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Pre-treatment proliferation and the outcome of conventional and accelerated radiotherapy

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ABSTRACT

This study investigated the influence of pre-treatment proliferation characteristics, assessed by Ki-67 staining, in patients treated in the CHART trial of accelerated radiotherapy in head and neck cancer. Histological material from 402 patients was collected and stained for the presence and pattern of Ki-67 staining. Locoregional control and overall survival were the main clinical endpoints. Increasing Ki-67 positivity was associated with decreasing differentiation ($P < 0.001$) and increasing N-stage ($P < 0.004$). Increasing N-stage was also associated with the progression of proliferation pattern from marginal to random ($P < 0.001$). Using a multivariate model, a trend was seen towards a greater benefit from CHART in the lower Ki-67 tumours ($P = 0.08$); this became significant by pooling the low and intermediate Ki-67 groups in comparison with the high Ki-67 group ($P = 0.032$). Tumours with marginal proliferation pattern showed a lower hazard ratio with CHART versus conventional for locoregional control ($P = 0.005$). The data presented in this study do not support that a high pre-treatment Ki-67 is associated with a therapeutic benefit from accelerated radiotherapy.

1. Introduction

Cellular repopulation is widely recognised as one of the most important determinants governing the clinical outcome of fractionated radiotherapy [1,2]. Over the past 10 years there has been a succession of clinical trials reported in head and neck squamous cell cancer (HNSCC) that explored the potential of altered fractionation to improve the results obtained with conventional, empirically-derived schedules [3–13]. These trials provided compelling evidence for the importance of overall treatment time [14]. A key feature of HNSCC patients is heterogeneity which is manifested in both their response to treatment and their tumour biology. It is evident that whilst modest gains can be made from accelerated treatment in the overall patient population, certain tumours may benefit more, or less, from altered fractionation. This concept is the impetus for research into predictive factors that can identify patients, based on tumour characteristics, that are better suited to non-conventional schedules.

The CHART (continuous, hyperfractionated, accelerated radiation therapy) schedule has been the most radical of accelerated treatments to be tested in a multi-centre randomised trial [15]. In the 918 cases entered into the head and neck trial, there was only a small, non-significant improvement in local tumour control. However, as a consequence of the strong acceleration in CHART where the treatment time was

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reduced from $6\frac{1}{2}$ weeks to 12 days, this trial represents a unique opportunity to resolve some of the underlying biology that dictates treatment outcome.

The commonly held hypothesis is that accelerated schedules are likely to benefit patients whose tumours possess the capacity for rapid regeneration. However, although repopulation is likely to be a multifactorial process, measurement of pre-treatment cell kinetic parameters is the most promising way of predicting the likelihood of patients failing conventional radiotherapy because they reflect the rate of cell production. In recent years, the application of flow cytometric analysis of dynamic cell kinetic measurements utilising halogenated pyrimidines administration to patients, has failed to provide a consistent measure to predict the repopulation of surviving clonogenic cells in fractionated schedules of radiotherapy [16]. In parallel, the assessment of proliferation using monoclonal antibodies recognising the cell cycle associated protein, Ki-67, has presented the opportunity to study proliferation in retrospective and prospective histological material [17–24]. In head and neck cancer a variety of investigations have assessed the clinical utility of Ki-67 labelling index with mixed results [25]. These studies fail to concur due to variable patient numbers and different sites in the head and neck and treatment modalities being investigated.

In this present study we have analysed both Ki-67 labelling index and its distribution in a cohort of 402 patients treated in the randomised setting of the CHART trial. The trial was designed to investigate the influence of proliferation on treatment outcome radically different overall treatment times of 12 days versus 61/2 weeks and represents an ideal opportunity to test the clinical utility of Ki-67 expression.

2. Patients and methods

2.1. Patients

The patients involved in this study were drawn from the randomised multi-centre trial of CHART versus conventional radiotherapy in head and neck cancer. Nine hundred and eighteen patients with squamous cell cancer (SCC) in the main sites within the head and neck region were included into the trial from 11 centres. The randomisation was 3:2 in favour of CHART, where a dose of 54 Gy was given in 36 fractions over 12 days (3 fractions of 1.5 Gy per day, 6 h interval between daytime fractions and 12 h overnight), or to conventional therapy where 66 Gy was given in 33 fractions over 6.5 weeks.

2.2. Histological material

Histological material was obtained, retrospectively, from the referring hospitals. Each Pathology Department was requested to either provide the original blocks for processing at Mount Vernon Hospital or to cut up to twelve 4 μm sections mounted onto poly-l-lysine coated slides. Each specimen was examined by a pathologist (PIR) to confirm the presence of SCC and that the specimen was assessable. A total of 402 specimens were obtained and stained and assessed for Ki-67 expression.

2.3. Immunohistochemical staining for Ki-67

Four micrometer sections were dried overnight at 37 °C. Endogenous peroxidase activity was blocked with a 3% solution of hydrogen peroxide in methanol for 30 min. Prior to antibody staining, the slides were pre-treated with microwave irradiation to unmask binding epitopes by immersion in 200 ml of 10 mM citrate (pH 6.0) for 4 min on high-power (800 w). This step was repeated twice. The slides were then left to stand for 10 min in buffer at room temperature before being washed thoroughly in tap water. After three washes in TBS, the slides were incubated in Dako Serum Free Protein block (Dako Ltd, High Wycombe, X0909) for 5 min. A 1:200 dilution of rabbit anti-Ki-67 polyclonal antibody (Dako Ltd, High Wycombe, A0047) in TBS was applied to the slides and incubated overnight at 4 °C. After three further washes in TBS, excess buffer was removed and a 1:400 dilution of biotinylated swine anti-rabbit antibody (Dako Ltd.), diluted in TBS, was added and incubated for 1 h at room temperature. After three further washes in TBS, Avidin Biotin Complex (Dako Ltd., K0355) was added. Finally after three further washes, the slides were exposed to DAB substrate (Vector Labs SK4100) for 5 min. The slides were washed in tap water and counterstained with Mayers Haematoxylin for 10 s to 1 min and then dehydrated, cleared and mounted in DPX. Specimens were stained in batches of 25–50 over a 4 week period. Each staining run used human tonsil as a positive control (proliferating cells in germinal centres) and a negative control was also assessed by omitting the primary antibody. The staining for Ki-67 was always clean and specific.

2.4. Assessment of staining

All slides were inspected for the presence of assessable tumour and semi-quantitatively analysed by a single consultant pathologist (PIR). Assessment was performed over a two month period with batches of 30–40 specimens being analysed in each session. Internal consistency was maximised by repeated evaluation of up to 5 specimens from the previous session prior to commencing assessment of a new batch of specimens. The slides were visually scanned and ascribed to one of three scores; 1 = less than 20% positive cells, 2 = 20–40% positive and 3 = greater than 40% positivity. In addition, the proliferation pattern was assessed as previously described [25] where 1 = marginal (most organised), 2 = intermediate (mainly organised), 3 = mixed (more than one pattern usually including random) and 4 = random (diffuse, disorganised staining).

2.5. Endpoints and statistical analysis

Tumour outcome was closely monitored at 8 weeks and 3 months after the first day of radiotherapy, subsequently 3 monthly to 2 years, 6 monthly to 5 years and annually thereafter. Loco-regional failure was calculated from the start of radiotherapy to the time when definitive clinical reappearance was detected in the primary site or in the nodes. Survival was calculated from the date of randomization until the time of death from any cause or, in patients who are still alive, the time of the last follow-up (minimum follow-up was 4 years).

Actuarial estimates of survival and freedom from local failure in subgroups of patients were obtained by the product-limit method of Kaplan and Meier. Differences between subgroups were tested using the Mantel–Cox log-rank test.

Multivariate analyses of time to death or time to locoregional failure were performed using Cox proportional hazards model. The proportional hazards assumption was tested graphically. Hazard ratios were estimated from a Cox proportional hazards model with treatment arm as the only covariate. Hazard ratios with confidence intervals were converted into a corresponding change in 5-year outcome with 95% confidence limits.

Associations between proliferation parameters and clinicopathological features were tested for statistical significance using Pearson's χ^2-test for independence.

All analyses were performed using the SPSS statistical software package release 10.0.5 running under Windows NT 4.0. All *P*-values are from 2-sided tests and statistical significance is claimed if $P < 0.05$.

3. Results

3.1. *Patient characteristics and distribution of proliferation parameters*

Table 1 summarises the salient clinicopathological features of the patient population included in this study. There were no significant differences between this cohort and the overall trial population (918 patients). The majority of patients were T2 or 3 and two thirds were node negative. The most prevalent sites were larynx and oropharynx. The scoring system for Ki-67 positivity was devised at the start of the study based on biological considerations; 46% of tumours were classified as having low proliferation (<20% positivity), 32% had intermediate proliferation (20–40% positivity) and 22% had the highest growth fractions (>40%). The pattern for proliferation was previously defined and based on the distribution of bromodeoxyuridine labelled cells observed in patients treated in the pilot study of CHART [26]. The classification scheme was applied to Ki-67 labelling with the caveat that more cells label with Ki-67 than the S-phase specific marker. In brief, the classification system has four categories in which "marginal" tumours retains a pattern similar to the normal mucosa with proliferation being observed in the basal and suprabasal layers; "intermediate" describes where proliferation is mainly in the basal layers but extends to other areas; "random" has no discernible pattern of proliferation; and "mixed" has combinations of three patterns usually including random areas. Seventeen percent of tumours were characterized by the most organized (marginal) proliferation pattern whilst a third of patients showed the intermediate pattern. Thirty six percent of patients had no discernible organization to the distribution of proliferating cells whilst 13% showed evidence of mixed patterns always including areas of random staining.

Table 1 – Clinicopathological characteristics and Ki-67 staining characteristics of the patient population

Variable	Category	Number of patients	% Of total patients
Age	Median = 63.8 years	402	100
Gender	Male	295	73
	Female	107	27
Treatment	CHART	240	60
	Conventional	162	40
T class	T1	12	3
	T2	185	46
	T3	131	33
	T4	74	18
Size of longest *T* axis	Median = 3 cm	356	89
N class	N0	255	63
	N+	147	37
Size of longest *N* axis	Median = 2.5 cm	144	36
Histological grade	Well differentiated	85	21
	Moderately differentiated	183	46
	Poorly differentiated	79	20
	Squamous, not specified	55	14
Site	Oropharynx	100	25
	Hypopharynx	41	10
	Larynx	190	47
	Oral cavity	56	14
	Nasal sinuses	2	1
	Nasopharynx	13	3
% of Ki-67 stained cells	Low (0–20%)	186	46
	Intermediate (20–40%)	129	32
	High (>40%)	87	22
Ki-67 staining pattern	Mostly organised	70	17
	Mainly organised	134	33
	Mixed	52	13
	Random	146	36

3.2. *Correlation of proliferation with clinicopathological features*

The percentage of Ki-67 positive cells correlated to Ki-67 pattern (Spearman's $\rho = 0.54$, $P < 0.001$). Eighty three percent of marginal tumours exhibited less than 20% Ki-67 positivity whereas 44% of randomly organized tumours expressed greater than 40% positivity; this represented almost three quarters of all tumours with the most rapid proliferation characteristics. Ki-67 positivity also correlated with N category (Spearman's $\rho = 0.14$, $P = 0.004$) with increased proliferation associated with the presence of positive nodes. Histological grade was correlated to Ki-67 positivity (Spearman's $\rho = 0.35$, $P = 0.001$) with increasing proliferating cells associated with decreasing differentiation (Fig. 1a). Ki-67 positivity was also correlated to a lesser extent to tumour subsite within the head and neck (Kruskal–Wallis $\chi^2 = 8.8$, $P = 0.119$) where larynx and oral cavity had the highest proportion of more slowly proliferating tumours. No significant correlation was seen between percentage of Ki-67 positive cells and T category or size of the longest *T* and *N* axis.

Similarly, Ki-67 pattern correlated to N category (Spearman's $\rho = 0.22$, $P < 0.001$) with increasing N-stage associated with the progression of proliferation pattern from marginal to random. Tumour sub-site within the head and neck was significantly associated with proliferation pattern (Kruskal–Wallis $\chi^2 = 27.6$, $P < 0.001$) where the incidence of tumours with random proliferation patterns was 48.8% and 49% in hypopharynx and oropharynx tumours whilst it was only 26.8% in both larynx and oral cavity tumours. As expected there was a strong correlation between proliferation pattern and histological grade (Spearman's $\rho = 0.35$, $P < 0.001$), but it should be noted that different proliferation patterns were represented, to a greater or lesser extent, in each of the differentiation categories (Fig. 1b); emphasising that these two classifications provide different information. Again, there was no association with T category or size of the longest *T* and *N* axis.

3.3. *Ki-67 positivity and the outcome of CHART or conventional fractionation*

Fig. 2 shows the locoregional control stratified by proliferation score in the conventional (Fig. 2a) and CHART (Fig. 2b) arm of the trial. Proliferation score had no effect on locoregional control in the conventional arm (log-rank, $P = 0.988$). In the

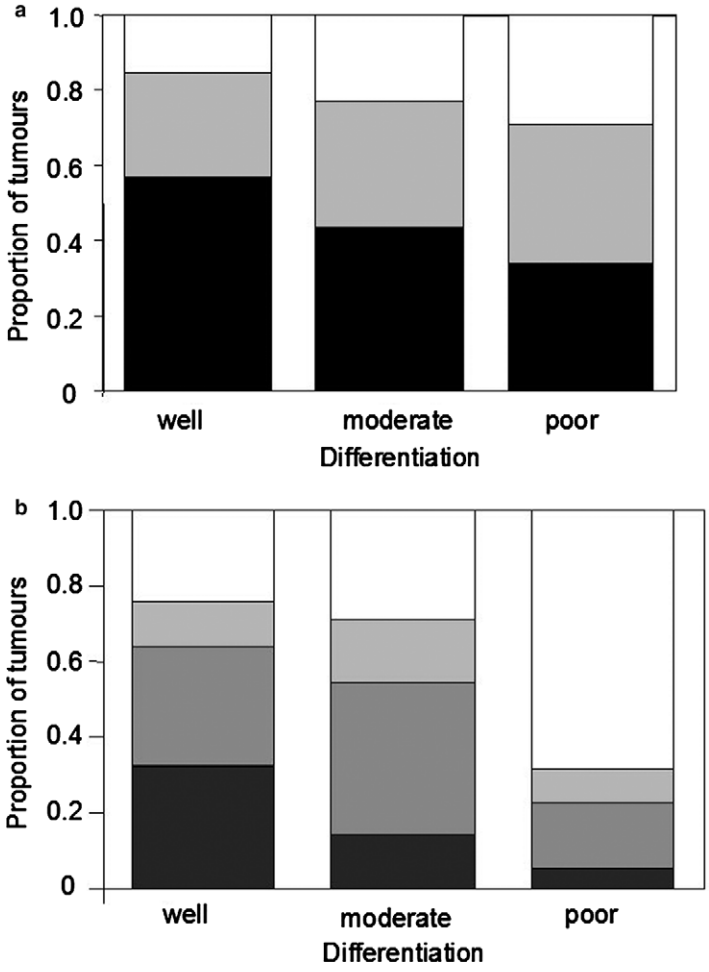

Fig. 1 – (a) The association between histological differentiation and Ki-67 positivity and (b) proliferation pattern. In (a), (■) represents tumours with Ki-67 positivity indices less than 20%, (■) >20%, <40% and (□) >40%. In (b), (■) represents marginal, (■) intermediate, (■) mixed and (□) random proliferation patterns.

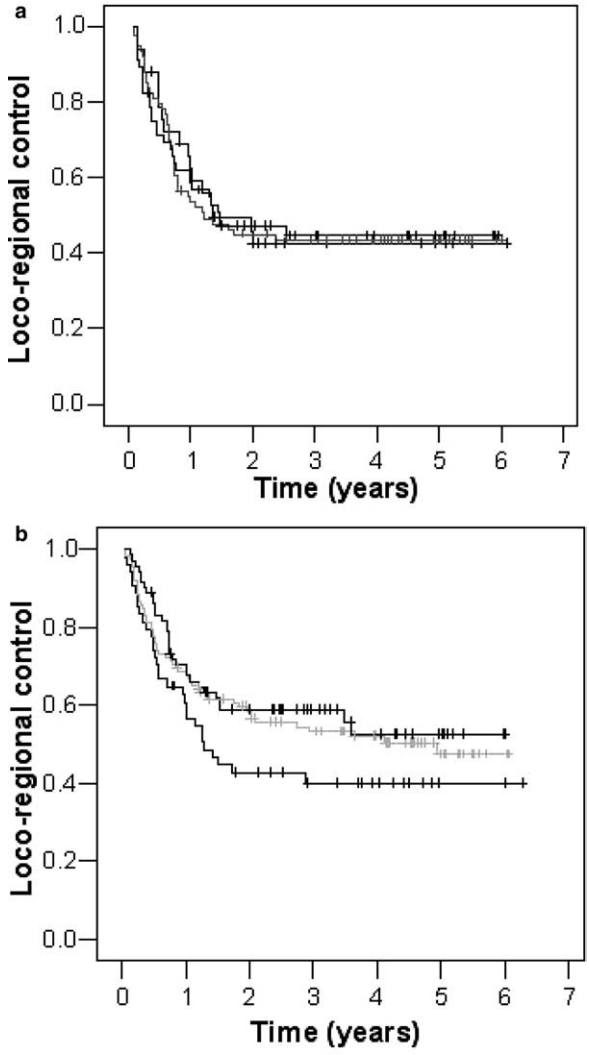

Fig. 2 – Locoregional control in the two arms of the trial as a function of Ki-67 percentage of stained cells. Grey lines are low Ki-67 (0–20% of stained cells), black dotted lines are intermediate Ki-67 (20–40% of stained cells) and black lines are high Ki-67 (>40% of stained cells). (a) The conventional arm and (b) the CHART.

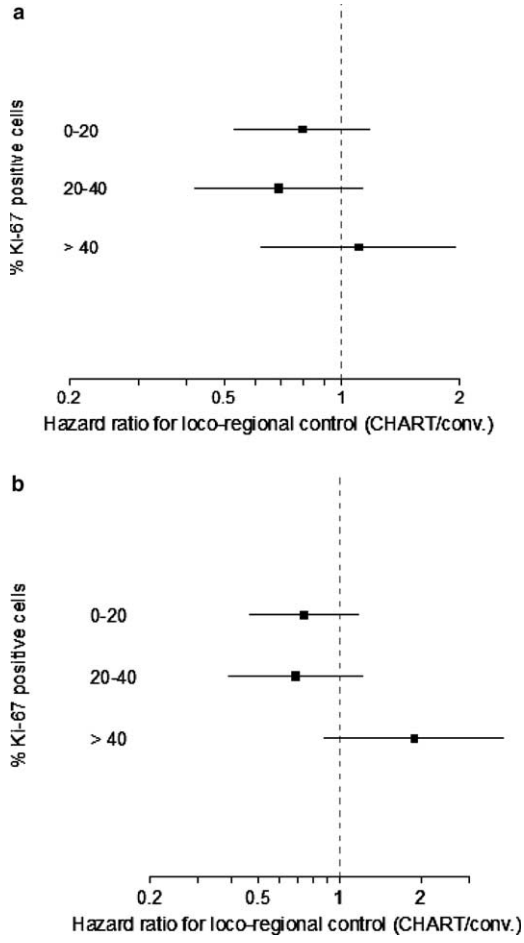

Fig. 3 – Hazard ratios for locoregional control after CHART compared with conventional fractionation as a function of the proportion of Ki-67 stained cells. (a) Ki-67 and treatment are the only covariates. (b) A multivariate model was used to correct for the effects of nodal and primary tumour burden (T category and N negative versus positive), nodal and tumour size (longest axis), Ki-67 pattern and tumour sub-site within the head and neck, patient age and gender.

CHART arm tumours with a low Ki-67 tended to do well although this was not significant (log-rank, $P = 0.183$). Pooling the data from the low and intermediate (pooled) versus high Ki-67, gave a log-rank P value of 0.08.

In Fig. 3a the hazard ratio for locoregional relapse after CHART, relative to conventional fractionation was estimated from a Cox model with treatment arm as the only covariate. When tumours are grouped according to Ki-67 expression, the benefit from CHART was seen in low rather than in high Ki-67 tumours but this was not significant in a heterogeneity test ($P = 0.478$). However, using a multivariate model accounting for the effects of nodal and primary tumour extent (T category, and N0 versus N+), nodal and tumour size (longest axis), Ki-67 pattern and tumour sub-site, patient age and gender, a trend was seen (Fig. 3b) towards greater benefit from CHART in the lower Ki-67 tumours (heterogeneity test, $P = 0.08$). When a post-hoc test was performed by pooling the low and intermediate Ki-67 groups in comparison with the high Ki-67 group, the heterogeneity test gave a $P = 0.032$.

In a Cox multivariate analysis of the interaction between Ki-67 positive cells and treatment, the interaction of Ki-67 and treatment arm was of borderline significance ($P = 0.06$). However, when Ki-67 and treatment with their interaction were introduced in a multivariate analysis together with nodal and tumour size (longest axis); age; gender; Ki-67 pattern as covariates and tumour site as stratification variable, the significance was lost.

3.4. *Ki-67 pattern and the outcome of CHART or conventional fractionation*

Proliferation pattern, measured as Ki-67 staining pattern, has no significant effect on locoregional control in the conventional arm (Fig. 4a); the log-rank P value was 0.532.

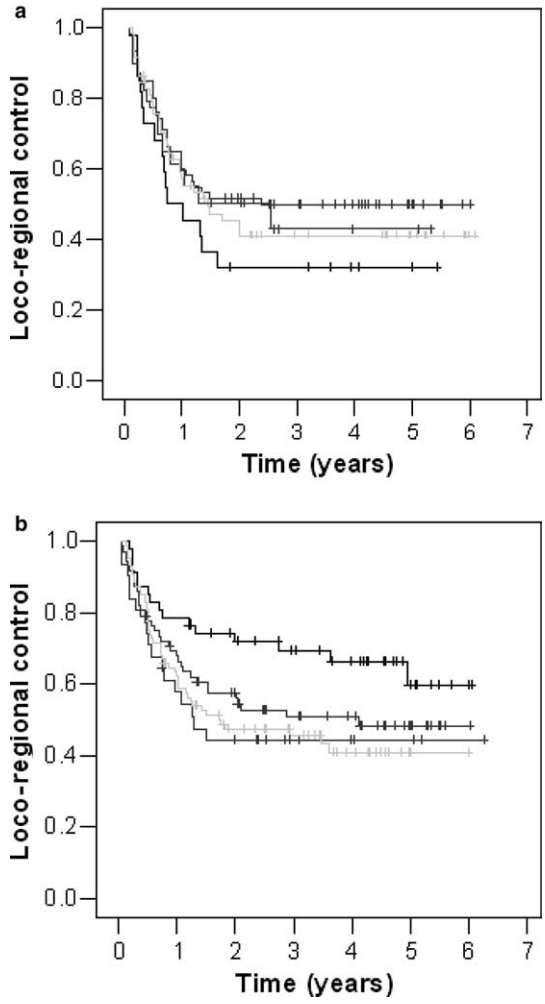

Fig. 4 – Locoregional control in the two arms of the trial as a function of Ki-67 pattern of staining. Light-grey lines are random pattern, dark-grey lines are intermediate pattern, dashed lines are mixed pattern and black lines are marginal pattern. (a) The conventional arm and (b) the CHART arm.

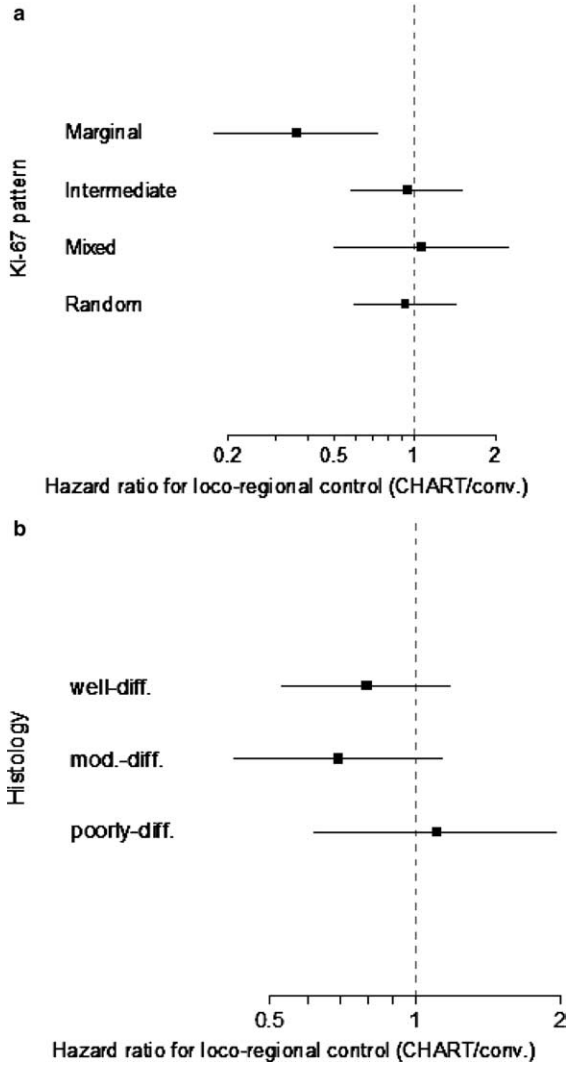

Fig. 5 – Hazard ratios of CHART versus conventional for locoregional control in the different proliferation pattern groups (a) from marginal to random and (b) histological differentiation.

In the CHART arm (Fig. 4b), tumours with a marginal pattern of Ki-67 staining tended to do better although this did not reach overall significance in a log-rank test ($P = 0.086$).

Tumours with marginal pattern showed a lower hazard ratio with CHART versus conventional (Fig. 5a) for locoregional control ($P = 0.005$), but an overall heterogeneity test did not reach significance ($P = 0.106$). No improvement was seen when correction for other factors was applied.

The interaction of Ki-67 pattern and treatment was significant in a Cox model together with the main treatment and pattern effects ($P = 0.009$ in a backward-likelihood-stepwise reduced model). This interaction term was still significant ($P = 0.013$ in a backward-likelihood-stepwise reduced model) in a multivariate analysis where pattern, treatment and their interaction was considered together with nodal and tumour size (longest axis), age, gender, Ki-67 pattern as covariates and tumour site as stratification variable.

3.5. *Histological differentiation and the outcome of CHART or conventional fractionation*

In this cohort of patients from the CHART trial, histological grade had no significant effect on locoregional control in either the conventional arm (log-rank, $P = 0.145$) or in the CHART arm (log-rank, $P = 0.472$). In Fig. 5b the hazard ratio was not significantly different from 1 in any of the differentiation groups and an overall heterogeneity test gave $P = 0.478$. No improvement was seen when correction for other factors was applied.

4. Discussion

Head and neck cancer represents a heterogeneous class of tumours characterized by variation in tumour histology, biology and response to treatment. It has been the subject of much clinical research in radiotherapy as it offers the tantalizing

prospect that advances in local and locoregional control may translate into more cures due to its relatively low propensity to metastasize at distant sites [13,27]. No other tumour site has experienced the diversity of fractionation schedules that have been attempted to improve the outcome of this disease [1].

The CHART regime represented a logical and interesting development in accelerated fractionation in which the overall treatment was drastically reduced from 6.5 weeks to only 12 days, achieved by delivering three fractions a day without treatment gaps. The aim of this strategy was to minimize the influence of repopulation during treatment. The lack of a statistically significant benefit from this very short accelerated schedule might at first appear clinically disappointing but there was significantly reduced late morbidity and the trial has yielded a wealth of important information from a clinical radiobiology point of view [2,28]. In addition, the data could be interpreted to support the notion that repopulation was the major issue as similar local controls could be achieved by a much lower total dose (54 versus 66 Gy), if it were given a reduced overall time. As so often in head and neck cancer, substantial variation in treatment schedule seems to produce only minor changes in clinical outcome. Considering the biological heterogeneity of head and neck cancer, the major conclusion that we draw is that individual tumours respond differently to different schedules because there are biological and genetic factors which we currently do not understand.

Proliferation characteristics are likely to play a key role in the response of tumours to radiation and the dogma has arisen that rapid proliferation may predispose tumours to worse outcome in conventional schedules due to repopulation. Unfortunately, proliferation and repopulation have become synonymous due to the similarity between the effective clonogenic cell doubling time (T_{eff}) during treatment and the cell kinetic parameter, potential doubling time (T_{pot}) [29]. It has been pointed out that T_{pot} is not a measure of predicted repopulation [24]; the same is true for Ki-67 which is a measure that equates to tumour growth fraction. Although Ki-67 is the most commonly used surrogate for proliferation in immunohistochemical studies, it may not be the most sensitive marker as its expression is found throughout the active cell cycle. Cell-cycle phase specific markers such as the cyclins may be a better alternative. Indeed, we have previously suggested that a combination of Ki-67 (growth fraction) and cyclin A (S+G2 phase) may be more informative combination of markers [30] and provide information that might be a surrogate for repopulation [31]. However, the intrinsic proliferation characteristics of tumours are likely to be important determinants of the repopulation process and an important parameter to study in the clinical setting.

The results presented in this study appear paradoxical; proliferation characteristics have no influence in the conventional treatment arm whilst there was a propensity for the more slowly proliferating tumours that retain an organized structure to benefit from acceleration. This is precisely the opposite of the expected result based on dogma. A similar conclusion was reached by Sakata and colleagues [24] who studied early (T1, T2) stage glottic tumours comparing a conventional schedule with accelerated treatment given as 2 fractions per day (1.72 Gy each), 5 days/week for 3.2–3.4 weeks with a total dose of 55–58.5 Gy. Using a cut-off of 50% to delineate high and low Ki-67 expressing tumours, they found that tumours with a lower LI did significantly better ($P = 0.018$) with the accelerated schedule. These data highlight that the mechanisms of failure/repopulation are more complex than can ever be represented by measuring a pre-treatment proliferation parameter alone.

There are clues from this study as to the likely cellular processes that might be involved in repopulation. It had previously been reported in the CHART study that well and moderately differentiated tumours did better with CHART and that poorly differentiated tumours did better with conventional radiotherapy [4]. We had also previously reported that tumours which retain the most organized proliferative structure have a favourable outcome when treated with CHART and that this parameter was superior to histological grading in discriminating the patients who did well [32]. This present study confirmed that observation and highlighted the lack of impact of proliferative organization in the conventional arm. Previously, Hansen and colleagues [33] had demonstrated a similar finding in conventional schedules based on differentiation status but had also discovered that when the overall treatment was extended to 9.5 weeks, by a split-course schedule, the outcome of moderately and well-differentiated tumours was compromised. Tumours which retain differentiation and have a low growth fraction will have a capacity to recruit cells into cycle after a radiation insult in a similar manner to normal mucosa. This mechanism of repopulation may be most apparent when the overall treatment time is extended but is controlled when the treatment is given in a short intense schedule.

Clearly, the multiple genetic changes that characterize head and neck cancer will influence the response of tumours to radiation treatment. Cell cycle, growth factor and differentiation-associated genes are commonly altered during oral carcinogenesis and deregulated in oral cancer [34–36]. A variety of molecular markers, involved in the control of recruitment into the active cell cycle, including epidermal growth factor receptor (EGFR) [37,38] and cyclin D1 [39,40] have shown clinical significance in head and neck cancer patients treated with radiotherapy. Interestingly, Eriksen and colleagues [38], in a study of different overall treatment times, noted that high EGFR expression was associated with a significantly poorer 5-year local control rate only in a 9.5 week split-course radiotherapy group; this was the same group of patients previously reported in which well or moderately differentiated tumours also did badly [33]. No significant effect of EGFR expression was detected in the 5.5 and 6.5 week groups [41]. In a recent analysis of the CHART trial patients [42], we have shown that tumours which overexpress EGFR gain significant benefit from accelerated radiotherapy and that EGFR overexpression is more prevalent in well differentiated tumours. These data strongly implicate recruitment during treatment as an important determinant of repopulation in different overall treatments and that acceleration may be of greatest benefit in the more slowly proliferating, differentiated tumours overexpressing EGFR where the capacity for recruitment is greatest.

The data presented in this study do not support a prognostic or predictive role for pre-irradiation Ki-67 index as an

independent marker of cellular proliferation influencing the outcome of radiotherapy. Instead, they point to a more complex interplay between cell cycle control deregulation and overall treatment time. In a recent analysis of multiple molecular markers in the CHART trial using unsupervised clustering methods, we demonstrated that phenotypes, based on Ki-67 and p53 expression, could be identified which predicted different responses in the two arms of the trial [43]. In the future, prescription of accelerated radiotherapy might be rationally allocated based on tumour histology and assessment of key cell cycle regulating genes.

Conflict of interest statement

None declared.

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