2-T4a bladder cancer referred to the Norwegian Radium Hospital (NRH). A pretreatment biopsy was taken from all patients at the time each patient was included in the treatment schedule. During this 15-year period, the radiotherapy schedule was changed on the strength of accumulated clinical experience and research results: up to about 1986, patients received 23 daily fractions of 2 Gy to two opposing pelvic fields during 4.5–5 weeks before radical cystectomy, which was performed after an interval of 4–5 weeks (time from start of radiotherapy to surgery: median = 77 days, range 62–101 days). From about 1986, the treatment policy was gradually changed, and 4 Gy × 5 was given during one week with similar fields and with the operation one week thereafter (time from start of radiotherapy to surgery: median = 8.5 days, range 7–23 days). In general, patients were followed-up at the outpatient department of the NRH for one year, and at their community hospital thereafter.

Patient selection

All patients with muscle-invasive TCC of the urinary bladder (T2-T4a) referred to the NRH in the period 1980–1995 for preoperative pelvic radiotherapy before subsequent cystectomy were evaluated for inclusion in the study. Eligibility criteria included: histological proof of tumour invasion of the muscular layer of the bladder wall in biopsy material obtained before the start of radiotherapy; confirmed residual tumour (by cystoscopy or x-ray) after transurethral resection of the bladder before start of radiotherapy; pre-cystectomy target radiation dose 20 or 46 Gy; no prior or concomitant chemotherapy; histological category pT0 (no residual tumour) or pT3b (residual tumour present) in the cystectomy specimen; and sufficient tumour tissue available in the paraffin-embedded blocks from the pretreatment biopsy. None of the patients had had systemic chemotherapy or pelvic radiotherapy prior to referral.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections were placed on silan-coated slides and immunostained using the biotin-streptavidin-peroxidase method (Super Sensitive Immunodetection System, LP000-UL, BioGenex, San Ramon, CA, USA). Deparaffinized sections were microwaved in 10 mM citrate buffer, pH 6.0 for 4 × 5 min to unmask p16 or p27 epitopes. Pressure cooking in 10 mM citrate buffer, pH 6.0 for 10 min was used to unmask p21 epitopes. After treatment with 1% H_2O_2 for 10 min to block endogen peroxidase activity, the sections were stained with monoclonal antibodies for p16 (Ab-4 (16P04), NeoMarkers, CA, USA, diluted 1:100), p21 (OP64, Calbiochem, UK, diluted 1:200), and p27 (Transduction Laboratories, KY, USA, diluted 1:200) for 30 min at room temperature. The slides were then incubated with biotinylated secondary antibodies (1:30) and horseradish peroxidase-conjugated streptavidin (1:30) for 20 min each, before development with 0.07% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, Saint Louis, Missouri, USA) freshly prepared in 0.05M tris(hydroxymethyl)aminomethane (Tris-) buffer at pH 7.6, containing 0.024% H_2O_2, and counterstaining with hematoxylin. Each step in the staining procedure was followed by thorough rinsing in OptiMax wash buffer (BioGenex). Positive control slides were processed in parallel with each batch of staining. Negative controls included replacement of the primary antibody with mouse myeloma protein of the same subclass and concentration. The IHC scoring was performed by two independent investigators (AaB, RH). The percentage of tumour cells showing nuclear staining was evaluated semiquantitatively and categorized as follows: 0/+, ⩽5%; ++, >5–50%; and +++, >50%.

Statistical analysis

The statistical program SPSS (release 9.0) was used for all calculations. Associations between variables were evaluated with χ^2 tests. The test for linear trend was used with ordinal variables when appropriate. Cancer-specific survival was assessed by the Kaplan–Meier method with the cut-off date being the date of patients' death or, for surviving patients, 1 July 2000. The median observation time was 25 months (range: 5–245) and the median observation time for surviving patients was 182 months (range: 98–245). The cause of death of each patient was retrieved from the NRH's medical records, the Norwegian Cancer Registry, or by contacting the community hospital associated with the case. Death from bladder cancer included death caused by the malignancy or death caused by intercurrent disease in patients with recurrent bladder cancer. Differences between the Kaplan–Meier curves were evaluated using the log-rank test with linear trend for factor levels. Possible differences in protein expression before and after treatment were calculated by the sign test. The level of significance was set at 0.05.

RESULTS

Fifty-nine eligible patients were included in the study (19 females, 40 males, age range 42–75 years, median 64 years). Of these, 42 patients had residual tumour in the cystectomy specimen (pT3b: 46 Gy: 14 patients; 20 Gy: 28 patients), while 17 had no residual tumour (pT0: 46 Gy: 9 patients; 20 Gy: 8 patients). When we compared patients *with* (pT3b) or *without* (pT0) residual tumour, no significant differences were found regarding sex, age, pretreatment haemoglobin value, s-creatinine, T-category, histological grade (20), or radiation schedule (Table 1). Ten years' cancer-specific rate of survival for pT0 was 58%, and 20% for pT3b (log-rank test, $p = 0.007$). Thirty-eight patients died of bladder cancer, whereas 9 died of other causes (complications: 1; other cancers: 5; benign conditions: 3). Twelve patients were still alive at the last follow-up, with a minimum observation time of 90 months. Seven patients developed local recurrence and 25 developed distant metastases. Three patients developed both local recurrence and distant metastases, whereas the exact site of relapse remained unknown for three patients. No significant association with survival was shown for sex, age above or below median, pretreatment haemoglobin value, s-creatinine, T-category, histological grade, or radiation schedule (log-rank test, $p > 0.05$) (data not shown).

Pretreatment biopsies

No association was found between expression of p16, p21, or p27 (χ^2 test; $p > 0.05$) (data not shown). Frequency distributions of immunostaining in pretreatment biopsies related to pT-category and results from cystectomy specimens are displayed in Table 2. No associations were found between the pretreatment expression of any of the investigated proteins and presence or absence of residual tumour after radiotherapy (χ^2 test, linear-by-linear association; $p > 0.05$) (Table 2, left part). None of the CDK inhibitors (p16, p21 or p27) showed any significant impact on survival when analysed separately (log-rank test, $p > 0.05$) (Fig. 1a–c).

Cystectomy specimens

Among patients with residual tumour in the cystectomy specimen, we compared expression of each of the inhibitors in pretreatment biopsies with expression in the corresponding cystectomy specimen (sign test). The expression of p21 and p27 was reduced in the cystectomy specimens ($p = 0.039$ and 0.004, respectively), whereas the expression of p16 did not change significantly. For p21, 10 patients had a lower score, 2 had a higher score and 30 had the same score after treatment compared with pretreatment levels. For p27, 24 patients had a lower score, 3 had a higher score and 11 had the same score. For p16, 7 patients had a lower score, 6 had a higher score and 26 had the same score (for a more detailed presentation of results from before and after treatment, see Table 2).

Unspecified cell cycle inhibitor expression (UCIE)

All the CDK inhibitors investigated in this study exert their cell cycle regulatory effect in G1/S. To examine the effect of unspecified cell cycle inhibition in G1 or S-phase, we constructed a new variable labelled 'Unspecified Cell

Table 1

Patient demographics

	pT0	pT3b	Total
Gender			
Female	4	15	19
Male	13	27	40
Age			
≤64 years	9	20	29
>64 years	8	22	30
Radiation dose			
20 Gy	8	28	36
46 Gy	9	14	23
Haemoglobin level, pre-radiotherapy			
Hb ≤ 11.0 g/dL	3	4	7
Hb > 11.0 g/dL	14	38	52
S-creatinine			
≤120 μmol/L	16	36	52
>120 μmol/L	1	6	7
T-category			
T2	5	5	10
T3/T4a	12	37	49
Histological grade			
Grade 2	0	3	3
Grade 3	17	39	56

Table 2

Protein expression in cystectomy specimens related to pretreatment IHC score

Variable	Pretreatment score	No. of patients		No. of patients with designated post-treatment IHC score (vertical), related to pretreatment score (horizontal)								
				pT0	pT3b	p-value[1]		pT3b				p-value[2]
								0/+	++	+++	non-eval.	
p16	0/+	41	→	11	30		→	23	5	0	2	
	++	13	→	6	7		→	2	3	1	1	
	+++	5	→	0	5		→	2	3	0	0	
						0.78						1.00
p21	0/+	37	→	11	26		→	25	1	0	0	
	++	21	→	6	15		→	9	5	1	0	
	+++	1	→	0	1		→	1	0	0	0	
						0.73						0.039
p27	0/+	9	→	2	7		→	6	0	0	1	
	++	33	→	11	22		→	14	3	3	2	
	+++	17	→	4	13		→	5	5	2	1	
						0.89						0.004
UCIE	Low	37	→	13	24							
	High	22	→	4	18							
						0.24						

[1] χ^2 test, linear-by-linear association, comparing protein expressions between patients with pT0 and pT3b.
[2] Sign test, comparing protein expressions before and after treatment in patients with residual tumour in the cystectomy specimen.
Abbreviations: UCIE = unspecified cell cycle inhibitor expression; IHC = immunohistochemistry.

cycle Inhibitor Expression' (UCIE score), based on the combined expression patterns of the studied CDK inhibitors: The 'high UCIE group' (n = 22) comprised samples with > 50% expression of one or more of the CDK inhibitors, and the 'low UCIE group' (n = 37) comprised the remaining samples.

UCIE in pretreatment biopsies

Pretreatment UCIE did not predict presence or absence of residual tumour after radiotherapy (p = 0.24, Fisher's exact test). However, a high UCIE score was significantly associated with unfavourable cancer-specific survival (p = 0.025, log-rank test) (Fig. 2a). Stratified for treatment modality, UCIE was not significantly associated with cancer-specific survival for patients treated with 20 Gy (p = 0.29, Fig. 2b), but for patients receiving 46 Gy, those with low UCIE had the best survival (p = 0.024, Fig. 2c).

UCIE in cystectomy specimens

UCIE assessed in cystectomy specimens did not correlate with survival, but the expression was significantly lower after treatment compared with pretreatment levels (sign test, p = 0.004). Considering the 59 pretreatment biopsies, 22 patients (37%) belonged to the high UCIE group. Considering the cystectomy specimens, 5 out of 42 patients (12%) displayed a high UCIE score. Only 2 patients displayed a high UCIE score in both the cystectomy specimen and the pretreatment biopsy (data not shown).

DISCUSSION

The expression profiles of three cyclin-dependent kinase inhibitors were examined to evaluate their possible influence on the outcome after radiotherapy of patients with muscle-invasive bladder cancer. Assessed individually, none of the CDK inhibitors displayed any significant correlation between expression in pretreatment biopsies and outcome after treatment, whether outcome was assessed as presence or absence of residual tumour in the cystectomy specimen, or as cancer-specific survival. However, when assessed together, patients with high expression (> 50%) of any of the CDK inhibitors (= high UCIE score) showed a significantly shorter cancer-specific survival than the others.

When stratified for different treatment modalities, the UCIE score displayed statistical significance only in the subpopulation of patients treated with 46 Gy in 23 fractions in 4.5–5 weeks, and not in those treated with 20 Gy in 5 fractions in one week (p = 0.024 and 0.29, respectively). The biological effect of these two fractionation regimens with respect to tumour response can be calculated using the linear-quadratic formalism (LQ-model), and expressed as the equivalent dose given in 2 Gy fractions, one fraction per day (Fig. 3). In the conventional LQ-model, total dose, dose per fraction and number of fractions are taken into account. However, a modification of this model is needed in order to take tumour cell proliferation into consideration during a course of fractionated radiation therapy:

$$BED = n \cdot d \cdot [1 + d/(\alpha/\beta)] - k \cdot (T - Tk)$$

where n is the number of fractions, d is dose per fraction, α and β are radiosensitivity parameters, T is overall treatment time, Tk is onset of tumour cell proliferation, and k is the dose per day lost as a result of proliferation; k is related to tumour doubling time (Td) and radiation sensitivity as $k = \ln 2/(\alpha \cdot Td)$. In the calculations presented in Fig. 3, k

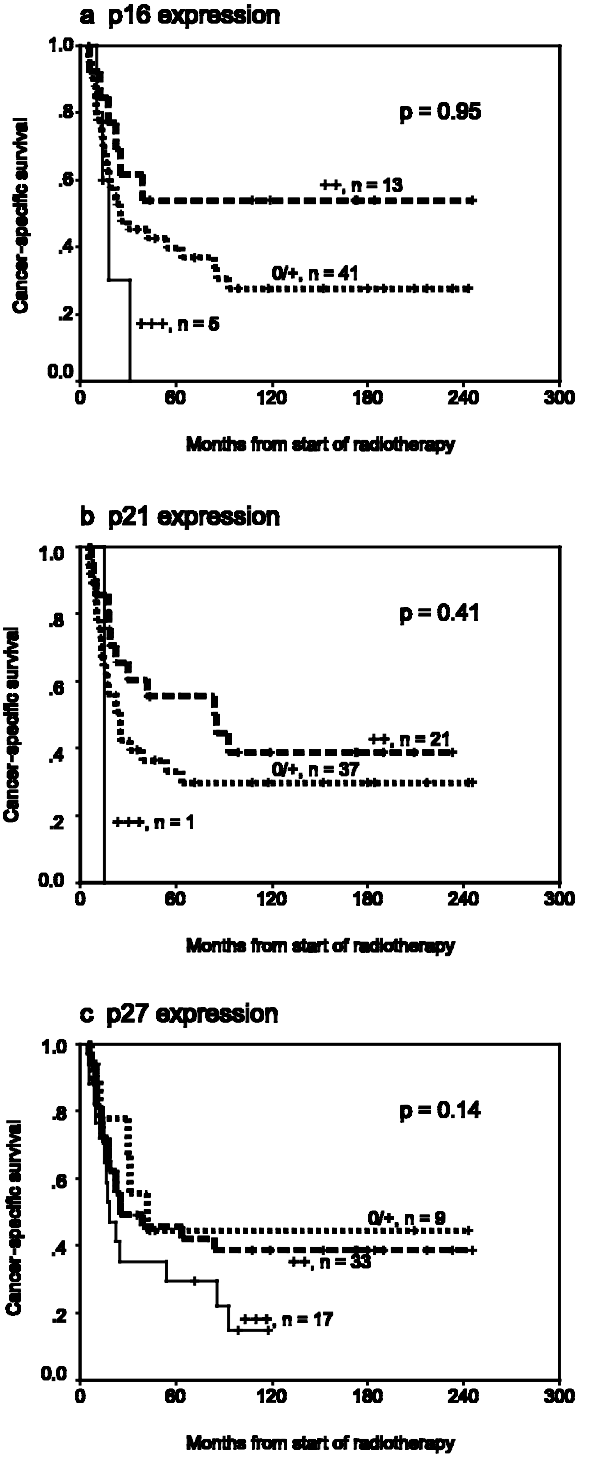

Fig. 1. Cancer-specific survival in patients with transitional cell carcinoma (TCC) bladder cancer treated with preoperative radiotherapy and cystectomy, in relation to expression of (a) p16; (b) p21; (c) p27.

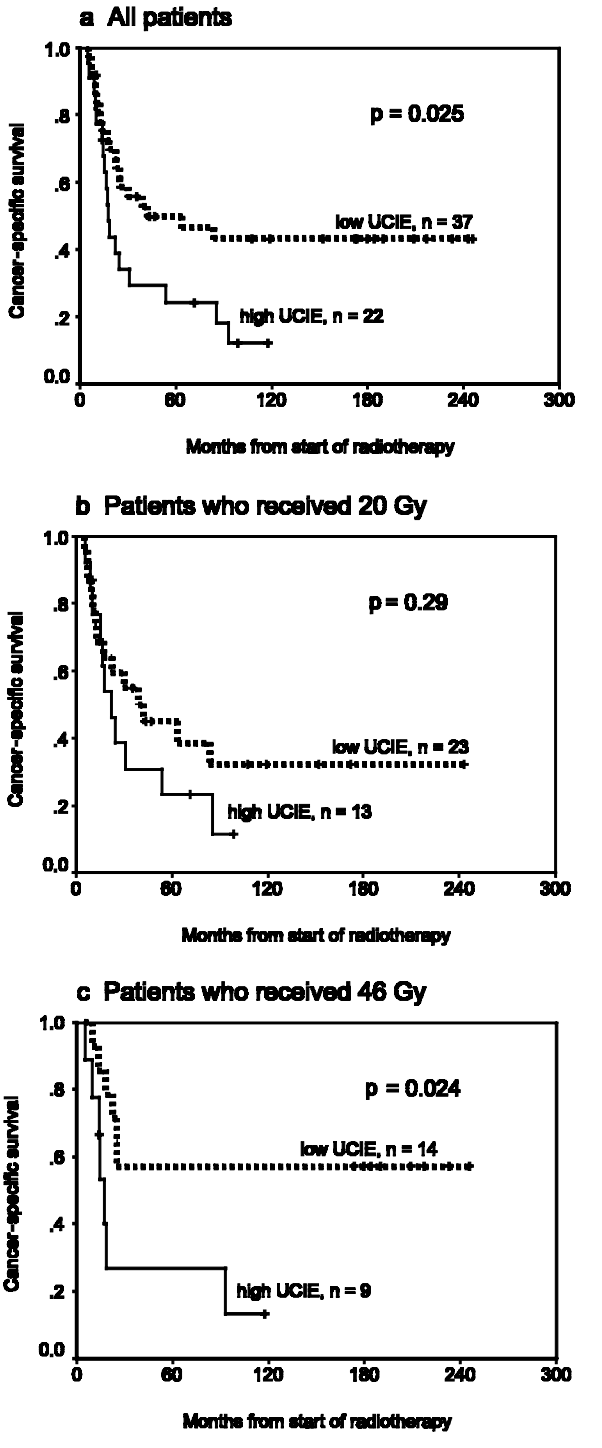

Fig. 2. Cancer-specific survival in patients with transitional cell carcinoma (TCC) bladder cancer treated with preoperative radiotherapy and cystectomy, in relation to unspecified cell cycle cnhibitor cxpression (UCIE) in (a) all 59 patients; (b) patients treated with 20 Gy preoperatively; (c) patients treated with 46 Gy preoperatively.

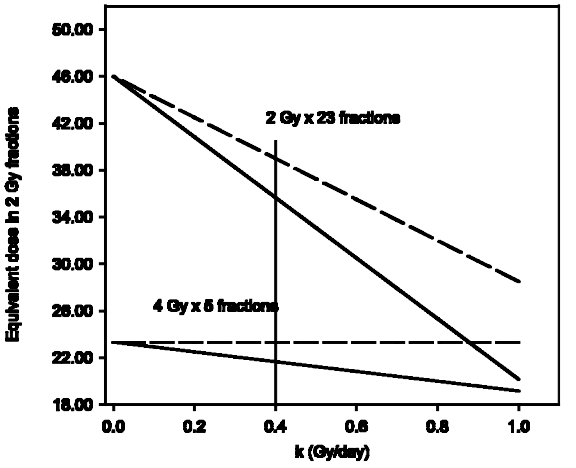

Fig. 3. Calculated biological effect of 20 Gy in 5 fractions vs. 46 Gy in 23 fractions, expressed as the equivalent dose given in 2 Gy fractions, and as function of tumour proliferation. Both regimens are given as 5 fractions per week. Calculations are based on $\alpha = 0.1$, $\beta = 0.01$; i.e. $\alpha/\beta = 10$, Tk = 0 days (solid line) and 10 days (dotted line).

varies between 1 and 0 Gy per day, corresponding to a tumour doubling time of approximately 7 days to infinity. It is evident from the calculations shown in Fig. 3 that the biological effectiveness of a standard fractionated regimen of 46 Gy is significantly higher than the hypo-fractionated regimen of 20 Gy, if tumour cell proliferation is negligible. However, as the k-value increases, i.e. tumour doubling time decreases, a marked reduction in the biological effectiveness of the standard fractionated regimen is seen. The corresponding reduction for the hypo-fractionated regimen is much less pronounced. Moreover, if onset of tumour cell proliferation (Tk) occurs later than the actual overall treatment time, i.e. Tk > T, no modification of biological response appears. The expected biological effect of these two regimens will be equal only for very short tumour doubling times. Previous published analyses of the relationship between total dose required to achieve 50% tumour control and overall treatment time indicate a value of k equal to approximately 0.4 Gy/day (21). This corresponds to a potential doubling time of approximately 17 days. Our calculations therefore demonstrate that, even after accounting for tumour proliferation in T3 tumours, the conventional fractionated regimen of 46 Gy exerts a higher biological effect on the tumour tissue than the hypo-fractionated regimen of 20 Gy. Moreover, these results are in accordance with the complete remission rates observed for the two regimens (Table 2). This implies that the observed association between the UCIE score and survival is somehow related to the treatment, even if a role for the UCIE score as a treatment-independent prognostic factor cannot be ruled out.

The increase in expression level of p21 in response to ionizing radiation (6 h after radiation) is well documented, but less is known about how the relative number of p21-expressing tumour cells before radiation affects radiosensitivity in the tumour. Murine carcinomas with low constitutive expression of p21 have shown increased radiocurability (22). Low levels or complete loss of p21 expression has previously been reported to be associated with increased radiosensitivity in several in vitro studies (Waldman, human colon cancer; Wouters, human colon cancer; Kokunai, human gliomas; Song, mouse epidermal and hair follicular cells) (23–26). In contrast, functional expression of the human p21 gene in rat glioma cells induced radiosensitivity (27), and p21 positivity tended to be associated (without reaching statistical significance) with increased survival in TCC bladder cancer patients treated with radiotherapy (28). On the other hand, patients with p21-negative pancreatic cancers treated with resection and adjuvant chemoradiation displayed decreased survival rates (29). With regard to p27, a high labelling index before radiotherapy has been associated with improved disease-free and metastasis-free survival in a study of squamous cell carcinoma of the cervix (30). Transfection of p16 genes increased the radiosensitivity in two human glioma cell lines (31), and in two lung adenocarcinoma cell lines (32), but showed no effect in a nasopharyngeal cell line (33). Expression of p16 protein induced increased radiosensitivity in human melanoma cell lines (34), but Valenzuela et al. found no relationship between p16 expression and radiosensitivity in a series of human tumour cell lines (35). Taken together, these results are conflicting. Possibly the genes influence the radiosensitivity in a different manner in different types of cancer.

Assuming that 1) CDKI expression predominantly reflects cell cycle arrest, and 2) arrested cells are relatively radioresistant, the displayed effect of UCIE on survival suggests that it may be the cell cycle arrest as such, irrespective of the cause of arrest, which is important for the radioprotective effect. Accordingly, assessment of one inhibitor at a time will identify only some of the patients with radioresistant tumours, whereas several others will not be identifiable. Under these assumptions, our results regarding the association between UCIE and survival are also in accordance with previous findings which suggest that CDK inhibitors may improve the cells' radioresistance by giving the cells more time to repair the damage before reaching mitosis (reviewed in (17, 18)). M-phase is often the most sensitive cell cycle phase, whereas interphase cells display varying, but generally higher radioresistance (4, 5). However, this effect is probably of minor clinical importance since the M-phase is believed to be too short to contribute to a change in radiosensitivity in the cell population. Furthermore, the variations in radiosensitivity throughout the interphase are considered small in comparison with the changes provided by the extended time for repair.

Tumours dominated by (presumably) cell cycle arrested cells are thus 'protected' from the cytotoxic effect of the radiation. The radioresistance profile, especially during the

interphase, seems to vary between cell types or even lines of the same type. As noticed by Hill et al., some of these reported differences may be due to methodological differences (5). Cell type specific characteristics and acquired genetic aberrations may also account for some of the observed variability.

At present, these explanations must be seen merely as speculation, since there is a paucity of information about the fundamental characteristics of the tumour cells. Regarding the first assumption, the CIP/KIP family of CDK inhibitors, here represented by p21 and p27, is known to be involved in assembly of cyclin D/CDK4-6 complexes without inhibiting the cell cycle progression. To what extent cells stained by p21 or p27 antibodies, or even p16 antibodies, in this series are in a state of cell cycle halt or arrest is not known. Furthermore, the relative radiosensitivity of TCC cells in different cell cycle phases has not been explored. If, however, there is an induced cell cycle arrest that is responsible for the displayed effect, it should be considered whether other G1/S inhibitors, or even inhibitors of other parts of the cell cycle, should be included in the panel for assessment of radioresistance.

Cystectomy samples

We found that expression of p21 and p27, but not p16, was decreased in the cystectomy specimens (Table 2). This decrease in protein expression after irradiation may, theoretically, be due 1) to selective removal of cells expressing the actual CDK inhibitor, and/or 2) to a general downregulation of the protein expression in surviving cells. Radiation-induced kill of cells with high expression of cell cycle inhibitors is less probable as these cells are assumed to be relatively radioresistant. We therefore believe that downregulation of protein expression is a more probable explanation of our findings. Future radiobiological work should specifically study the expression of cell cycle inhibitors before and after radiotherapy.

Methodological considerations

In order to be absolutely certain about the radiotherapy response, only those patients were included in this present study who were deemed to have residual tumour after their TUR-B. Small size of this residual mass and high radiosensitivity may in part explain why 8 patients became tumour free after the short post-radiation interval following the 20 Gy regimen. In this retrospective study it was not possible to quantitate the impact of this confounding factor.

By setting the cut-off limit to 50% for the factors involved in the UCIE classification, only samples dominated by marked cells were considered as positive. This approach was used in an attempt to increase the probability of detecting a possible influence of the CDK inhibitors on survival or local treatment outcome. The clinically relevant limit for CDK inhibitor expression may well be below this limit. In more detailed studies, it may be possible to define this limit more accurately.

CONCLUSION

In our series, patients with tumours displaying high expression ($> 50\%$) of any one of the CDK inhibitors p16, p21 or p27, had a shorter cancer-specific survival time after treatment, compared with patients displaying low expression ($\leqslant 50\%$) of all the investigated CDK inhibitors ($p = 0.025$). This effect was more pronounced in patients receiving 46 Gy ($p = 0.024$) than in those treated with 20 Gy ($p = 0.29$). When analysed individually, the CDK inhibitors did not display any significant effect on locally assessed treatment outcome or survival. Assessment of the possible impact of other CDK inhibitors on treatment outcome and survival is warranted, together with a more precise determination of the appropriate cut-off value for clinical application.

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