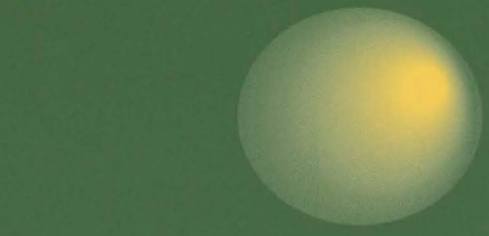
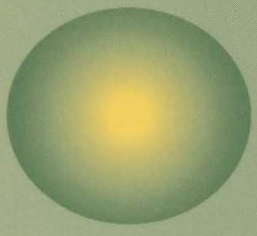
Prognosis in brain and head & neck tumours





Clinical relevance of cell proliferation markers and DNA-index

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Prognose van hersen- en hoofd-halstumoren

Klinische relevantie van celproliferatie-markers en DNA-index

(met een samenvatting in het Nederlands)

Proefschrift
ter verkrijging van de graad van doctor in de geneeskunde aan de Universiteit Utrecht, op gezag van de Rector Magnificus, Prof dr. J.A. van Ginkel, ingevolge het besluit van het College van Dekanen te verdedigen op dinsdag 11 februari 1997 om 14.30 uur

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Contents

Chapter 1

Introduction

Cancer

Cancer is defined as growth of tissue resulting from a continuous proliferation of abnormal cells having the ability to invade and destroy surrounding tissues and to metastasize to other organs or tissues. Cancer is not one specific disease but encompasses a large number of different malignancies classified according to tissue and cell type of origin.

In adults, cancer is the second greatest cause of death in Western Europe and the U.S.A. In children, it is the leading cause of death. Between 1975 and 1990 in the U.S.A. cancer death rates rose by 7% accounting for about 500,000 deaths annually [1]. In the Netherlands, the number of new cancer patients was approximately 54,000 in 1992 [2]. In that same year, 36.000 patients died of this disease. The total number of head and neck tumours and malignant brain tumours appeared to be 1800 and 900, respectively [2].

Survival rates of cancer patients have improved remarkably since 1920 when less than 20% of U.S. white cancer patients survived for more than 5 years. In 1960, 39% survived and survival rates further improved to 43% in 1970 and 50% in 1980 [1]. Most cancer cures may be attributed to treatment options including surgery and/or radiotherapy [3,4,5,6]. Treatment may be given with either curative or palliative intent. Palliation is of major importance in the management of cancer patients. In the Netherlands, radiation therapy is given with curative intent to 49% of the radiotherapy patients [7].

Radiotherapy

X-rays were discovered by Wilhelm Conrad Röntgen in November 8th 1895 in his laboratory at the Physical Institute of the Julius-Maximilians University of Würzburg in Bavaria. At that time, Röntgen was investigating phenomena caused by the passage of an electrical discharge from an induction coil through a partially evacuated glass tube. In spite of the fact that the tube was covered with black paper and the whole room was in complete darkness, he observed that, elsewhere in the room, a paper screen covered with the fluorescent material barium platinocyanide became illuminated. He then discovered that a silhouette of the bones of his hand could be projected upon the screen. The great discovery was made [9]. For this work, Röntgen was awarded the first Nobel Prize in physics. The identity of the person who introduced the term X-ray is, however, not known [8].

One of the first documented therapeutic uses of the X-ray technique was that of Freund in December 1896, who treated a five-year-old girl with a non malignant disease, i.e. hirsuites (Naevus pigmentosus pilosus) [9]. Early X-ray treatment applications included cutaneous affections, constitutional diseases, miscellaneous affections, and malignant growths [9].

In those early days, radiation regimen were based on empirical data. Initially, preference was given to the delivery of large fraction doses, however, damage to the normal tissues was not taken into consideration. Schwartz in 1914 [9] was one of the first authors to report on the efficacy of a fractionated course of irradiation administered to a mediastinal tumour that had previously been irradiated unsuccessfully by the single dose method. In 1922, Coutard presented evidence that advanced laryngeal cancer could be cured by irradiation alone. Experiments, performed by Regaud and Ferroux in 1927 [10], demonstrated the therapeutic benefit of a fractionated regimen. In 1928, Baclesse stressed the importance of daily dose fractions in order to prevent normal tissues damage [11]. The competition between the use of large single doses versus fractionated irradiation lasted until 1932 when Coutard published his results on the treatment of head and neck tumours with fractionated therapy. He showed that X-ray treatment consisting of one or two low-dose fractions per day could cure deep-seated tumours, particulary of the larynx and the tonsillar fossa [12].

Before 1950, radiotherapists had been using the Coolidge X-ray tube, developed in 1913. The voltage of this tube, with an accelerating potential of 140 kV, was increased in 1922 to 200 kV and again to 300 kV. In the sixties, orthovoltage therapy was largely replaced by Cobalt 60 machines and later by linear accelerators.

These machines provided the opportunity for

- a more homogeneous delivery of the intended radiation dose in the target volume,
- a smaller penumbra,
- a greater skin sparing ability,
- less bone absorption.

Nowadays, patients are treated 4-5 times a week once a day. In the case of palliation, treatment time is limited to 1-3 weeks. Curative treatment schedules are characterised by low fraction doses, and an overall treatment time of 5-7 weeks.

Methods of improving local tumour control probability by radiotherapy, as stipulated by J. Fowler [6], include

- improved fractionation schedules,
- improved physical dose distribution,
- (chemical) radiosensitization of tumour cells,
- radio-immunotherapy,
- combination with surgery,
- combination with chemotherapy.

According to Fowler, one of the most exciting areas of current development is in the first of the above mentioned methods, which happens to be the oldest. Improvements are most likely to be achieved by adapting schemes directed to specific characteristics of a specific tumour.

Radiobiology

Radiobiological experiments and theories began very early and in 1906 Bergonié and Tribondeau stated what was to become known as their Law 'that radiosensitivity varies directly with the reproductive capacity of the cell and inversely with its degree of differentiation' [9]. However, other subjects of research were given priority. Initially, the rationale for fractionation in radiation therapy was investigated. This gave rise to the Strandqvist plot in which effective total dose was plotted as a function of the overall treatment time [13]. It appeared that a prolonged overall treatment time was associated with decreased treatment efficacy. An important contribution in this area was made by Ellis and his colleagues with the introduction of the nominal standard dose (NSD) system which recognised separating overall time from the number of fractions [14]. Thereafter, the action of ionizing radiation on experimental tumour models was investigated [8]. It was demonstrated that DNA damage from X-rays was caused by a direct (physical) and an indirect (chemical) effect. In both situations, it was found that, apart from other biological damage, ionizing X-rays produce chromosome aberrations. These aberrations consist of a variety of exchanges and deletions which can be classified as chromatid or chromosome damage. These may ultimately lead to cell death, sometimes after a number of cell divisions [15].

Sensitivity to ionizing X-rays appeared to vary with cell cycle stage [8]. The cell cycle consists of four phases, i.e. G_1 (gap 1), S (DNA-synthesis phase), G_2 (gap 2) and M (mitosis or cell division) (Figure 1). From every cell cycle phase, cells may escape into a quiescent stage (Q fraction), e.g. G_1-phase cells into G_0-phase [15]. Cells in or close to the mitotic phase are most radiosensitive whilst cells in S-phase appear to be most radioresistant [16]. Irradiation of cells in G_2-phase leads mainly to chromatid damage. Radiation damage of cells induced in G_1-phase, if unrepaired, leads, after DNA duplication in S-phase, to defects involving both chromatids and thus to chromosome aberrations. Irradiation of cells in S-phase can lead to either type of damage, depending on whether or not the affected chromosome sites have undergone replication [15]. If the radiation dose is sufficiently high, the DNA is damaged to such an extent that adequate repair is not possible, thereby impairing the ability of cells to proliferate and cell death will ultimately follow [15]. Furthermore, radiation causes a delay in cell cycle progression whereby cells in the G_2-phase experience the longest delay [17,18,19]. The duration of the mitotic delay, as well as the fraction of cells which die in mitosis, depends on total dose and dose rate [19]. After radiation, cells may survive for some time and may complete a number of cell divisions before cell death [20].

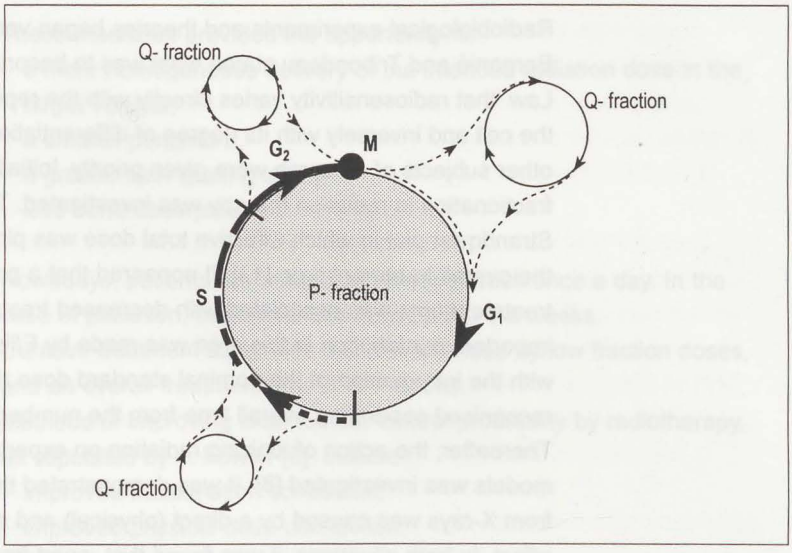


Figure 1 *Schematic presentation of the cell cycle. Before commencing DNA-synthesis (S) the proliferating cells (P-fraction) traverse the G_1-phase (G_1) of the cell cycle. After reduplication of the DNA, cells progress through the G_2-phase (G_2) and finally divide during the mitosis (M). Non-cycling or quiescent cells (Q-fraction) reside in early G_1, called the G_0-phase. Furthermore non-cycling cells in S- and G_2-phase can be present also.*

The radiobiological aspects of ionizing radiation were described by Withers et al. [21] by the four R's of radiobiology, i.e. i) Repair of sublethal damage, ii) Redistribution of cells through cell cycle, iii) Reoxygenation of hypoxic cells, iv) Repopulation by accelerated proliferation of surviving cells.

The width of the "shoulder" of a cell-survival curve reflects repair capacity of cells and the slope of the exponential part of the curve reflects the intrinsic radiosensitivity (Figure 2).

When doses of radiation are fractionated, cell survival rate increases with increasing time between the fractions. The "shoulder" varies with cell type and with physiological conditions, such as hypoxia. In the terminology of the linear-quadratic (α:β) description of the cell survival curve, it is the quadratic component ß that dominates the curve at higher doses. A large "shoulder" corresponds to a small α/β ratio, i.e. ß is important relative to α [8]. The "Linear Quadratic" model was introduced by Douglas and Fowler [22]. Separating the total radiation dose in a number of fractions leads to sparing of normal tissues, since the time interval necessary for Repair of sublethal damage between two

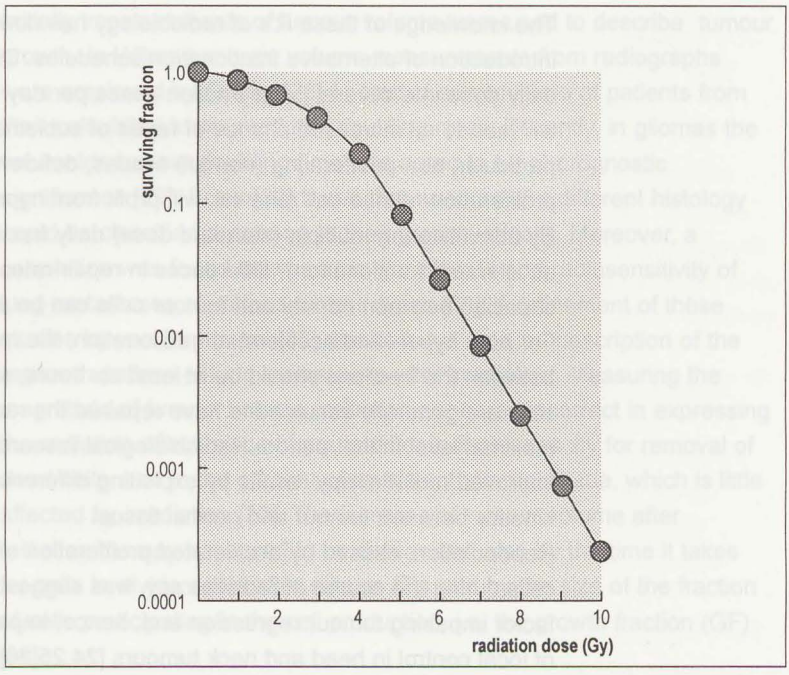


Figure 2 *A typical cell survival curve in tissue culture plotted on a logarithmic scale.*

fraction doses is, in general, sufficient in normal cells (24 hours). At the same time, fractionation leads to Reoxygenation of hypoxic cells in the tumour because oxygenated cells are more radiosensitive and will be killed preferentially. Repopulation is characterised by accelerated proliferation of surviving cells during the course of radiotherapy. Redistribution, as defined by Withers [21] results from the higher radiosensitivity of G_2-phase cells. It must be emphasized however, that the phenomenon of Redistribution differs from that of Reassortment. Reassortment appears to be caused by a radiation-induced arrest of cells in G_2-phase [18,19,23]. The cells will be inhibited from further cell cycle progression after entering the G_2-phase, irrespective of the position of the cells in the cell cycle at the moment of radiation. Since the duration of inhibition will decrease in the sequence of G_2-, S-, and G_1-phase cells, the result will be a synchronous release of cells from the G_2-block [15]. The duration of this G_2-arrest is primarily dose and radiosensitivity dependent. The moment after irradiation, at which a maximal number of cells is blocked in the G_2-phase, was found to be related to the intermitotic or cell cycle time, i.e. T_c [18]. Thus, the shorter the cell cycle duration, the sooner the accumulation of cells in the G_2-phase will be maximal [18,19].

The knowledge of these R's of radiobiology has now led to the introduction of alternative fractionation schedules. Dividing the normal daily dose into several lower fraction doses per day (hyperfractionation) will lead to an increased chance of repair of sublethal damage. In particular, non-proliferating (normal) tissues, deficient in tissue repair by proliferation-related cell renewal, will profit from hyperfractionation.
By administering multiple (standard dose) daily fractions (multi- or accelerated fractionation), differences in repair-rates (instead of repair capacity) between normal and tumour cells can be exploited.
For both hyper- and accelerated fractionation, the minimum interval between the fractions should be at least six hours, since the normal cells are generally assumed to have repaired the radiation damage to a maximal level in that period. Radiobiological research may lead to improved radiotherapy results by exploiting differences in cell and repair kinetics between tumour and normal tissue.
Repopulation, caused by accelerated proliferation of surviving tumour cells during the course of radiotherapy, was suggested to be a major factor impairing tumour regression and, hence, impairing the probability of local control in head and neck tumours [24,25,26]. Theoretically, it seems more effective to treat tumours, characterized by a high proliferative capacity, with accelerated fractionation in an attempt to complete the course of radiotherapy before repopulation adversely affects the treatment efficacy [27]. Attempts to characterize tumour growth have been the subject of numerous investigations in the past and present.

Tumour growth-rate

Volume measurements

Initially, measurements of tumour volume were used to describe tumour growth. In Wilms tumours, volume measurements from radiographs were employed by Collins [28] to predict survival time of patients from their calculated tumour volume doubling rates. Recently, in gliomas the volumetric tumour doubling time was noted to be of prognostic significance [29]. Breur [30] found that tumours of a different histology could be classified according to their growth velocity. Moreover, a correlation was found between the growth-rate and radiosensitivity of lung metastases of various tumour types [31]. A refinement of these studies by van Peperzeel [32] resulted in a detailed description of the regrowth pattern of lung metastases after irradiation. Measuring the magnitude of tumour volume reduction appeared incorrect in expressing the radiation effect, since tumours differ in their capacity for removal of dead cells, and in the amount of persistent stromal tissue, which is little affected by irradiation [33]. The increase of tumour volume after radiation therapy appeared to be determined also by the time it takes for cells to divide again after mitosis (T_c) and by the size of the fraction of cells participating in the cell production, i.e. the growth fraction (GF) [34].

Cell proliferation markers

The investigation of cell proliferation was greatly improved by the possibility to use radioactively labelled compounds. Mitotic figures were counted in histologic sections from serial biopsies. The studies of Taylor [35] have shown, that the DNA can be labelled selectively with ^{3}H-thymidine (^{3}HTdR). This technique facilitated the determination of the duration of cell cycle (T_c), S-phase (T_s), G_1-phase (T_{G1}), and G_2-phase (T_{G2}). After labelling with ^{3}HTdR, labelling index (LI) is taken as the fraction of cells in S-phase [15].

With continuous labelling the influx-rate of cells entering S-phase can be measured. If GF=1, LI will be 100% after the duration of approximately T_c minus T_s. However, if the GF is less than 1.0, LI will increase until all the proliferating cells are labelled. Due to proliferation of labelled cells, the LI will increase further but at a much lower rate. It appears, that variation in T_c is such that there is often difficulty in recognizing the inflection point on the LI-curve. Taking into account the production of labelled cells by proliferation, the results may allow for accurate determination of GF, T_s and T_c [18].

The utilisation of a labelling technique to investigate cell cycle kinetics, by composing a curve of the percentage of labelled mitoses (PLM) after flash labelling, was introduced by Quastler [36]. The calculation of T_c from a PLM curve is sometimes hampered by T_c variability.

Furthermore, GF cannot be determined by this method. Disturbance of the cell cycle may occur, due to a ^{3}H-radiation-induced inhibition of cell cycle progression, resulting in a misinterpretation of the proliferation markers [37,38].

With autoradiography, estimation of T_c can also be obtained by counting the grains over the nuclei of labelled cells in serial biopsies. The time it takes for the mean grain count to halve is approximately equal to the T_c. However, complications may arise with this method also, i.e.

- from an uneven distribution of the labelling over the cells,
- from entry of labelled cells in the non-proliferating Q-fraction, and
- from low labelled nuclei, which are difficult to distinguish from the background grain count.

Another autoradiographic method, the technique of double labelling, can be used to investigate cell proliferation markers [15]. The cells are flash labelled twice, once with $^{3}HTdR$ and once with $^{14}CTdR$, separated by an interval not longer than T_{G2}. The T_c can then be calculated, since the ^{3}H-label can be distinguished from the ^{14}C-label by a special technique of autoradiography exploiting the differences in range of β-particles from ^{3}H (1.4 µm) and from ^{14}C (10-40 µm) [39]. However, the results obtained by this method are often not comparable with those obtained by other methods. This is caused by difficulty in the distinction of the two labels from each other and in the different detection levels of the two labels.

The use of radioactive labelled compounds can be avoided by applying stathmokinetic agents, as mitotic inhibitors [15]. After blocking cells in mitosis, mitotic figures are counted in histologic sections from serial biopsies. Extrapolation of the time it takes for all cells to reach mitosis gives an estimation of the T_c. This estimate is less accurate since some cells appear to escape from inhibition and some cells degenerate during this arrest. First to employ this method was Kipp [41], using vinblastin as a mitotic inhibitor. This method allowed for the determination of the GF and of the duration of the cell cycle phases in exponentially growing cell cultures. Comparable experiments in vivo, however, were complicated by a greater variation in T_c and/or by an incomplete inhibition by the blocking agent.

By using synchronised cells in culture, progression of the synchronised cohort of cells through the cell cycle can be monitored with flow cytometry and T_{G1}, T_s, T_{G2+M}, and T_c can be determined [40].

It is also possible to inhibit exit of cells from a cell cycle phase and to determine the rate of decrease of cells in following cell cycle phases. From this, cell kinetic parameters can be calculated. For many years,

ionizing radiation has also been known to reduce the mitotic index [23]. Radiation as blocking agent has the advantage that, unlike cytostatic drugs administered in vivo, all the cells are targeted equally and systemic toxicity can be avoided.

The moment after irradiation at which a maximal amount of cells are blocked in the G_2-phase, was found to be related to T_c [18]. Thus, the shorter the T_c, the sooner the accumulation of cells in the G_2-phase will be maximal. The magnitude of the G_2-block depends largely on the size of GF. A relatively good estimation of the T_c and the GF can be obtained by monitoring the accumulation pattern of cells in G_2 [18,19].

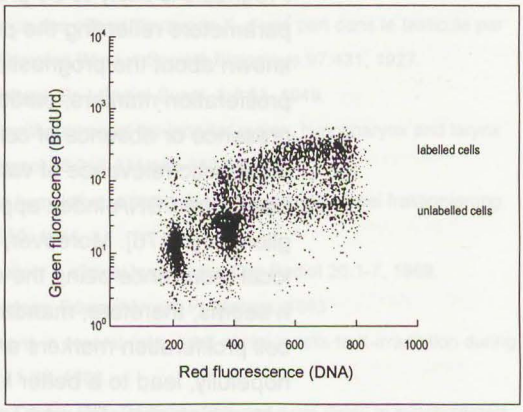
Not all the methods mentioned above, however, are suitable for a reliable assessment of proliferation markers in human tumours. Especially, those methods for which radioactively labelled compounds and serial biopsies are needed, appear not be suitable. A range of approaches have recently evolved to overcome this problem. The proliferative potential of tumours can be quantified both directly and indirectly, in vitro as well as in vivo [42].

Cell proliferation markers, representing the distribution of cells over cell cycle phases in human tumours, can be determined by flow cytometry. Identification, by the use of monoclonal antibodies, of S-phase cells labelled with the (non-radioactive) thymidine analog 5-bromo-2'-deoxyuridine (BrdUrd) has enabled further determination of specific markers of cell proliferative activity [43,44]. After intravenous infusion, BrdUrd is incorporated into DNA by cells in S-phase. By flow cytometry or immunohistochemistry, cell proliferation markers can then be assessed [28,45,46,47]. Other cell proliferation markers can also be obtained, e.g. Ki-67, representing all non-G_0 cells [48].

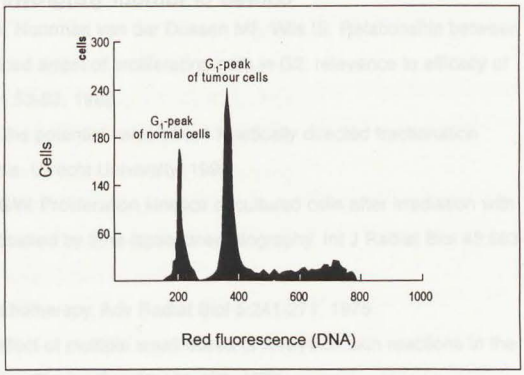
Flow cytometry

A detailed description of the method of flow cytometry (FCM) is given in each chapter. Some details are discussed here.
Single cell suspensions were obtained by an enzymatic preparation. Since it was found that the coefficient of variation was higher and that cell clumping was more extensive, we did not use the mechanical method [49]. Nevertheless, cell clumping remains a potential problem. Cell aggregations can be produced by i) cells which are not dissociated by the cell suspension preparation procedure, ii) cells which reaggregate after cell suspension preparation, iii) cells passing through the flow cytometer close together and can, therefore, not be measured separately.
A FACS-analyzer (Becton Dickinson) flow cytometer was used. Fifty thousand cells were analyzed from each tumour sample. Correction for cell debris counts, the so called "background", was carried out by drawing a straight line from the left-side basis of the G_1-peak to the right-side basis of the G_2+M-peak on a log-log scale. Furthermore, it was found necessary to correct for cell clumps, since doublets are measured as G_2-phase cells causing the number of cells in the G_2-phase to be overestimated. In order to facilitate a correct determination of the number of triplets (and from that a correct calculation of the number of doublets), the background subtraction was extended underneath the peak of the triplets [50].
The histogram analysis was carried out by an iterative program [51]. With dual parameter FCM, both cell cycle distribution and (BrdUrd) LI were determined (Figure 3a +3b).
These investigations were supervised by the radiobiologist (DHR), in order to avoid interobserver variability. The green signal (BrdUrd detection) was log amplified. Only if a distinct separation between BrdUrd labelled and unlabelled population was obtained, the measurements were used in the analysis. The furthest left peak of the DNA-histogram was assumed to correspond to the G_1-phase normal cells always present in tumours (leucocytes, endothelial, stromal cells). The G_1-peak of tumour cells was assumed to be accompanied by a corresponding G_2-peak, positioned at approximately twice the G_1-peak value. If the position of the G_2-peak was distinctly shifted from twice the G_1-peak value, often accompanied by a broadened or a non-Gaussian G_1-peak, then a shift from DNA-diploidy was assumed. DNA-index (DI) was then calculated from the ratio of DNA-index of G_1-phase tumour cells to that of G1-phase of normal cells. DNA-diploid tumours were defined by DI=1, tetraploid tumours by DI=2, and aneuploid tumours by DI>1.

The alternative calculation methods of T_{pot} (White et al. [52], Durand et al. [53], and Ritter et al. [54]) are, to date, not common practise in clinical studies. We, therefore, opted for the standard approach suggested by Begg et al. [44]. For calculations of T_s and T_{pot}, the mean DNA-value of BrdUrd-labelled S-phase cells, during BrdUrd infusion was assumed to be half of G_2-peak minus G_1-peak value (RM_0 = 0.5), and progression rate of cells through S-phase was assumed to be constant. The potential doubling time (T_{pot} in days) was calculated using the formula $T_{pot} = 0.8\ T_s/LI$. Ultimately, DNA-index, G_1-phase fraction (G_1PF), S-phase fraction (SPF), G_2-phase fraction (G_2PF), BrdUrd-labelling index (LI), duration of S-phase (T_s), and potential doubling time (T_{pot}) were obtained. T_{pot} represents the doubling time of the tumour in the absence of cell loss [3].



3a: the 2-dimensional display of red-green fluorescence



3b: the derived distribution of DNA contents

Figure 3a+3b *Flow cytometry of a DNA-aneuploid tumour some hours after labelling with BrdUrd.*

Motivation for the present investigation

In head and neck tumours, treated with a conventionally fractionated scheme of radiotherapy, a decreased local control capacity was observed in tumours characterised with a high proliferative capacity (e.g. low T_{pot} values) [55,56]. In these tumours prognostic relevance was also reported for DNA-index [57-63]. Moreover, an increased loco-regional tumour control rate will lead to a longer survival in this patient group, since most of them die from loco-regional recurrent disease [64]. It seems, therefore, mandatory to evaluate the prognostic relevance of cell proliferation markers and DNA-index in head and neck tumours. This will, hopefully, facilitate the ability to adequately adjust radiation treatment schemes.

The biological behaviour of brain tumours cannot always be adequately assessed by the use of cytomorphological features alone [65-67]. Prognosis is likely to be predicted more adequately by taking account of parameters reflecting the proliferative activity [68-71]. In gliomas, little is known about the prognostic relevance of flow cytometrically derived proliferation markers, particulary LI and T_{pot}, compared with that of the presence or absence of cells in mitoses. Little is known also about the prognostic relevance of various cell proliferation markers compared with each other. DNA-index appeared to be of prognostic relevance in gliomas [72-76]. Moreover, most glioma patients ultimately die, with local recurrence being the cause of death in 90% of patients [65-67]. It seems, therefore, mandatory to evaluate the prognostic relevance of cell proliferation markers and DNA-index in brain tumours. This will, hopefully, lead to a better knowledge on the ability to identify tumours with a high risk of (early) local recurrent disease. Eventually, this may lead to a proper adjustment of the treatment to the expected clinical course of tumour (re)growth.

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Chapter 2

Interrelationships of cell proliferation markers, DNA-ploidy, T-stage, and N-stage in primary laryngeal tumours

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Summary

The purpose of this study was to evaluate the interrelationships between cell proliferation markers, DNA-ploidy status, T-stage, and N-stage in 64 primary laryngeal tumours.
DNA-index, S-phase fraction (SPF), 5-bromo-2'-deoxyuridine (BrdUrd)-labelling index (LI), duration of S-phase (T_s), and potential doubling time (T_{pot}) were determined by flow cytometry. T-stage and N-stage were assessed in accordance with the TNM classification system (UICC 1987).
T_{1-2} , when compared with T_{3-4} stage tumours, had significantly higher LI values (independent from N-stage) resulting in lower T_{pot} values. No such relationship was found with respect to N-stage. N_{1-3} tumours, as opposed to N_0 tumours, appeared to be characterised by a significantly shorter T_s (and hence a shorter T_{pot}). T_s values appeared to vary considerably (range 1.9-6.2). For DNA-aneuploidy, as opposed to DNA-diploidy, a significantly higher mean LI was noted. Locally advanced tumours, when compared with T_{1-2} tumours, were characterised by a significantly higher percentage of DNA-aneuploidy.
The reported lack of prognostic relevance of cell proliferation markers to predict radiation treatment efficacy may relate to a decreased mean LI in locally advanced (T_{3-4}) tumours, as found in our series. In laryngeal tumours, the predictive value of cell proliferation markers (LI, T_{pot}) should, therefore, be evaluated after stratification for T-stage. The existence of higher LI values in DNA-aneuploidy tumours was confirmed in our series, stressing the need for distinction of DNA-diploid tumour cells from DNA-diploid normal cells. The prognostic potential of DNA-index was confirmed.

Introduction

In head and neck tumours, repopulation, due to accelerated proliferation of surviving tumour cells during the course of radiotherapy, has been suggested to be a major factor determining tumour regression and local control probability [11,27,34]. Theoretically, it would be more effective to treat tumours, characterized by a high proliferative potential (short T_{pot}), with accelerated fractionation in an attempt to minimize the probability of accelerated tumour cell proliferation occurring during treatment [3]. If proliferation rate is the governing factor of impaired outcome after conventionally fractionated radiation therapy, higher proliferation potentials should be found in locally advanced (T_{3-4}) tumours, when compared with those of T_{1-2} tumours. However, the reliability of cell proliferation markers in the prediction of radiation treatment efficacy has not been established unequivocally. Some authors could not demonstrate a statistical significant relationship between proliferative potentials and prognosis [8,9,33], while others found statistical significance [10] or a trend towards significance [4]. In these studies, head and neck tumours at various sites and of various T- and N-stages, were included.

DNA-index has also been associated with prognosis in head and neck tumours [12,13,14,17,19,24,26,28,29,30,31]. Tumours at various sites and of various T- and N-stages were included in these studies.

The purpose of the present study was to investigate the interrelationships between DNA-ploidy, cell proliferation markers, T-stage, and N-stage in a single tumour site, i.e. in primary laryngeal tumours. S-phase fraction (SPF), 5-bromo-2'-deoxyuridine (BrdUrd) labelling-index (LI), duration of S-phase (T_s), and potential doubling time (T_{pot}) were used as indicators of the proliferative potential.

Materials and methods

Patients

From 1989 to 1994, 64 patients (57 males and 7 females) with a primary squamous cell carcinoma of the larynx were admitted to the department of ENT of the University Hospital Utrecht. All agreed to participate in cell kinetic studies involving BrdUrd-infusion. The mean age was 61 years and all had a minimum Karnofsky performance status of 80%. T-stage and N-stage were assessed in accordance with the TNM classification system (UICC 1987).

Flow cytometry

Fresh tumour samples were obtained after 100 mg BrdUrd was administered intravenously as a bolus, in 5-10 minutes. The interval between BrdUrd infusion and each tumour sampling was noted. The mean interval in 41 evaluable cases for T_{pot} was 240 minutes and differed neither with T- and with N-stage nor between DNA-diploid and DNA-aneuploid tumours.

Tumour samples were fixed in 70% ethanol for a minimum of 24 hours. After removal of the ethanol, single cell suspensions were prepared using pepsin (Sigma P-7012, 0.4 mg/ml 0.1 N HCl) over 30 minutes at 37° C. The pepsin digestion stripped the cells to nuclei for the vast majority of cells. The resulting suspensions were filtered through plastic tissue with a pore size of 60 µm. BrdUrd staining was carried out by the method of Schutte et al. [21] using a monoclonal anti-BrdUrd antibody (Eurodiagnostics, clone IIb5) and a fluorescein isothiocyanate (FITC) labelled secondary antibody (Dakopatts F313). Prior to flow cytometric analysis DNA was stained by 0.5 ml propidium iodide solution (0.01 mg/ml, Calbiochem 537059) containing 0.1 mg/ml RNAse (Sigma R-5503) for a minimum of 15 minutes. A FACS-analyser (Becton Dickinson) flow cytometer was used. Fifty thousand cells were analysed from each tumour sample. After subtraction of cell debris counts and correction for cell doublets, DNA-histograms were analyzed using an iterative program [15]. Triplets were counted and used to calculate doublet counts [1] and then to recalculate G1PF, SPF, and G2+MPF, taking into account the calculated number of doublets and the measured number of triplets. The cell cycle distribution (including SPF) and BrdUrd-LI were determined with dual parameter flow cytometry. The green signal (BrdUrd detection) was log amplified. Only if a distinct separation between BrdUrd labelled and unlabelled population was obtained, the measurements were used in the analysis.

The furthest left peak of the DNA histogram was assumed to correspond to the G_1-phase normal cells which are always present in tumours (leucocytes, endothelial, stromal cells). The G_1-peak of tumour

cells was assumed to be accompanied by a corresponding G_2-peak, positioned at approximately twice the G_1-peak value. If the position of the G_2-peak was distinctly shifted from twice the G_1-peak value, accompanied by a broadened or a non Gaussian G_1-peak, then a shift towards DNA-aneuploidy was assumed. In our study no standard cells were used to assess DI. However, instrument setting was standardized using either murine thymocytes or fluorescent latex particles. DNA-index (DI) was calculated from the ratio of DNA-content of G_1-phase tumour cells to that of G_1-phase of normal cells. DNA-diploid tumours were defined by DI=1 and aneuploid tumours by DI>1. We have opted to use the standard approach, as suggested by Begg et al. (2), to calculate T_s and T_{pot}. For calculations of T_s and T_{pot}, the mean of DNA-value of BrdUrd-labelled S-phase cells was assumed to be half of G_2-peak minus G_1-peak value during BrdUrd infusion. Progression rate of cells through S-phase was assumed to be constant. The potential doubling time was calculated using the formula $T_{pot} = 0.8\ T_s/LI$.
DNA-index and SPF values could not be obtained in 12 and 20 cases, respectively. Determination of LI, and hence analysis of T_s and T_{pot} was impossible in one case, since BrdUrd was not administered. In eight cases LI, but no T_s and T_{pot} values could be determined, because the interval between BrdUrd administration and tumour sampling was unrecorded. T_s and T_{pot} could not be adequately analysed in another 14 cases (Table 1).

Table 1 *Success rates of the determination of DNA-ploidy, LI, T_s, and T_{pot}*

Parameter	Percent
DNA-ploidy	81
SPF	69
LI	78*
T_s	75**
T_{pot}	75**

*in case BrdUrd was administered.

**in case BrdUrd was administered and interval time was registered.

Statistical analysis

A chi-square test was used to compare tumour groups with respect to their DNA-ploidy. The cell proliferation markers SPF, LI, T_s, and T_{pot} all showed a skewed distribution. The statistical analysis was, therefore, performed on their logarithmically transformed values. For these (continuous) variables an analysis of variance (ANOVA) was carried out. By the use of interaction terms, we evaluated the differences of the means of the cell proliferation markers and DI between T-stages in relation to N-stages (and vice versa).

Results

In T_{1-2} tumours, when compared with T_{3-4} tumours, the percentage of DNA-diploid tumours was significantly higher ($p = 0.05$) (Table 2). With respect to N-stage such a relation was not found (data not shown).

Table 2 *Cross tabulation of DNA-ploidy and T-stage*

	DI>1	DI=1
T_{1-2}	11	14
T_{3-4}	20	7

Chi-square test with continuity correction: $p = 0.05$

The geometric means of SPF did not differ significantly for T-stage, N-stage and DNA-ploidy (Table 3).

Table 3 *Analysis of variance of SPF (n=44) for T-stage, N-stage, and DI with 95% confidence interval for the ratio (95% CI)*

	Mean SPF*	P-value	Ratio	95% CI
T_{1-2}	0.084	ns**	1.1	na***
T_{3-4}	0.075			
N_0	0.077	ns**	0.9	na***
N_{1-3}	0.089			
DI=1	0.071	ns**	0.8	na***
DI>1	0.089			

*geometric mean **$p > 0.10$ ***not analysed

The mean LI was significantly higher ($p = 0.02$) in T_{1-2} tumours, compared with that in T_{3-4} tumours (Table 4). With respect to T-stage, the observed differences for T_s showed borderline significance ($p = 0.06$) (Table 5). The mean T_{pot} appeared to be significantly lower in T_{1-2} tumours, compared with that in T_{3-4} tumours ($p = 0.004$) (Table 6). Significantly higher mean LI values were observed in DNA-aneuploid tumours ($p = 0.001$) (Table 4). In N_{1-3} tumours, compared with N_0 tumours, the mean T_{pot} was significantly shorter ($p = 0.043$) (Table 6). Similar results were noted for T_s ($p = 0.02$) (Table 5), whereas no

statistically significant difference was noted for mean LI values (Table 4). Interrelationships of T-stage and N-stage for SPF, LI, T_s, and T_{pot} are given in table 7. By the use of interaction terms, we found that the observed differences of the means of the cell proliferation markers and DI between T-stages, were independent from N-stage and vice versa (data not shown). The ratios of the differences of the means of T_s and T_{pot}, with respect to N-stage, appeared to be more pronounced in T_{1-2}-stage tumours, when compared with those in T_{3-4}-stage tumours. Statistical significance was, however, not reached.

Table 4 *Analysis of variance of LI (n=49) for T-stage, N-stage, and DI with 95% confidence interval for the ratio (95% CI)*

	Mean LI*	P-value	Ratio	95% CI
T_{1-2}	0.081	0.02**	1.9	1.1;3.3
T_{3-4}	0.043			
N_0	0.056	ns**	0.9	na***
N_{1-3}	0.062			
DI=1	0.037	0.001	0.4	1.5;4.1
DI>1	0.093			
*geometric mean		**p > 0.10		***not analysed

Table 5

Analysis of variance of T_s (n=41) for T-stage, N-stage, and DI with 95% confidence interval for the ratio (95% CI)

	Mean T_s* (hours)	P-value	Ratio	95% CI
T_{1-2}	3.3	0.06	0.6	1.0; 2.5
T_{3-4}	5.9			
N_0	6.0	0.02	1.8	1,1; 3.0
N_{1-3}	3.2			
DI=1	3.5	0.07	0.6	na**
DI>1	5.5			
*geometric mean				**not analysed

Table 6 *Analysis of variance of T_{pot} (n=41) for T-stage, N-stage, and DI with 95% confidence interval for the ratio (95% CI)*

	Mean T_{pot}* (days)	P-value	Ratio	95% CI
T_{1-2}	1.4	0.004	0.3	1.4; 5.1
T_{3-4}	4.1			
N_0	3.5	0.043	2.1	1,1; 4.1
N_{1-3}	1.7			
DI=1	2.8	ns**	1.4	na***
DI>1	2.0			
*geometric mean		**p > 0.10		***not analysed

Table 7 *Interrelationships of T-stage and N-stage for SPF, LI, T_s, and T_{pot}*

	N_0	N_{1-3}
SPF*		
T_{1-2}	0.082	0.087
T_{3-4}	0.073	0.077
LI*		
T_{1-2}	0.072	0.090
T_{3-4}	0.043	0.043
T_s* (hours)		
T_{1-2}	5.8	1.9
T_{3-4}	6.2	5.6
T_{pot}* (days)		
T_{1-2}	2.5	0.8
T_{3-4}	4.8	3.5
*geometric mean		

Discussion

In 64 primary tumours of the larynx, we evaluated the interrelationships between DNA-ploidy, cell proliferation markers, T-stage, and N-stage. We found that the success rates of the determination of DNA-ploidy and cell proliferation markers were in accordance with the literature data [3].

DNA-ploidy

In locally advanced (T_{3-4}) tumours, when compared with T_{1-2} tumours, we found a significantly higher percentage of DNA-aneuploidy. The higher fraction of prognostically unfavourable DNA-aneuploidy tumours in T_{3-4} tumours reflects the adverse prognosis of patients with locally advanced disease [12,13,14,17,24,28,29,30,31]. With respect to N-stage, no significant association with DNA-ploidy was found. However, if adequate therapy, i.e. extensive surgery with or without radiotherapy, is given nodal stage appeared a less important prognostic factor concerning the probability of locoregional recurrent disease [16,18].

Proliferation markers

In our series, the differences of the means of the cell proliferation markers between T-stages, were found to be independent from N-stage (and vice versa). Literature data, with respect to the relation between T- or N-stage and cell proliferation markers, are not conclusive. In 123 head and neck tumours, located at different sites, Bennet et al. [6] did not find a relation between proliferation markers on the one hand and tumour site, histological grade, T-stage, and N-stage on the other. In 52 patients with head and neck tumours located at different sites, Benazzo et al. [5] found a significantly shorter T_{pot} in larger tumours. In our study involving laryngeal tumours only, however, we found in T_{3-4}-stage tumours, compared with T_{1-2}-stage tumours, a significantly lower proliferative potential expressed by a decreased mean LI and an increased mean T_{pot} value. Similar results were reported by Schultz et al. [20], with respect to cell kinetics for small and large tumours derived from three separate cell lines. They state that i) small tumours show little or no necrosis, ii) large tumours develop fairly extensive areas of necrosis and are surrounded by a zone of hypoxic but viable cells. Since a retarding effect of hypoxia on cell cycle progression has been observed both in vitro [7] and in vivo [22], the tumour size related decrease of proliferative capacity might be explained by increased hypoxia. In our series, the lower proliferative potential in locally advanced (T_{3-4}) tumours probably relates to an increased degree of hypoxia in larger tumours. These findings may explain in part the reported lack of prognostic significance of cell proliferation markers in head and neck tumours [4,8,9,10,33], since an increase of proliferation capacity with increasing tumour size was expected. We found higher

proliferation rates, i.e. a lower mean T_s and T_{pot} value, in the presence of neck node metastases. These results are in concordance with the data of Benazzo et al. [5]. A potential drawback of dual parameter flow cytometry is the unknown proportion of normal cells in DNA-diploid tumours. In diploid tumours this leads to an underestimation of the proliferation rate of tumour cells because in diploid tumours the fraction of G_1-phase tumour cells is contaminated by normal G_1-phase cells such as inflammatory, endothelial, and stromal cells [23,25].
Due to small numbers, we were not able to perform a separate and reliable analysis of the cell proliferation markers in DNA-diploid and DNA-aneuploid tumours in relation to T- and N-stage. However, the proliferative capacity in T_{1-2} tumours is likely to be even underestimated, since the proportion of DNA-diploidy in T_{1-2} tumours, when compared with that in T_{3-4} tumors, was higher in our series.

DNA-ploidy and proliferation markers

We observed significantly higher LI values in DNA-aneuploid cases. Other authors have reported similar findings regarding LI values in head and neck tumours, located at different sites [4,5,9]. These findings can be explained as caused by the underestimation of LI in DNA-diploid tumours. By comparing data derived by immunohistochemistry and flow cytometry Bennet et al. [6] demonstrated clearly that LI and hence T_{pot} was underestimated in diploid tumours.

T_s

In our series, the reported geometric means of T_s (and, hence, T_{pot}), in T_{1-2} tumours, may appear to be short since (mean) T_s values in human tumours were reported to be 7-18 hours [4,32]. This assumption may need revision since i) T_s values of head and neck tumours appear to vary considerably, i.e. T_s values as low as 5.4 and as high as 24.3 hours have been reported [5], ii) data on T_s values in T_{1-2} laryngeal tumours are, to date, scarce. We found T_s to be shorter in diploid specifically T_{1-2} tumours. However, only a trend towards significance could be demonstrated. Begg et al. [3], and Bennet et al. [6] reported similar (and significant) differences in various head and neck tumours. Cooke et al. [9], however, were unable to confirm these findings.

Conclusions

In laryngeal tumours we found

I T_{1-2} tumours when compared with locally advanced (T_{3-4}) tumours, had a significantly higher proliferation rate (LI, T_{pot}), (independent from N-stage) explaining in part the reported lack of prognostic significance of cell proliferation markers and implying that the predictive value of LI and T_{pot} needs to be evaluated after stratification for T-stage (T_{1-2} versus T_{3-4}),

II T_s values varied considerably with T- and N-stage (range 1.9-6.2).

III N_{1-3} tumours, as opposed to N_0 tumours, were characterised by significantly lower means of T_s and T_{pot} values.

IV A higher mean LI was noted in DNA-aneuploidy tumours, emphasising the need for distinction of DNA-diploid tumour cells from DNA-diploid normal cells.

V Locally advanced (T_{3-4}) tumours, when compared with T_{1-2} tumours, were characterised by a significantly higher percentage of DNA-aneuploidy, thus supporting with the finding of impaired prognosis in aneuploid tumours.

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Chapter 3

Prognostic relevance of cell proliferation markers and DNA-index in head and neck tumours

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Summary

The purpose of this study was to evaluate the prognostic significance of cell proliferation markers and DNA-index in relation to clinical parameters in 103 patients with a head and neck tumour.
With flow cytometry, DNA-index, G_1PF-phase fraction (G_1PF), S-phase fraction (SPF), 5-bromo-2'-deoxyuridine (BrdUrd)-labelling index (LI) and potential doubling time (T_{pot}), were determined. T-stage and N-stage were assessed in accordance with the TNM classification system (UICC 1987).
After multivariate analysis, it appeared that the locoregional recurrence rate was increased by i) T_4-stage, ii) high values of the ratio SPF to LI, iii) DNA-tetraploidy. Prognostic significance for SPF, LI, and T_{pot} was not demonstrated. However, the treatment regimen was not uniform for the total group of 103 patients.
After univariate analysis, in 45 patients treated with radiation as single treatment, we observed that prognoses was significantly decreased by i) T_4-stage, ii) DNA aneuploidy, iii) DNA-tetraploidy. In 49 patients, treated by surgery and postoperative radiotherapy, i) T_4-stage and ii) DNA-tetraploidy appeared to be associated with an increased rate of locoregional recurrent disease. Prognostic significance for SPF, LI, and T_{pot} could not be demonstrated in these subgroups either. Separate statistical analysis was not executed for nine patients, treated by surgery alone.
We concluded that prognostic significance could not be demonstrated for T_{pot}, LI, SPF, and G_1PF in our series. The ratio of SPF to LI was found to be a promising prognosticator and is regarded to be indicative of the size of the hypoxic fraction. Prognostic significance for DNA-index was confirmed.

Introduction

Withers et al. [35] found that the probability of locoregional recurrent disease in oropharyngeal tumours was increased by a prolonged overall treatment time. Thereafter, tumour cell repopulation, caused by accelerated proliferation of surviving tumour cells during the course of radiotherapy, was suggested to be a major factor determining tumour regression and local tumour control probability [10,28,35]. The purpose of evaluating proliferation markers is to predict the phenomenon of repopulation and, hence, to predict treatment outcome [3]. However, no conclusive data exist, to date, on the prognostic significance of cell proliferation markers in head and neck tumours [4,5,7,8,34]. DNA-index was reported to be associated with prognosis in this group of tumours [13,14,18,25,29,30,31,32].

In 103 patients with a squamous cell carcinoma of the head and neck, treated with curative intent, we investigated the correlation between cell proliferation markers and DNA-index on the one hand, and the probability of locoregional recurrent disease on the other. G_1-phase fraction (G_1PF), S-phase fraction (SPF), 5-bromo-2'-deoxyuridine (BrdUrd)-labelling index (LI), and potential doubling time (T_{pot}) were used as indicators of the proliferative potential.

Materials and methods

Patients

Between 1989 and 1994, 103 patients with a squamous cell carcinoma of the head and neck admitted to the department of Ear, Nose & Throat (ENT) of the University Hospital Utrecht agreed to participate in cell kinetic studies involving BrdUrd infusion. The mean age was 62 years. All patients had a minimum Karnofsky performance score of 80%. Histologic examination of all tumour specimen was performed by the same pathologist (PJS). T-stage and N-stage both were assessed in accordance with the TNM classification system (UICC 1987).

Treatment

In 45 patients treated with radiation alone, total doses of 66-70 Gy in 33-35 fractions (mean overall treatment time 56 days) were given. One patient did not complete the irradiation, because of sudden death from cardiac infarction.
Surgery and postoperative radiotherapy was performed in 49 patients. In 40 patients, treated with postoperative radiotherapy, a high total dose of 60-70 Gy in 30-35 fractions (mean overall treatment time 55 days) was given, in the expectance of microscopic residual disease. In eight patients a lower total dose was administered, i.e. 50 Gy in 25 fractions (mean overall treatment time 40 days), as elective postoperative radiotherapy. In one patient postoperative radiotherapy was not completed because of sudden death from cardiac disease. Surgical treatment alone was carried out in nine patients.

Flow cytometry

Tumour samples were obtained after 100 mg BrdUrd was administered intravenously as a bolus, in 5-10 minutes. The interval between BrdUrd infusion and each tumour sampling was registered. The mean sampling time in 74 evaluable cases for T_{pot} was 230 minutes and appeared to differ for neither tumour site, T-stage, nor for N-stage.
Tumour samples were fixed in 70% ethanol for a minimum duration of 24 hours. After removal of the ethanol, single cell suspensions were prepared using pepsin (Sigma P-7012, 0.4 mg/ml 0.1 N HCl) during 30 minutes at 37°C. The pepsin digestion stripped the cells to nuclei for the vast majority of cells. The resulting suspensions were filtered through plastic tissue with a pore size of 60 µm. BrdUrd staining was performed by the method of Schutte et al. [22] using a monoclonal anti-BrdUrd antibody (Eurodiagnostics, clone IIb5) and a fluorescein isothiocyanate (FITC) labelled secondary antibody (Dakopatts F313). Prior to flow cytometric analysis, DNA was stained by 0.5 ml propidium iodide solution (0.01 mg/ml, Calbiochem 537059) containing 0.1 mg/ml RNAse (Sigma R-5503) during a minimum of 15 minutes. A FACS-analyzer (Becton Dickinson) flow cytometer was used. Fifty thousand cells were analyzed from each tumour sample. After subtraction of cell debris

counts and correction for cell doublets, DNA-histograms were analyzed using an iterative program [15]. Triplets were counted and used to calculate doublet counts [1] and then to recalculate G_1PF, SPF and G_2+MPF taking into account the calculated number of doublets and the measured number of triplets.

With dual parameter flow cytometry (FCM), both the cell cycle distribution and LI were determined. The green signal (BrdUrd detection) was log amplified. Only if a distinct separation between BrdUrd labelled and unlabelled population was obtained, the measurements were used in the analysis. The furthest left peak of the DNA histogram was assumed to correspond to the G1-phase normal cells which are always present in tumours (leucocytes, endothelial and stromal cells). The G_1-peak of tumour cells was assumed to be accompanied by a corresponding G_2-peak, positioned at approximately twice the G_1-peak value. If the position of the G_2-peak was distinctly shifted from twice the G_1-peak value, accompanied by a broadened or a non-Gaussian G_1-peak, then a shift towards DNA-aneuploidy was assumed.

Since DI depends partly on the stainability of DNA, comparison with standard cells (chicken or trout erythrocytes) must be regarded with caution [36]. This difference in stainability between standard cells and cells in tumour biopsies is often accredited to variations in accessibility of DNA to the dye [6,9,16]. In our study, therefore, no standard cells were used to assess DI. However, instrument setting was standardized using either murine thymocytes or fluorescent latex particles. DNA-index (DI) was calculated from the ratio of DNA-index of G_1-phase tumour cells to that of G_1-phase of normal cells. DNA-diploid tumours are defined by DI=1, tetraploid tumours by DI=2, and aneuploid tumours by DI>1.

Since the calculation methods of White et al. [33] and Ritter et al. [17] have not been common practice in clinical studies, we opted to use the standard approach, as suggested by Begg et al. [2], to determine T_{pot}. The mean DNA-value of BrdUrd-labelled S-phase cells was assumed to be half of G_2-peak minus G_1-peak value, during BrdUrd infusion. Progression rate of cells through S-phase was assumed to be constant. The potential doubling time (T_{pot}) was calculated using the formula

$$T_{pot} = 0.8\ T_s/LI.$$

G_1PF (and SPF) and DI values were not obtained in 24 and 14 cases, respectively. Determination of LI and, hence, calculation of T_{pot} could not be performed in five cases because BrdUrd was not administered. In 11 cases, only LI and not T_{pot} could be determined because the interval between BrdUrd-administration and tumour sampling was not recorded. T_{pot} values were unable to be adequately obtained in another 13 cases (Table 1).

Table 1 *Success rates of determining DNA-index, G_1PF, SPF, LI*, and T_{pot}***

Parameter	Percent
DNA-index	86
G_1PF/SPF	77
LI*	86
T_{pot}**	85

*in case BrdUrd is administered

**in case BrdUrd is administered and interval time is registered

Statistical analysis

The prognosticators were evaluated with respect to the locoregional recurrence free survival. In the majority of cases only the mean (and not the median) recurrence free survival could be assessed. We used the logarithmically transformed data of G_1PF, SPF, LI, SPF/LI, and T_{pot} for the analyses, because these continuous variables all showed skewed distributions. Differences between levels of a categorical variable were assessed by the log-rank test. The prognostic significance of continuous variables was assessed by Wald's test. For multivariate analysis Cox proportional hazard regression analysis was performed with SPSS for Windows (version 6.01). Results are presented as p-values and relative risks (RR). We also evaluated whether the prognostic significance of cell proliferation markers was influenced by DNA-ploidy (diploidy versus aneuploidy). These analyses included the use of interaction terms.
To evaluate the influence of treatment modality, the prognostic significance of T-stage, N-stage, cell proliferation markers (including SPF/LI values), and DNA-index (including DNA-tetraploidy) was separately evaluated both in 45 patients treated by radiation therapy alone and in 49 patients treated by surgery followed by radiotherapy. A separate statistical analysis of four recurrences in the group of nine patients treated by surgery alone was, in our opinion, not appropriate.

Results

After a mean follow up period of 47 months (range 2-72), 35 locoregional recurrences were diagnosed. The results of univariate analysis of all 103 patients for categorical variables are given in table 2a.

Table 2a *Univariate analysis for categorical variables with respect to locoregional recurrence free survival*

	Number	Mean locoregional recurrence free survival (months)	P-value
Male	90	42.9	0.39
Female	13	56.5	
Larynx	64	51.0	0.005
Hypopharynx	17	24.1	
Oropharynx	13	27.3	
Oral cavity	9	37.0	
Grade I&II	68	48.3	0.17
Grade III&IV	24	34.1	
T_1	18	50.9	0.03
T_2	27	49.9	
T_3	20	41.7	
T_4	38	36.8	
N_0	59	47.5	0.11
N_{1-3}	44	43.4	
Radiotherapy	45	43.1	0.91
Surgery	9	32.3	
Surgery & RT	49	49.5	
DI=1	32	52.6	0.09
DI >1	57	44.8	
DI=1	32	52.6	0.005
DI>1 & DI<2	39	46.9	
DI=2	8	11.0	
DI>2	10	41.7	

T-stage, DNA-tetraploidy, and tumour site appeared to be of significance. A trend towards significance for DNA-ploidy ($p = 0.09$) was noted. With respect to the continuous variables (Table 2b), we observed for high LI values a trend towards an increased local control probability ($p = 0.22$). For high SPF values, however, we noted a trend towards significance ($p = 0.06$) for a decreased local control probability. We, therefore, decided to include the ratio of SPF to LI in the analyses. It appeared, that SPF/LI was significantly associated with the locoregional recurrence rate. No significance was noted for T_{pot} and the other cell proliferation markers. By the use of interaction terms, it appeared that DNA-ploidy did not influence these results (data not shown).

Table 2b *Univariate analysis for continuous variables with respect to locoregional recurrence free survival*

	Number	Mean	P-value
Age	103	62 years	0.23
G_1PF	79	0.78*	0.26
SPF	79	0.08*	0.06
LI	84	0.07*	0.22
SPF/LI	79	1.1 *	0.01
T_{pot}	74	2.5 days*	0.32

*geometric mean

In the multivariate analysis, we noted that the probability of locoregional recurrent disease was increased in T_4-stage tumours. DNA-tetraploidy and high SPF/LI values each showed additional prognostic relevance, i.e. $p = 0.043$, RR 2.7 and $p = 0.047$, RR 2.6, respectively (Table 3). The results of the univariate analysis of categorical and continuous variables for the primary irradiated patients are given in table 4a and 4b, respectively. A higher probability of locoregional recurrent disease was found to be associated with T_4-stage. DNA-aneuploidy and DNA-tetraploidy also were found to be of prognostic significance (Figure 1). For patients treated by postoperative radiotherapy, the results for categorical and continuous variables are given in table 5a and 5b, respectively.

T_4-stage and DNA-tetraploidy appeared to be associated with a higher probability of locoregional recurrent disease. The prognostic relevance of DNA-ploidy for these patients is illustrated in figure 2. For high SPF/LI values a trend towards significance was found (p = 0.07). For these two treatment groups, we also noted, by the use of interaction terms, that DNA-ploidy did not influence the level of significance for the cell proliferation markers (data not shown). In both treatment groups, the geometric means of G_1PF, SPF, LI, and T_{pot} were calculated both for DNA-diploid and for DNA-aneuploid tumours. We could not demonstrate that locoregional recurrent tumours, compared with non recurrent tumours, were characterized by a higher proliferation rate, as represented by any of these proliferation markers. Of these analyses, only the data with respect to T_{pot} are given. The geometric means T_{pot} of tumours in patients with locoregional recurrent disease, i.e. T_{pot} 2.6 days for DI≠1 (n=21); T_{pot} 2.0 days for DI=1 (n=4), were not significantly lower than the geometric means of T_{pot} of tumours in patients without locoregional recurrent disease, with a minimum follow-up period of 2 years, i.e. T_{pot} 1.5 days for DI≠1 (n=14); T_{pot} 3.4 days for DI=1 (n=16).

Table 3 *Multivariate analysis with respect to locoregional recurrence free survival (n=79)*

	Univariate analysis P-value	1st Step *entered T-stage* P-value	2nd Step *entered SPF/LI** P-value	2nd Step *entered DI* P-value
T_{1-3}	0.002	0.004	0.013	0.015
T_4				
N_0	0.06	0.4	0.52	0.53
N_{1-3}				
Larynx	0.05	0.2	0.28	0.19
Oral cavity				
Hypopharynx				
Oropharynx				
SPF/LI*	0.01	0.047	0.047	0.12
DI≠2	0.003	0.035	0.13	0.043
DI=2				

*geometric mean

Table 4a *Univariate analysis for categorical variables with respect to locoregional recurrence free survival after radiotherapy alone*

	Number	P-value
T_{1-3}	40	0.002
T_4	5	
N_0	37	0.12
N_{1-3}	8	
DI≠2	30	0.03
DI=2	4	
DI=1	16	0.02
DI>1	18	

Table 4b *Univariate analysis for continuous variables with respect to locoregional recurrence free survival after radiotherapy alone*

	Number	Mean *	P-value
SPF	28	0.08	0.15
LI	30	0.07	0.39
SPF/LI	28	1.1	0.79
T_{pot}	25	2.6 days	0.95

*geometric mean

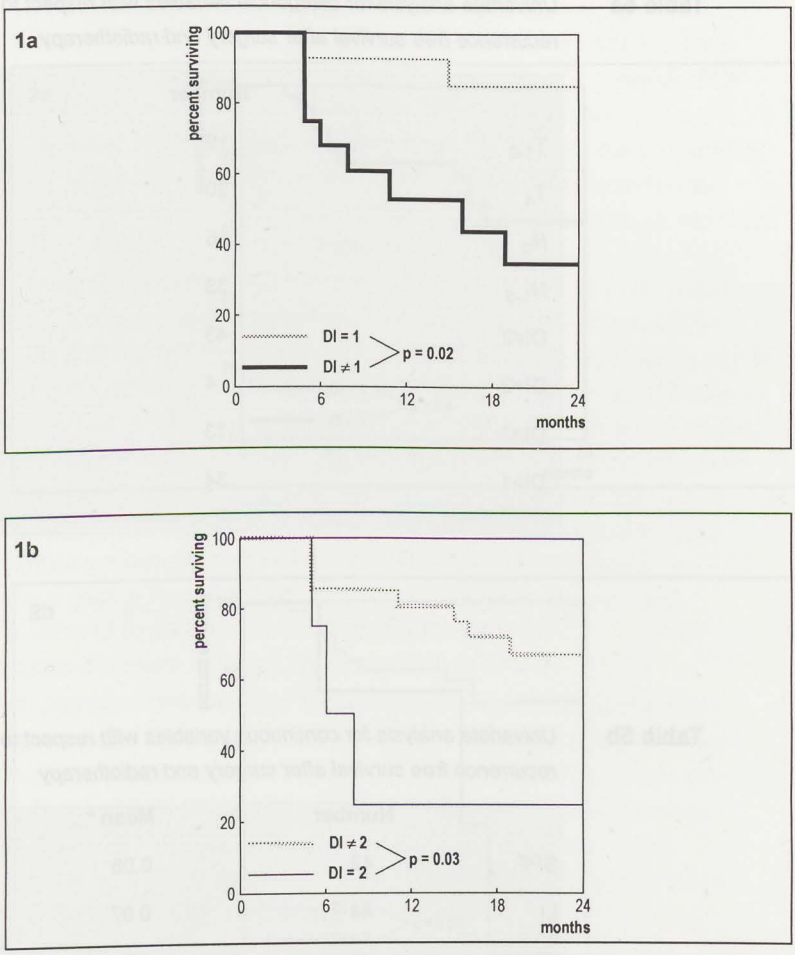

Figure 1a+1b *Locoregional recurrence free survival according to DNA-ploidy after radiotherapy alone.*

Table 5a *Univariate analysis for categorical variables with respect to locoregional recurrence free survival after surgery and radiotherapy*

	Number	P-value
T_{1-3}	19	0.045
T_4	30	
N_0	16	0.17
N_{1-3}	33	
DI≠2	43	0.05
DI=2	4	
DI=1	13	0.4
DI>1	34	

Table 5b *Univariate analysis for continuous variables with respect to locoregional recurrence free survival after surgery and radiotherapy*

	Number	Mean *	P-value
SPF	42	0.08	0.31
LI	44	0.07	0.21
SPF/LI	42	1.2	0.07
T_{pot}	39	2.5 days	0.71

*geometric mean

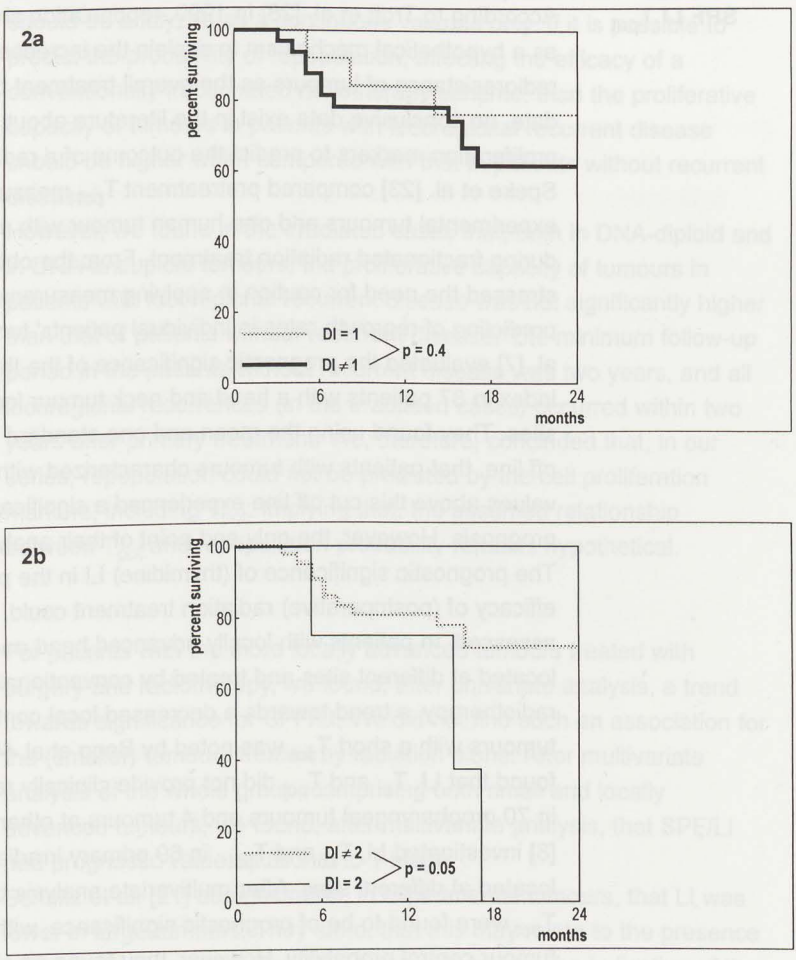

Figure 2a+2b *Locoregional recurrence free survival according to DNA-ploidy after surgery and postoperative radiotherapy*

Discussion

In 103 patients with a head and neck squamous cell carcinoma and treated with curative intent, we evaluated the prognostic significance of cell proliferation markers and DNA-index. The success rates of obtaining LI and T_{pot} values were of the same magnitude as reported by Begg et al. [4].

SPF LI T_{pot}

According to Trott et al. [28] in 1990, repopulation should be regarded as a hypothetical mechanism to explain the increase in the radioresistance of tumours as the overall treatment time is increased. To date, no conclusive data exist in the literature about the efficacy of cell proliferation markers to predict the outcome of a radiation treatment. Speke et al. [23] compared pretreatment T_{pot} measurements of four experimental tumours and one human tumour with measurements during fractionated radiation treatment. From the obtained results they stressed the need for caution in applying measurements of T_{pot} for prediction of regrowth rates in individual patients' tumours. Chauvel et al. [7] evaluated the prognostic significance of the thymidine labelling index in 87 patients with a head and neck tumour located at various sites. They found using the mean and one standard deviation as the cut off line, that patients with tumours characterized with (thymidine) LI values above this cut off line experienced a significantly impaired prognosis. However, the only end point of their analysis was survival. The prognostic significance of (thymidine) LI in the prediction of the efficacy of (postoperative) radiation treatment could, therefore, not be assessed. In patients with locally advanced head and neck tumours, located at different sites and treated by conventionally fractionated radiotherapy, a trend towards a decreased local control probability for tumours with a short T_{pot} was noted by Begg et al. [4]. Bourhis et al. [5] found that LI, T_s, and T_{pot} did not provide clinically relevant information in 70 oropharyngeal tumours and 4 tumours at other sites. Corvo et al. [8] investigated LI, T_s, and T_{pot}, in 69 primary irradiated tumours, located at different sites. After multivariate analysis both T-stage and T_{pot} were found to be of prognostic significance, with respect to local tumour control probability. However, they found no significance for T_{pot} in a subgroup of 32 patients treated by concomitant boost accelerated radiotherapy. Moreover, no separate analysis for DNA-ploidy status was performed. The proliferation parameters, BrdUrd-LI, T_s, and T_{pot} of tumours, treated according to a continuous hyperfractionated accelerated radiotherapy (CHART) regimen, showed no dependence on local tumour control probability [34]. Most of these tumours were located

in the oral cavity and oropharynx. Failures could not be predicted by proliferation rate. These authors suggested that the CHART scheme might overcome repopulation. These clinical studies all comprised tumours of various T-stages.

We did not find prognostic significance for the cell proliferation markers, when analyzed as a continuous variable. It is, however, questionable whether the prognostic significance of the cell proliferation markers should be analyzed as a continuous variable only. If it is possible to predict the probability of repopulation, affecting the efficacy of a conventionally fractionated radiotherapy scheme, than the proliferative capacity of tumours in patients with locoregional recurrent disease should be higher when compared with that in patients without recurrent disease.

However, we found in the irradiated cases that, both in DNA-diploid and in DNA-aneuploid tumours, the proliferative capacity of tumours in patients with locoregional recurrent disease was not significantly higher than that of patients without recurrent disease. The minimum follow-up period in the patients without recurrent disease was two years, and all locoregional recurrences (in the irradiated cases) occurred within two years after primary treatment. We, therefore, concluded that, in our series, repopulation could not be predicted by the cell proliferation markers, including T_{pot}. Implying that, the assumed relationship between T_{pot} and repopulation probability remains hypothetical.

SPF/LI

For patients with the more locally advanced tumours treated with surgery and radiotherapy, we found, after univariate analysis, a trend towards significance for SPF/LI. We did not find such an association for the (smaller) tumours treated by radiation alone. After multivariate analysis of the whole group, comprising both small and locally advanced tumours, we found, after multivariate analysis, that SPF/LI had prognostic value additional to T-stage.

Schultz et al. [21] demonstrated, in experimental tumours, that LI was lower in large tumours. They state, that this may relate to the presence of hypoxia in large tumours. SPF/LI may, therefore, be indicative of the hypoxic fraction of these tumours, since hypoxia may decrease the fraction of actually proliferating cells (i.e. lower LI values in large tumours). Rutgers et al. [19] found that hypoxic cells in necrotic parts of an experimental tumour did progress through the cell cycle, but at a much lower rate. Furthermore, Gabbert et al. [11] demonstrated that labelling of S-phase cells was decreased by an increasing distance of

S-phase cells to the capillaries. These findings may explain in part the reported lack of the prognostic significance of cell proliferation markers, since small tumours were included in these studies also [4,5,7,34]. Due to contamination of the G_1-phase tumour cell fraction with, normal G_1-phase cells, values of SPF and LI are underestimated in diploid tumours [26]. This disadvantage of a general application of SPF and LI as prognosticator, is overcome by using the quotient of SPF and LI in the assessment of prognosis in head and neck tumours. The relationship of SPF/LI with prognosis of patients with head and neck tumours should be confirmed in future studies.

DNA-index

After multivariate analysis, we demonstrated that DNA-tetraploidy had additional prognostic significance to T-stage. We found, in primarily irradiated (and predominantly) laryngeal tumours, a higher probability of local recurrent disease in DNA-aneuploid and DNA-tetraploid tumours. In patients treated by surgery and radiotherapy, the locoregional tumour control probability appeared to be specifically impaired in DNA-tetraploid tumours. The total number of DNA-tetraploid tumours, however, was only eight. Additional prognostic significance of DNA-index (to T-stage and/or N-stage) was reported in studies involving more than 450 patients [12,13, 29,31]. It appeared that the probability of recurrent loco-regional disease was decreased in DNA-diploid tumours. These findings were confirmed, by univariate analysis, in studies on laryngeal tumours of specific T-stages treated either by radiotherapy alone [25,30,32] or treated by radiotherapy with or without surgery [18]. Moreover, Sakr et al. [20] found that DNA-aneuploidy correlated with the frequency of histologically malignant features. In other studies of mainly oral cavity and/or oropharyngeal tumours, the prognostic significance of DNA-index could not be established by others [8,34]. Toffoli et al. [27] found that the probability of locally recurrent disease and/or local persistent tumour in 152 irradiated T_{1-2} laryngeal tumours was decreased with distinct DNA-aneuploidy, as opposed to DNA-peridiploidy (DI<1.2). However, this analysis was based on only 72 of 152 cases.The majority of studies, however, are in support of our data. Furthermore, it was shown that remission-rate after chemotherapy was increased in DNA-aneuploid tumours [12,24]. Tennvall et al. [24] concluded that this response was a strong predictor for survival of patients with DNA-aneuploid tumours. In head and neck tumours, the efficacy of induction chemotherapy followed by radiation therapy in DNA-aneuploid tumours needs to be evaluated in clinical trials.

Conclusions

In head and neck tumours:

I Prognostic significance could not be demonstrated for T_{pot}, LI, SPF, and G_1PF.

II The ratio of SPF to LI was found to be a promising prognosticator.

III The ratio of SPF to LI may be indicative of the size of the hypoxic fraction.

IV The impaired prognosis in DNA-aneuploidy tumours was confirmed and could be largely ascribed to DNA-tetraploidy.

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Chapter 4

Regional heterogeneity and intra-observer variability of DNA-content and cell proliferation markers determined by flow cytometry in head and neck squamous cell carcinomas

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Summary

In 69 samples from 23 malignant head and neck tumours, we analysed regional heterogeneity of DNA-index (DI), G_1-phase fraction (G_1PF), S-phase fraction (SPF), and BrdUrd-labelling index (LI).
In 13% of all cases, DNA-diploid and DNA-nondiploid tumour cell populations both were found to be present in the separate tumour samples. The average coefficient of variation (CV) of DI appeared to be 2%. The CV, with regard to G_1PF, LI, and SPF, was 4%, 20%, and 25%, respectively. The CV of the regional heterogeneity both for S-phase duration (T_S) and for potential doubling time (T_{pot}) was determined in 14 samples out of 7 cases and appeared to be 17% and 30%, respectively. The ratio of the intratumoural variability to the intertumoural variability, was lowest for DI.
The intraobserver variability was evaluated in 45 tumour samples from 19 randomly selected cases. In one tumour sample, the first analysis showed a DNA-diploid tumour cell population whilst in the second analysis a DNA-aneuploid tumour cell population was found. The correlation coefficient (CC) for LI, DI, and RM was 0.99, 0.98, and 0.71, respectively. Due to a large difference within a tumour of one patient the mean difference between the first and second analysis of the intraobserver variability was of statistical significance for RM.
We thus concluded that, in head and neck tumours, the conditions for a prognosticator were better for DI, as opposed to those for SPF, LI, G_1PF, T_s, and T_{pot}.

Introduction

Recurrent disease in head and neck cancer occurs at the primary site and or in regional lymph nodes in the majority of cases [12,13,19,20,22,25,39,47]. Furthermore, patients with N_{1-3} disease who later showed recurrent disease in the lymph nodes also failed in more than 90% at their original site of disease [28].

The influence of the overall treatment time and proliferative potential in affecting the efficacy of fractionated radiotherapy in head and neck tumours was suggested [3,15,42,52]. Determination of the proliferative potential by flow cytometry has been shown to be promising in the selection of patients for differing radiotherapy regimens [2].

A range of techniques have evolved to quantify this proliferative potential, both directly and indirectly, in vitro and in vivo [8]. DNA content, as expressed by the DNA-index (DI), fraction of G_1-phase cells (G_1PF), and fraction of S-phase cells (SPF) can be determined by flow cytometry. After pre-operative infusion of the thymidine analogue 5-bromo-2'-deoxy-uridine (BrdUrd), it is also possible to estimate the BrdUrd-labelling index (LI), duration of S-phase (T_s) and, hence, potential doubling time (T_{pot}) of tumour cells [3]. T_{pot} is the doubling time of the tumour in the absence of cell loss [36].

However, since these proliferation markers are estimated from a single biopsy, regional heterogeneity may interfere with the accuracy of the parameter determination. Intraobserver variability may also influence the accuracy of the parameter determination. As a consequence, the clinical application of proliferation markers for the selection of patients for adapted irradiation schemes could be insufficient reliable.

We, therefore, evaluated the regional heterogeneity of DNA-index and proliferation markers in 69 samples from 23 malignant head and neck tumours.

The intraobserver variability was evaluated for DNA-index, LI, and RM in 45 tumour samples from 19 randomly selected cases. Here, we decided to evaluate beside DI, LI and RM because these parameters are essential to calculate T_s and T_{pot}.

Materials and methods

Tumour samples

Between 1989 and 1993, 139 head and neck patients admitted to the department of ENT of the University Hospital Utrecht agreed to participate in cell kinetic studies involving BrdUrd infusion. DI and proliferation markers were assessed in 69 tumour samples obtained from 23 patients who underwent primary surgical treatment. The characteristics are listed in table 1.

Table 1 *Characteristics of patients with respect to the analysis of regional heterogeneity*

Tumour site	Male/ female	Mean age (years)	T_{1-2}	T_{3-4}	N_0	N_{1-3}
Larynx	6/0	68	2	4	4	2
Hypopharynx	2/1	69	0	3	0	3
Oropharynx	4/0	48	2	2	3	1
Oral cavity	9/1	62	6	4	6	4

Since in 16 cases the interval between BrdUrd-infusion and tumour sampling varied within each case, it was only possible to adequately evaluate regional heterogeneity of T_s and T_{pot} in seven cases. Due to poor quality of tissue samples, regional heterogeneity of LI could not be evaluated in five tumour samples of three cases. G_1PF and SPF could not be evaluated in six tumour samples of five cases. The intraobserver variability was analysed in 45 tumour samples of 19 randomly selected cases. The characteristics are listed in table 2. The second analysis was performed by the same flow cytometrist (DHR), 6-12 months after the first analysis. The intraobserver variability could not be evaluated for RM in two cases due to poor quality tissue samples.

Table 2 *Characteristics of patients with respect to the analysis of intraobserver variability*

Tumour site	Male/ female	Mean age (years)	T_{1-2}	T_{3-4}	N_0	N_{1-3}
Larynx	4/1	69	1	4	4	1
Hypopharynx	0/1	46	0	1	0	1
Oropharynx	4/0	48	2	2	3	1
Oral cavity	8/1	62	6	3	5	4

Flow cytometry

Pre-operatively, 100 mg BrdUrd was administered intravenously as a bolus, in 5-10 minutes. The interval between BrdUrd-infusion and tumour sampling was registered. Fresh tumour samples were fixed in 70% ethanol for a minimum duration of 24 hours. After removal of the ethanol, single cell suspensions were prepared using pepsin (Sigma P-7012, 0.4 mg/ml 0.1 N HCl) for 30 minutes at 37° C. The pepsin digestion stripped the cells to nuclei for the vast majority of cells. The resulting cell suspensions were filtered through plastic tissue with a pore size of 60 μm. BrdUrd staining was performed by the method of Schutte et al. [33], using a monoclonal anti-BrdUrd antibody (Eurodiagnostics, clone IIb5) and a fluorescein isothiocyanate (FITC) labelled secondary antibody (Dakopatts F313). Prior to flow cytometric analysis, DNA was stained by 0.5 ml propidium iodide solution (0.01 mg/ml, Calbiochem 537059) containing 0.1 mg/ml RNAse (Sigma R-5503) over a minimum of 15 minutes. A FACS-analyser (Becton Dickinson) flow cytometer was used. Fifty thousand cells from each tumour sample were analysed. After subtraction of cell debris counts and correction for cell doublets, DNA-histograms were analysed using an iterative program [23], resulting in G_1PF, SPF, and G_2+MPF. The green signal (BrdUrd detection) was log amplified. With bivariate flow cytometric analysis, cell cycle distribution and BrdUrd-LI were determined. Only if a distinct separation between BrdUrd labelled and unlabelled population was obtained, the measurements were used in the analysis. In our study, no standard cells were used to assess DI. However, instrument setting was standardized using either murine thymocytes or fluorescent latex particles. The furthest left peak of the DNA-histogram, was assumed to correspond to the G_1-phase normal cells which are always present in tumours (leucocytes, endothelial, stromal cells). The G_1-peak of tumour cells was assumed to be accompanied by a corresponding G_2-peak, positioned at approximately

twice the G_1-peak value. If the position of the G_2-peak was distinctly shifted from twice the G_1-peak value, accompanied by a broadened or a non-Gaussian G_1-peak, then a shift towards DNA-aneuploidy was assumed. DI was calculated from the ratio of DNA-content of G_1-phase tumour cells to that of G_1-phase normal cells. Diploid tumours were defined by DI=1, and aneuploid tumours by DI>1. Calculation of S-phase duration (T_s in hours) was made according to the method of the relative movement (RM) [3]. The mean DNA-value of BrdUrd-labelled S-phase cells was assumed to be half of G_2-peak value minus G_1-peak value at the time of BrdUrd-labelling ($RM_0 = 0.5$). Progression rate of cells through the S-phase was assumed to be constant. The potential doubling time was calculated by

$$T_{pot} = 0.8\ T_s/LI.$$

Statistical analysis

Regional heterogeneity and intraobserver variability were assessed with Analysis of Variance (ANOVA). In diploid tumours, the admixture of inflammatory and stromal cells tends to influence markedly the values of G_1PF, SPF, LI, and T_{pot}, and will result in an underestimate of the proliferation rate of the tumour cells [37,41]. Therefore, the analysis of regional heterogeneity and intraobserver variability for the cell proliferation markers was performed separately in either diploid or aneuploid tumour samples of a specific case. The regional heterogeneity or the intratumoural variability was represented by the square root of the variance within tumours. The intertumoural variability was calculated from the square root of the difference between the mean square between tumours and the mean square within tumours, divided by two. With respect to DI, the coefficient of variation (CV), representing the relative regional heterogeneity, was determined by the ratio of the mean of all standard deviations (SD) of all sample values in each case and the mean value of DI for all cases. Here we used the mean, since in the majority of cases no differences with regard to DI were observed and, consequently, the median SD (and CV) would then be zero. The CV's, representing the relative regional heterogeneity for G_1PF, SPF, LI, T_s, and T_{pot}, were determined by the ratio of the median of all SD's of all sample values in each case and the mean value of these variables in all cases. The intraclass correlation coefficient (CC) R, representing the relative magnitude of the intertumoural variability to the intratumoural variability, was calculated both for DI and for proliferation markers. The intraclass correlation coefficient R is 1.0, in case the intratumoural variability is neglectable.

The intraobserver variability was determined by the square root of the variance within observations. The mean differences, the p-values and the 95% confidence limits (CL) between the first and the second assessment of DI, LI, and RM were given. To quantify the association between the first and second assessment the product-moment correlation coefficient was calculated.

Results

Regional heterogeneity

The regional heterogeneity of DI was analysed in 69 tumour samples obtained from 23 cases. Co-existence of a DNA-diploid and a DNA-nondiploid (DI#1) tumour clone was found in three cases (13%). In one of these cases, a DNA-diploid (DI=1) and a DNA-tetraploid (DI=2) tumour cell population co-occurred. The CV, representing the regional heterogeneity for DI, appeared to be 2% (Table 3). The regional heterogeneity, expressed by the corresponding CV of the intratumoural heterogeneity for G_1PF, LI, and SPF, appeared to be 4% (range of SD 0-19%), 20% (range of SD 0.2-70%), and 25% (range of SD 0-53%), respectively (Table 3). The regional heterogeneity for T_{pot} and T_s, was determined in 14 tumour samples from seven cases. The CV for T_{pot} and T_s appeared to be 30% and 17%, respectively (Table 3).

Table 3 *Regional heterogeneity, represented by the intratumoural variability (Intra V), the corresponding coefficient of variation (CV), the intertumoural variability (Inter V) and the intraclass correlation coefficient (R) for DI, G_1PF, SPF, LI, T_s (hours) and T_{pot} (days)*

	Number of cases	Number of samples	Mean	Intra V	CV	Inter V	R
DI	23	69	1.4	0.09	2%	0.72	0.89
G_1PF	20	56	82.2%	4.7	4%	6.0	0.56
SPF	20	56	7.4%	2.2	25%	3.2	0.59
LI	20	56	9.3%	2.4	20%	6.2	0.72
T_s	7	14	5.1	1.1	17%	1.8	0.62
T_{pot}	7	14	3.6	1.2	30%	2.0	0.62

Inter- and intratumoural variability

The intratumoural variability, the corresponding intertumoural variability, and the intraclass correlation coefficient R for DI and the proliferation markers are given in table 3 and 4.

Intraobserver variability

The intraobserver variability was assessed for DI, LI, and RM in 45 tumour samples of 19 randomly selected cases. In one sample, the first analysis showed a DNA-diploid tumour cell population, whilst in the second analysis a DNA-aneuploid tumour cell population was found. This case was excluded for further analysis, with regard to the

intraobserver variability for LI. The CC for LI, DI and RM was 0.99, 0.98, and 0.71, respectively. The mean difference between the first and the second assessment for DI, LI, and RM appeared to be 0.005 (95% CL-0.025;0.036), 0.095 (95% CL-0.112;0.03), and -0.034 (95% CL-0.058;-0.010), respectively. For RM, this difference was statistically significant, with a p-value of 0.01. However, a pronounced variation of RM, as well as of DI, was revealed after the second analysis of one case. In the first analysis the mean values of RM and DI in this tumour were 0.94 and 1.3, respectively. After repeat analysis these values were found to be 0.63 and 1.8, respectively.

Table 4 *Intraobserver variability (Intra V), the corresponding coefficient of variation (CV), the intertumoural variability (Inter V) and the intraclass correlation coefficient (R) for DI, LI, and RM*

	Number of cases	Number of samples	Mean	Intra V	CV	Inter V	R
DI	19	45	1.4	0.06	4%	0.63	0.92
LI	18	44	9.9%	0.5	5%	5.6	0.92
RM	17	43	0.76	0.046	6%	0.07	0.62

Discussion

DNA-index and proliferation markers were reported to be promising prognosticators in squamous cell carcinomas of the head and neck [2,12,16,19,40,45,46,49,50]. Determination of (DI and) cell proliferation markers is essential to estimate the proliferation rate of a tumour and hence, to select patients for either conventional or accelerated radiotherapy [4]. However, the existence of regional heterogeneity has been reported to occur in several tumours. For instance, differences of DI were reported to occur in human gliomas [10,14], prostatic adenocarcinomas [48], breast carcinomas [17], carcinomas of the biliary tract and pancreas [38], colorectal carcinomas [34], and adenoid cystic carcinomas [44]. Different T_{pot} values within one tumour were found in colorectal cancer [51], cervix tumours [7], and canine osteosarcomas [21].

Also interobserver variability on analysis of DI and cell proliferation markers, as determined by flow cytometry for various tumour sites was demonstrated to be substantial and may be caused by a lack of standardization [1,18,35,43,51]. From this, we conclude that since standardization of the determination of DI and cell proliferation markers by flow cytometry is, to date, not perfected, comparison of the results of different studies is hazardous.

We analysed the regional heterogeneity and intraobserver variability, with respect to DNA-index and proliferation markers in head and neck squamous cell carcinomas.

Regional heterogeneity

We evaluated the regional heterogeneity of DNA content, with respect to both DNA-ploidy status (diploid, polyploid, aneuploid) and DI. In many studies the prognostic significance of DNA content was evaluated for diploid versus non-diploid tumours [12,16,19,32,40,45,46,49,50]. According to some authors, however, prognosis appeared to depend on various DNA populations [19,24]. In some tumours prognostic significance of DI<1.6 versus DI>1.5 was found [11,30]. On the other hand, prognosis also could relate to DI=1+DI=2 versus aneuploidy [31]. A small shift in DI-value, therefore, may cause a shift of tumour classification into another prognostic subgroup. These data emphasise the necessity of evaluating the regional heterogeneity of DNA content, with respect both to DNA-ploidy status and to DI. We found both DNA-diploid and DNA-aneuploid tumour cell population in 13% of 23 cases. Although, in one case the absolute difference in DI was small, i.e. 0.1. In our study, we found the CV, reflecting the regional heterogeneity for DI to be low, i.e. 2% also. These data are in accordance with the literature. Kearsly at al. [19] found of 60 samples, which originally

appeared diploid only two demonstrated aneuploid peaks when another sample from the same head and neck tumour was analysed. Bennet et al. [5] found both diploid and non-diploid tumour clones in two out of eight head and neck tumours.
Regional heterogeneity of G_1PF, SPF, LI, T_s and T_{pot} was analysed only in diploid or only in non-diploid tumour samples of specific cases. In our study, we found the CV reflecting the relative regional heterogeneity of the cell proliferation markers, to be lowest for G_1PF, i.e. 4%. For LI, we found an average CV of 20%. Bennet et al. [5] reported an average CV for LI of 26.8% as determined in eight cases. Begg et al. [4] found similar values, regarding CV for LI, in five malignant head and neck tumours.
With respect to T_s and T_{pot}, we analysed regional heterogeneity in 14 tumour samples of seven separate tumours. Bennet et al. [5] found, in 48 samples of eight cases, an average CV for T_s and T_{pot} of 15.1% and 30.3%, respectively. This supports our data of 17% and 30%, respectively. Begg et al. [4] reported similar data in five malignant head and neck tumours. The regional heterogeneity for T_s and T_{pot}, in our series, is also in range of published data for other malignancies such as bladder cancer [4], colorectal cancer [51], and breast cancer [29].

Inter- and intratumoural variability

For DI and LI the intraclass correlation coefficient R appeared to be most pronounced, i.e. 0,89 and 0.72, respectively. This means that the measured variability for DI, can be ascribed for 89% to the intertumoural variability and for 11% to the intratumoural variability. This infers that, from this point of view, DI is the most favourable candidate as a prognosticator. We also found a larger intertumoural variability for T_s and T_{pot}, compared with the intratumoural variability. Similar findings were reported by Bourhis et al. [9] in oropharyngeal cancer, by Perez et al. [26] in human tumour xenografts and by LaRue et al. [21] in canine osteosarcomas. All these findings reflect the potential prognostic relevance both of T_s and of T_{pot}.

Intraobserver variability

The intraobserver variability of DI, LI, and RM was analysed in 45 fresh tumour samples obtained from 19 randomly selected cases. The second analysis showed a different DNA-ploidy status in only one out of 45 samples. The intraobserver variability of DI was reported to be low in breast cancer [6]. In our study, the CC for LI and DI, appeared to be high, i.e. 0.99 and 0.98, respectively.
Similar results, regarding LI, obtained from five tumour samples of one

squamous cell carcinoma of the tongue, were reported by Begg et al. [4]. For RM, the CC was found to be lower i.e. 0.71. The mean difference between the first and second analysis of the intraobserver variability appeared to be of significance for RM and, hence, affected importantly the calculated CV for both T_s and T_{pot}. The calculated CV for T_s and T_{pot}, based on the mean differences of LI and RM, appeared to be 17% and 18%, respectively. However, the observed mean difference between the first and the repeat assessment of RM could be ascribed largely to a pronounced different result of the second assessment in only one tumour sample. For DI and LI, the intraclass correlation coefficient R appeared to be pronounced, i.e. 0.92 and 0.92, respectively.

Conclusions

I With regard to regional heterogeneity, DNA-ploidy status and DI, when compared with that of LI, and SPF, showed less variation.

II The ratio of the intratumoural variability to the intertumoural variability, was lowest for DI.

III The regional heterogeneity for T_s and T_{pot} was moderate.

IV The intraobserver variability was low for DNA-ploidy status, DI, and LI, and moderate for RM.

We thus concluded that, in head and neck tumours, the conditions for a prognosticator were better for DI, when compared with those for SPF, LI, G_1PF, T_s, and T_{pot}.

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Chapter 5

S-phase fraction, BrdUrd-labelling index, duration of S-phase, potential doubling time, and DNA-ploidy status in benign and malignant brain tumours.

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Summary

Seventy one histologically malignant brain tumours, 52 histologically benign brain tumours, and 14 intracerebral metastases were characterised according to DNA-index (DI), and cell proliferation markers, i.e. S-phase fraction (SPF), BrdUrd-labelling index (LI), duration of S-phase (T_s), and potential doubling time (T_{pot}), assessed by flow cytometry (FCM)

In histologically benign tumours, a high percentage of DNA-diploid tumours and a low proliferative potential in DNA-diploid tumours were found. DNA-aneuploid, benign tumours appeared to be characterised by a high proliferation rate. Histologically malignant tumours and cerebral metastases were both found to be characterized by a low percentage of DNA-diploid tumours and a high proliferation rate in DNA-diploid tumours. The observed differences of DNA-index and proliferative potential fully agree with differences in clinical behaviour in these groups of tumours. The duration of S-phase appeared to be low (range 4.5-4.7 hours). T_s values appeared not to differ between the three groups, nor did T_s values differ with ploidy status. From this, the observed differences in T_{pot} values should be accredited mainly to differences in LI. Long term survival of low grade glioma patients was reported to be low and these tumours appeared to be characterised by malignant (FCM) features, i.e. i) a high percentage DNA-aneuploidy, ii) a high mean DI (for DI>1), iii)) a high proliferative potential. Low grade gliomas should, therefore, be considered to be a malignant disease.

Introduction

In low grade gliomas, no histologically malignant characteristics, i.e. mitoses, necrosis or endothelial proliferation are present. According to a review article of Vecht [1], prognosis varies considerably and appears to depend mainly on age. Five year survival rates for patients of 20-50 years vary between 35 and 60%. For patients over 50 years it falls to 30% or less [1]. Most high grade glioma patients die within 2 years, with local recurrence being the cause of death in 90% of patients, but long term survival has been reported [2,3]. Local recurrence rate in histologically benign brain tumours, such as meningiomas is low and cannot be predicted adequately by histological features [4,5,6,7,8,9].
In patients with gliomas of low or moderate grade, the 5-bromo-2'-deoxy-uridine (BrdUrd) labelling-index (LI) has been shown to correlate with survival time [10,11,12,13]. Prognosis in primary brain tumours is, therefore, likely to be predicted more adequately by taking into account parameters reflecting the proliferative potential. Direct and indirect techniques have evolved to quantify the proliferative potential, both in vitro and in vivo [14].
In the present study, human brain tumours with histologically benign (n=52) and malignant characteristics (n=71) were characterised with respect to differences in, i) DNA-ploidy status (diploid versus aneuploid), ii) mean DNA-index (DI) of DNA-aneuploid tumours, and iii) proliferative potential. Fourteen patients with intracerebral metastases of malignant epithelial tumours were also included in this evaluation. The objective was also to evaluate whether some tumours, with histologically benign characteristics, show malignant (FCM) features, such as high proliferation-rate, or aneuploidy.
The DI, as well as distribution of cells through the cell cycle were determined by flow cytometry (FCM), resulting in fractions of cells in G_1-phase (G_1PF), in S-phase (SPF), and in G_2+M-phase (G_2+MPF). In 1982, Gratzner [15] demonstrated, that the thymidine analogue BrdUrd, after incorporation into DNA during S-phase, could be recognised by the use of monoclonal antibodies. BrdUrd-labelling indices can be obtained after both in vitro [16,17,18] and in vivo labelling [10,11,19,20]. After in vivo labelling, we calculated the duration of S-phase (T_s) and the potential doubling time (T_{pot}) from a single tumour biopsy, using flow cytometry [21]. T_{pot} is the doubling time of the tumour in the absence of cell loss [22]. The T_{pot} is also potentially useful in the selection of patients for different radiotherapy regimens [23].

Materials and methods

Patients

Consecutive patients admitted to the department of Neurosurgery of the University Hospital of Utrecht for treatment of a brain tumour, were asked to participate in this study in the period 1990 through 1994. Eventually 137 patients agreed to participate. All patients had a minimum Karnofsky performance status of 80%. The histological diagnosis, gender, age, primary or recurrence of malignant brain tumours are given in table 1. The characteristics of the histologically benign primary brain tumours are listed in table 3. The characteristics of 14 cerebral metastases of are shown in table 3.

Grading

Grading of the 43 astrocytomas was established according to the criteria of the revised WHO classification [24]. Gemistocytic astrocytomas, containing at least 20% gemistocytes, were considered to be of high grade malignancy since the prognosis of these tumours was reported to be comparable with that of high grade astrocytomas [25]. In 14 patients with pure oligodendrogliomas, grading of the tumour was established according to the criteria of Smith [8]. Oligo-astrocytomas were diagnosed in 10 patients. The malignancy grade of these mixed tumours was ascribed to the highest grade of malignancy of either histological component. The tumours were then divided into a group of low grade gliomas and a group of high grade gliomas (table 4).

Table I *Malignant brain tumours*

Histology	Total number	Male/ female	Mean age (yrs)	Recurrent/ primary
Astrocytoma*	43	32/11	50	9/34
Oligodendroglioma	14	8/6	42	6/8
Oligo-astrocytoma	10	4/6	41	4/6
Ependymoma	2	1/1	27	0/2
Medulloblastoma	1	1/0	23	0/1
Neuroblastoma	1	0/1	21	1/0
Total	71	46/25	46	20/51

* including 1 ganglioglioma and 1 pilocytic astrocytoma.

Table 2 *Benign brain tumours*

Histology	Total number	Male/ female	Mean age (yrs)	Recurrent/ primary
Meningioma	42	16/26	58	12/30
Craniopharyngioma	3	3/0	44	1/2
Acoustic neurinoma	3	1/2	42	0/3
Neurilemnoma	1	1/0	58	0/1
Neurocytoma	1	0/1	21	0/1
Pineocytoma	1	0/1	56	0/1
Pituitary adenoma	1	1/0	38	0/1
Total	52	22/30	55	13/39

Table 3 *Cerebral metastases*

Primary tumour	Total number	Male/female	Mean age (yrs)
Breast carcinoma	8	3/5	63
Bronchus carcinoma	4	2/2	64
Melanoma	1	1/0	43
Metastases nos*	1	1/0	36
Total:	14	7/7	60

* not otherwise specified

Table 4 *Malignancy grading of gliomas*

	Low grade	High grade
Astrocytoma	12*	31
Oligodendrogliomas	6	8
Oligo-astrocytomas	6	4
Total	24	43

* including 1 ganglioglioma and 1 pilocytic astrocytoma

Flow cytometry

Preoperatively, 100 mg BrdUrd was administered intraveneously as a bolus, in 5-10 minutes. Fresh tumour samples were obtained during surgery. The interval between BrdUrd infusion and tumour sampling was noted. The mean interval in 73 evaluable cases for T_{pot} was three hours and was similar in the three groups. Tumour samples were fixed in 70% ethanol for a minimum of 24 hours. After removal of the ethanol, single cell suspensions were prepared using pepsin (Sigma P-7012, 0.4 mg/ml 0.1 N HCl) over 30 minutes at 37° C. The pepsin digestion stripped the cells to nuclei in the vast majority of cells. The resulting suspensions were filtered through plastic tissue with a pore size 60 µm. BrdUrd staining was performed by the method of Schutte et al. [26], using a monoclonal anti-BrdUrd antibody (Eurodiagnostics, clone IIb5) and a fluorescein isothiocyanate (FITC) labelled secondary antibody (Dakopatts F313). Prior to flow cytometric analysis, DNA was stained by 0.5 ml propidium iodide solution (0.01 mg/ml, Calbiochem 537059) containing 0.1 mg/ml RNAse (Sigma R-5503) for a minimum of 15 minutes. A FACS-analyser (Becton Dickinson) flow cytometer was used. Fifty thousand cells from each tumour sample were analysed. After subtraction of cell debris counts DNA-histograms were analysed using an iterative program [27]. Triplets were counted and used to calculate doublet counts [28] and then to recalculate G_1PF, SPF, and G_2+MPF taking into account the calculated number of doublets and the measured number of triplets. With bivariate flow cytometric analysis, cell cycle distribution and LI were determined. The green signal (BrdUrd detection) was log amplified. Only if a distinct separation between BrdUrd labelled and unlabelled population was obtained, the measurements were used in the analysis. The furthest left peak of the

DNA-histogram was assumed to correspond to the G_1-phase normal cells which are always present in tumours (leucocytes, endothelial and stromal cells). The G_1-peak of tumour cells was assumed to be accompanied by a corresponding G_2-peak, positioned at approximately twice the G_1-peak value. If the position of the G_2-peak was distinctly shifted from twice the G_1-peak value, accompanied by a broadened or a non-Gaussian G_1-peak, then a shift towards DNA-aneuploidy was assumed. Since DI depends partly on the stainability of DNA, comparison with standard cells (chicken or trout erythrocytes) should be regarded with caution [29]. This difference in stainability between standard cells and cells in tumour biopsies is often accredited to variations in accessibility of DNA to the dye [30,31]. In our study, therefore, no standard cells were used to assess DI. However, the instrument setting was standardized using either murine thymocytes or fluorescent latex particles. DI was calculated from the ratio of DNA-content of G_1-phase tumour cells to that of G_1-phase of normal cells. Diploid tumours were defined by DI=1, and aneuploid tumours by DI>1. Since the calculation methods of White et al. [32] and Ritter et al. [33] have not been commonly used in clinical studies, we opted to use the standard approach as suggested by Begg et al. [21] to determine T_s and T_{pot} values. For calculations of T_s and T_{pot}, the mean DNA-value of labelled S-phase cells was assumed to be half of G_2-peak value minus G_1-peak value at BrdUrd infusion. Progression rate of cells through S-phase was assumed to be constant. The potential doubling time (T_{pot}) was calculated using the formula

$$T_{pot} = 0.8\ T_s/LI.$$

Standardisation is lacking and, therefore, the exact data of our series may differ from that of others. These differences could be related to e.g. differences in gate setting, in stainability of the BrdUrd caused by the use of different monoclonal antibodies. However, our calculations have been executed in a consequent way. The results of the comparison of DI, SPF, LI, T_s, and T_{pot} between malignant and benign brain tumours are not influenced by the calculation method. Determination of LI, and analysis of T_s and T_{pot} could not be performed in 12 cases because BrdUrd was, erronously, not administered. In four cases only LI and no T_s and T_{pot} values could be determined, because the interval between BrdUrd administration and tumour sampling was not recorded. Poor quality of tumour samples, or a very low LI precluded the adequate calculation of T_s and T_{pot} for the remainder of the missing values.

Statistical analysis

The proliferation markers showed a skewed distribution. In the assessment of the significance of differences between groups, the geometric method was, therefore, used. Analysis of variance was performed on continuous variables to test differences between groups of tumours, with additional pairwise comparison of specific groups. A chi-square test was used to compare the tumour groups with respect to their DNA-ploidy status, i.e. DNA-diploidy or DNA-aneuploidy.

Results

In table 5, the means of flow cytometrically derived parameters in malignant and benign brain tumours are given. In comparing these two groups of tumours, means of DNA-ploidy status, SPF, LI, and T_{pot} were significantly different ($p<0.001$). The mean DI of DNA-aneuploid tumours differed at the $p=0.01$ level. The same level of significance was observed in the differentiation of parameters between benign brain tumours and intracerebral metastases, except for the percentage of DNA-diploid tumours and for the mean DI of DNA-aneuploid tumours (table 6). Duration of S-phase appeared to be insignificantly different between the three groups (table 5 and 6). In table 7, the geometric means of the various parameters are given for a more restricted group of malignant tumours (including astrocytomas, oligodendrogliomas, and oligo-astrocytomas), for meningiomas, and for a restgroup of tumours with benign histology (excluding meningiomas). The percentages of DNA-diploid tumours and the geometric means of SPF, LI, T_s, and T_{pot} of these groups, when compared with those in the larger group, appeared to be of the same magnitude.In the restricted group of benign tumours, when compared with meningiomas, the percentage of DNA-aneuploid tumours was higher and a tendency towards a more active proliferation, as expressed by a higher mean LI and lower mean T_{pot} value, was noted. We evaluated SPF, LI, T_s, and T_{pot} in relation to the DNA-ploidy status, i.e. diploid or aneuploid, for malignant and benign tumours (table 8). In diploid tumours, statistically significant differences were found for SPF ($p<0.001$), LI ($p<0.001$), and T_{pot} ($p<0.001$). In case of aneuploidy statistical significance could not be demonstrated and only a trend towards significance was noted for LI ($p=0.07$).

Table 5 *DI, geometric means of DI for DI>1, SPF, LI, T_s, T_{pot} in malignant and benign brain tumours*

	Malignant brain tumours (n=71)	Benign brain tumours (n=52)	P-value
DI=1 (%)	46 (n=66)	85 (n=48)	< 0.001
DI>1 (mean)	1.9 (n=35)	1.4 (n= 7)	0.01
SPF	0.05 (n=58)	0.02 (n=47)	< 0.001
LI	0.029 (n=43)	0.007 (n=44)	< 0.001
T_s (hours)	4.5 (n=35)	4.7 (n=33)	> 0.10
T_{pot} (days)	5.4 (n=35)	20.9 (n=33)	< 0.001

Low grade gliomas, when compared with high grade gliomas, tended to have a longer T_{pot} (p=0.05). The percentages of DNA-diploid tumours in low and high grade gliomas were 39% and 50%, respectively. The mean DI for DNA-aneuploid tumours was of the same magnitude both for low and for high grade gliomas. Mean values of SPF, LI, and T_s were of the same magnitude in low and in high grade gliomas (Table 9).

Table 6 *DI, geometric means of DI for DI>1, SPF, LI, T_s, T_{pot} in benign brain tumours and cerebral metastases*

	Benign brain tumours (n=52)	Cerebral metastases (n=14)	P-value
DI=1 (%)	85 (n=48)	58 (n=12)	0.05
DI>1 (mean)	1.4 (n= 7)	2.0 (n= 5)	ns*
SPF	0.02 (n=47)	0.05 (n=11)	< 0.001
LI	0.007 (n=44)	0.03 (n= 8)	< 0.001
T_s (hours)	4.7 (n=33)	3.9 (n= 5)	ns*
T_{pot} (days)	20.9 (n=33)	3.9 (n= 5)	< 0.001

* p > 0.10

Table 7 *DI, geometric means of DI for DI>1, SPF, LI, T_s, T_{pot} in gliomas, meningiomas, and benign brain tumours (excluding meningiomas)*

	Astrocytomas Oligodendrogliomas Oligo-astrocytomas n=67	Meningiomas n=42	Benign brain tumours (excl. meningiomas) n=10
DI=1 (%)	46 (n=63)	90 (n=40)	62.5 (n= 8)
DI>1 (mean)	1.9 (n=34)	1.5 (n= 4)	1.4 (n= 3)
SPF	0.05 (n=55)	0.02 (n=40)	0.02 (n= 7)
LI	0.028 (n=40)	0.006 (n=37)	0.011 (n= 7)
T_s (hours)	4.5 (n=32)	4.7 (n=26)	4.5 (n= 7)
T_{pot} (days)	5.5 (n=32)	23.5 (n=26)	13.3 (n= 7)

Table 8 *Geometric means of SPF, LI, T_s and T_{pot} in malignant brain tumours and benign brain tumours according to DNA- ploidy status*

	Malignant brain tumours (n=71)	Benign brain tumours (n=52)	P-value
SPF for DI=1	0.039 (n=30)	0.017 (n=41)	< 0.001
SPF for DI≠1	0.064 (n=28)	0.049 (n= 6)	ns*
LI for DI=1	0.026 (n=21)	0.006 (n=38)	< 0.001
LI for DI≠1	0.031 (n=22)	0.016 (n= 6)	0.07
T_s (hours) for DI=1	3.8 (n=17)	4.6 (n=28)	ns*
T_s (hours) for DI≠1	5.4 (n=18)	4.6 (n= 5)	ns*
T_{pot} (days) for DI=1	4.9 (n=17)	25.8 (n=28)	< 0.001
T_{pot} (days) for DI≠1	6.0 (n=18)	6.3 (n= 5)	ns*

* p > 0.10

Table 9 *DI, and geometric means of DI for aneuploidy (DI>1), SPF, LI, T_s, T_{pot}, in low grade and high grade gliomas*

	Low grade gliomas (n=24)	High grade gliomas (n=43)	P-value
DI=1 (%)	39 (n=23)	50 (n=40)	ns*
DI>1 (mean)	1.9 (n=14)	2.0 (n=20)	ns*
SPF	0.05 (n=18)	0.05 (n=37)	ns*
LI	0.02 (n=11)	0.03 (n=29)	ns*
T_s (hours)	5.3 (n= 8)	4.3 (n=24)	ns*
T_{pot} (days)	8.7 (n= 8)	4.7 (n=24)	0.05

* p > 0.10

Discussion

Data in the literature [1,2,3,4,5,6,7,8,9] illustrate, that the prognosis in primary brain tumours can not be predicted by histological features alone. Prognosis is likely to be more accurately predicted by taking into account parameters reflecting the proliferative potential of tumours [10,11,12,13]. This aspect has been the subject of several studies over the years. Various methods have been used to determine the proliferation rate of human brain tumours, including immunohistochemistry with Ki-67 and MIB-1 antibodies [35,36,37,38,39], antibodies to the proliferating antigen (PCNA) [40,41,42,43,44], silver staining for nucleolar organizer regions (AgNOR) [35,45,46,47], BrdUrd-LI by immunohistochemistry [11,12,13,16,18,20,48,49,50,51,52,53] and by flow cytometry [10,19,49,54,55,56]. In the majority of studies investigating BrdUrd-labelling indices, immunohistochemical techniques were used. It was concluded, however, that both the flow cytometrically derived LI and the immunohistochemically derived LI may provide relevant information in the prediction of biological behaviour of brain tumours [49]. Lapham [57] was the first to report on DI of gliomas.

DNA-index

In our study, DNA-ploidy status of benign and malignant brain tumours differed significantly. The majority (85%) of histologically benign primary brain tumours appeared to be DNA-diploid. In meningiomas and in some other tumours with benign characteristics, the percentages of DNA-diploid tumours appeared to be 90% and 62.5%, respectively. With regard to meningiomas, Assietti et al. [19] found 96% (n=25) to be DNA-diploid. We found 46% of all malignant primary brain tumours to be DNA-diploid. Ganju et al. [56] found 36% to be DNA-diploid in 51 astrocytic tumours. They also found 51% of 25 oligodendrogliomas and pilocytic tumours to be DNA-diploid. Similar results were reported by Assietti et al. [19] in astrocytomas and by Danova et al. [54] in tumours of neuro-epithelial origin. The differences that we noted in mean DI, between DNA-aneuploid tumours with benign and malignant characteristics, were also reported by Nishizaki et al. [58].
In our study, we observed a high percentage DNA-aneuploidy and a high mean DI (for DI>1) both in low and in high grade gliomas. These findings confirm the potential malignant nature of low grade gliomas [1].
In our study, the percentage of DNA-diploid tumours in cerebral metastases was 58%, being in concordance with the data of Kiss et al. [59] although these results were obtained by image cytometry. The observed high percentage of DNA-diploidy in the group of tumours with histologically benign characteristics accords with the reported

favourable prognosis of DNA-diploid tumours and tumours with benign histology [4,5,6,54,56,59,60]. From this we can conclude that histologically benign tumours with DNA-aneuploidy may behave more malignantly than as deduced from the histological characteristics.

SPF LI

We found geometric mean values of SPF and LI to be significantly lower in histologically benign primary brain tumours, compared with those in histologically malignant primary brain tumours. With respect to malignant brain tumours, similar SPF values, as determined by flow cytometry, were found by Coons et al. [55] on 230 astrocytomas and by Ganju et al. [56] on 95 gliomas. With respect to BrdUrd-LI, results of the same magnitude were reported by Danova et al. [10], Assietti et al. [19], and Nishizaki et al. [58] on gliomas, using flow cytometric techniques. These results were in support with the data obtained by the use of immunohistochemical techniques [16,18,53,61,62]. Using flow cytometry, Assietti et al. [19] noted lower LI values (mean 0.02) in 25 meningiomas compared with LI values derived from 14 gliomas (mean 0.04). We found a geometric mean LI of 0.006 in 37 meningiomas. Comparison of these absolute values, however, is hazardous because of the FCM procedure, with respect to the determination of BrdUrd-LI, is not yet standardized and interlaboratory differences were reported [63,64,65]. Furthermore, it must be stressed that we used the geometric means. Using immunohistochemistry a low mean of LI was found by Iwaki et al. [66] in 40 meningiomas. Assietti et al. [19] reported a mean SPF of 0.02 in 19 meningiomas, being in support with our data.
In the present study, the observed higher values of SPF and LI both in intracerebral metastases and in malignant brain tumours, as opposed to those in benign brain tumours, are reflecting the expected poor prognosis of these patients [1,2,10,11,12,13,55]. The method of dual parameter flow cytometry implicates, however, that the G_1-phase peak of normal cells of the DNA-histogram, contaminates the G_1-phase peak of DNA-diploid tumour cells. Since normal cells in tumours are predominantly in G_1-phase, the calculated mean values of SPF and LI in DNA-diploid tumours are, as a consequence, underestimated [67,68]. We, therefore, decided to perform a separate analysis of all cell proliferation markers in diploid and aneuploid tumours. We found a significant lower proliferative capacity (SPF, LI) in diploid benign tumours, when compared with that of diploid malignant tumours. In aneuploid tumours, these differences appeared to be less pronounced, i.e. only a trend towards significance was noted for LI. However, the number of aneuploid benign tumours was small.

T_s T_{pot}

The mean BrdUrd sampling time (ST) of three hours, in our series, is relatively short. A ST longer than T_{G2+M} has been recommended by Begg et al. [21,69]. But T_{G2+M} is, a priory, not known in malignant brain tumours. These tumours are generally characterised by very rapid growth and invariably by a short T_{G2+M}. A ST longer than T_{G2+M} leads to progression of cells through G_2+M-phase into G_1-phase and thus leads, if uncorrected to overestimation of the proliferative capacity. Höyer et al. [70] stated that ST should be adjusted to tumour type and should generally be between 60% and 100% of the expected T_s. Therefore, a ST of 3 hours seems to be appropriate in malignant brain tumours. Since proliferation of labelled cells has not yet occurred, the reliability of LI values may even be greater than that after longer intervals. To date, the optimal ST in malignant and in benign brain tumours, however, is not known. In our series, the mean T_s values appeared not to differ significantly between histologically malignant (4.5 hours), histologically benign primary brain tumours (4.7 hours), and intracerebral metastases (3.9 hours). T_s was not influenced by ploidy status. The reported values of T_s, in our series, were short relative to reported T_s values of 7-18 hours for various tumour sites [69,70,71,72,73]. These T_s values may need to be reconsidered since i) T_s values of human tumours appear to vary considerably, i.e. values as low as 4.0 and as high as 37.6 hours have been reported [70], ii) in experimental mammalian tumour systems (both in vivo and in vitro) T_s values of 4 hours and less have been demonstrated [74], iii) data on T_s values in human benign and malignant brain tumours are, to date, scarce.

The mean T_{pot} of 5.4 days, of 35 histologically malignant primary brain tumours, appeared to be significantly shorter when compared with the mean T_{pot} of benign tumours. We noted a significantly higher proliferative capacity (T_{pot}) in diploid malignant tumours when compared with that of diploid benign tumours. In case of aneuploidy, proliferation rate appeared to be the same in benign and malignant brain tumours. However, the number of aneuploid benign tumours was small. We found in low grade gliomas, when compared with high grade gliomas, a lower proliferative potential. This was expressed by a significantly higher mean T_{pot} value. These findings reflect the longer survival time for patients with low grade gliomas [1,2]. In five intracerebral metastases, the estimated T_{pot} was short, i.e. 3.9 days. The mean T_{pot} of 26 meningioma's appeared to be 23.5 days. In the literature, data on T_{pot} values in primary brain tumours are scarce. Danova et al. [10] found a median T_{pot} of 12.5 days in 68 gliomas. Assietti et al. [19] found no differences in mean T_{pot} between nine neuroepithelial tumours and two

meningiomas. The mean T_{pot} in our series, both in histologically malignant primary brain tumours and in brain metastases were shorter than the mean T_{pot} reported both by Assietti et al. [19] and by Danova et al. [10]. However, these differences can be explained by the observed lack of standardization of the method of T_{pot} calculation [63,64,65]. In addition the formula for the T_{pot} calculation used by Assietti differed from ours. We used the λ-value of 0.8, as opposed to λ-value of 1.0 in their calculations. However, the observed discrepancies in geometric mean values of T_{pot} between histologically benign and malignant tumours, as observed in our study, fully agree with the expected differences in clinical behaviour [1,2,4,5,6,59]. As T_s showed no significant difference between the three groups, differences in T_{pot} values must be accredited mainly to differences in LI.

Conclusions

I Malignant brain tumours, as opposed to histologically benign brain tumours, were found to be characterised by malignant FCM features, as expressed by a significantly
- higher proportion of DNA-aneuploid tumours,
- higher mean DI (for DI>1),
- higher proliferative capacity (SPF, LI and T_{pot}) in diploid tumours.

II Geometric mean T_s values appeared to be low, i.e. 4.5-4.7 hours and not to differ between benign and malignant brain tumours.

III Low grade gliomas were found to be characterised by low survival rates and malignant FCM features and should, therefore, be considered as a malignant disease.

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Chapter 6

Prognostic relevance of cell proliferation markers and DNA-index in gliomas

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Summary

The prognostic significance of clinically, histologically and flow cytometrically derived parameters was assessed in 49 glioma patients. With flow cytometry, DNA-index, S-phase fraction (SPF), 5-bromo-2'-deoxyuridine (BrdUrd)-labelling index (LI), and potential doubling time (T_{pot}) were determined.

After univariate analysis of the clinical variables such as, age, seizures as initial symptom, and duration of first symptom were found to be significantly associated both with proliferation rate and with local progression free survival (LPFS).

The cytomorphological features such as, the presence or absence of mitosis, necrosis, and endothelial proliferation, appeared to be significantly associated with LPFS.

With respect to the cell proliferation markers, we observed a longer LPFS to be associated with low SPF, low LI, and high T_{pot} values. We did not observe a significant association between DNA-ploidy status and LPFS. After multivariate analysis both of high and of low grade tumours, we found that neither LI, SPF, nor age had additional prognostic significance to cells in mitoses. We also demonstrated, that necrosis and endothelial proliferation had no additional prognostic significance to that of cells in mitoses. In the subgroup of low grade gliomas, in contrast to high grade gliomas, we noted prognostic significance for LI.

We concluded, that

- the presence or absence of cells in mitoses, representing the proliferation rate, was the strongest single prognosticator in gliomas,
- in low grade gliomas, LI holds prognostic significance.

Introduction

Gliomas are the most common type of primary intracerebral neoplasms [1]. Despite appropriate treatment, almost all patients with intracranial gliomas die due to local progressive disease [2,3,4,5]. Established prognosticators are malignancy grading, age, and performance status [4,6,7,8,9,10,11]. However, the biological behaviour of these tumours cannot always be adequately predicted [4,5,12]. Prognosis in gliomas is likely to be predicted more adequately by taking account of parameters reflecting the proliferation rate. This aspect has been the subject of several studies. Different approaches have been used to determine the proliferation rate of human brain tumours, including immunohistochemistry with Ki-67 and MIB-1 antibodies [13,14], antibodies to the proliferating cell nuclear antigen [15,16], silver staining for nucleolar organizer regions [17,18], antibodies to 5-bromo-2'-deoxy-uridine (BrdUrd) [19,20,21,22,23,24] and flow cytometry with S-phase fraction (SPF), BrdUrd labelling-index and potential doubling time (T_{pot}) [10,11,25,26]. However, little is known about the additional prognostic significance of these experimental cell proliferation markers to the proven prognostic significance of the presence or absence of cells in mitoses, as determined by histological examination. Prognostic significance for DNA-ploidy (diploidy versus aneuploidy) was also reported [10,27,28].

We, therefore, decided to evaluate the prognostic significance of clinically, histologically, and flow cytometrically derived parameters in 49 glioma patients with a long-term follow up period. Fraction of S-phase cells (SPF), BrdUrd-labelling index (LI), and potential doubling time (T_{pot}), were used as indicators of the proliferative potential. T_{pot} represents the doubling time of the tumour in the absence of cell loss [29].

Materials and methods

Patients

Between 1989 and 1994, consecutive patients admitted to the department of Neurosurgery of the University Hospital Utrecht for treatment of a primary supratentorial glioma were asked pre-operatively to participate in cell kinetic studies, involving BrdUrd infusion. Eventually, 49 patients, all adults, agreed to participate. All patients had a minimum Karnofsky performance status of 80%.

Grading

Grading of intracranial gliomas is based on the presence or absence of a number of cytomorphological features, i.e. nuclear atypia, mitoses, necrosis and endothelial proliferation [7,30]. It was decided to analyze these features separately. All histological slides were reviewed (GHJ). Adequate information with respect to all the separate cytomorphological features was obtained in 48 cases. The number of mitotic figures was counted per square millimetre in areas of the tumour sample with the highest mitotic rates. Nuclear atypia was present in the vast majority (>90%) of cases and it was, therefore, decided not to analyze this parameter separately. Grading of 35 astrocytomas and eight pure oligodendrogliomas was established in accordance with the criteria of the WHO-II classification [30]. Since the prognosis of gemistocytic astrocytomas, containing at least 20% gemistocytes, was reported to be comparable with the prognosis of high grade astrocytomas, these tumours (n=5) were considered to be of high grade malignancy [31]. The malignancy grade of six oligo-astrocytomas was ascribed to the highest grade of malignancy of either histological component.

Treatment

After surgical treatment, patients were referred for radiation therapy. Radiotherapy consisted of external beam irradiation without concomitant or adjuvant chemotherapy. Patients with high grade gliomas received whole brain irradiation followed by a boost, with a margin of 2 cm, to the primary tumour site. Fraction doses varied from 2.0-2.35 Gy, resulting in total doses to the primary tumour site of 60 and 56 Gy, respectively. In 14 high grade glioma patients, irradiation could not be adequately given, due to rapid tumour progression. The median LPFS of these patients was one month. Patients with low grade gliomas received focal irradiation with a margin of 2-3 cm. Fraction doses varied from 1.8-2.0 Gy, resulting in total doses of 54-60 Gy. Four low grade glioma patients did not receive postoperative radiotherapy. Two of these patients developed local recurrent disease after a period of 19 and 22 months, respectively. The other two patients appeared to be free of progressive disease 74 and 75 months, respectively, after surgery. All patients were included in the study.

Flow cytometry

Preoperatively, 100 mg BrdUrd was intravenously administered as a bolus, in 5-10 minutes, and tumour samples were obtained during craniotomy. The mean time between BrdUrd infusion and tumour sampling was three hours in the 26 cases evaluable for T_{pot}. Fresh tumour samples were fixed in 70% ethanol for a minimum of 24 hours. After removal of the ethanol, single cell suspensions were prepared using pepsin (Sigma P-7012, 0.4 mg/ml 0.1 N HCl) for 30 minutes at 37° C. The pepsin digestion stripped the cells to nuclei for the vast majority of cells. The resulting suspensions were filtered through plastic tissue with a pore size of 60 µm. BrdUrd staining was performed by the method of Schutte et al. [32], using a monoclonal anti-BrdUrd antibody (Eurodiagnostics, clone IIb5) and a fluorescein isothiocyanate (FITC) labelled secondary antibody (Dakopatts F313). Prior to flow cytometric analysis, DNA was stained by 0.5 ml propidium iodide solution (0.01 mg/ml, Calbiochem 537059) containing 0.1 mg/ml RNAse (Sigma R-5503) over a minimum of 15 minutes. A FACS-analyser (Becton Dickinson) flow cytometer was used. Fifty thousand cells from each tumour sample were analysed. After subtraction of cell debris counts and correction for cell doublets, DNA-histograms were analysed using an iterative program [33]. Triplets were counted and used to calculate doublet counts and then to recalculate G_1PF, SPF, and G_2+MPF, taking into account the calculated number of doublets and the measured number of triplets [34]. With bivariate flow cytometric analysis, cell cycle distribution and BrdUrd-LI were determined. The green signal (BrdUrd detection) was log amplified. Only if a distinct separation between BrdUrd labelled and unlabelled population was obtained, the measurements were used in the analysis. The furthest left peak of the DNA-histogram was assumed to correspond to the G_1-phase normal cells which are always present in tumours (leucocytes, endothelial and stromal cells). The G_1-peak of tumour cells was assumed to be accompanied by a corresponding G_2-peak, positioned at approximately twice the G_1-peak value. If the position of the G_2-peak was distinctly shifted from twice the G_1-peak value, accompanied by a broadened or a non-Gaussian G_1 peak, then a shift towards DNA-aneuploidy was assumed. Since DI depends partly on the stainability of DNA, comparison with standard cells (chicken or trout erythrocytes) must be regarded with caution [35]. This difference in stainability between standard cells and cells in tumour biopsies is often accredited to variations in accessibility of DNA to the dye [36,37]. In our study, therefore, no standard cells were used to assess DI. However, instrument setting was standardized using either murine thymocytes or fluorescent latex particles. DI was calculated from the ratio of DNA-

content of G_1-phase tumour cells to that of G_1-phase of normal cells. Diploid tumours were defined by DI=1, tetraploid tumours by DI=2, and aneuploid tumours by DI>1.

Since the calculation methods of Ritter et al. [37] and White et al. [38] have not been common practice in clinical studies, we opted for the standard approach, as suggested by Begg et al. [39] to determine T_s and T_{pot}. For calculations of T_s and T_{pot}, the mean DNA-value of BrdUrd-labelled S-phase cells was assumed to be half of G_2-peak value minus G_1-peak value at the time of BrdUrd-labelling. Progression rate of cells through S-phase was assumed to be constant. T_{pot} was calculated by the formula

$$T_{pot} = 0.8\ T_s/LI.$$

DNA-histograms could not be adequately analysed for DI and SPF in 4 and 11 cases, respectively. Determination of LI and, hence, analysis of T_{pot}, could not be carried out in four cases, because BrdUrd was not administered. In one case LI but not T_{pot} was determined, because the interval between BrdUrd administration and tumour sampling was unrecorded. In another three cases LI, but no T_{pot} values were obtained due to very short BrdUrd interval times. In eight cases, LI and, hence, T_{pot} could not be adequately obtained due to the absence of a distinct separation of labelled and unlabelled populations. An unevaluable DNA/BrdUrd-plot was the reason why LI and T_{pot} could not be adequately analysed in 5 and 7 tumours, respectively (Table 1).

Table 1 *Success rate of the determination for DNA-index, SPF, LI, and T_{pot}*

Parameter	Percent
DNA-index	92
SPF	78
LI	71*
T_{pot}	63**

*in case BrdUrd administered
**in case BrdUrd administered (excluding very short interval times)

Statistical analysis

At the time of analysis, three patients were alive after the onset of local progressive disease and another four patients had died from non-tumour related causes. Treatment differed in a number of patients after the occurrence of local progressive disease. It is for these reasons that we found it more appropriate to perform the analysis with respect to the local progression free survival (LPFS) instead of to the overall survival. Categorical variables were tested by the log-rank method. The continuous variables, derived from flow cytometry, all showed a skewed distribution and, therefore, logarithmically transformed values were used. Differences in the (geometric) means of the cell proliferation markers in patients with or without seizures and short or long duration of symptoms were tested by the T-test (for the continuous variables) and the chi-square test (for the categorical variables). The prognostic significance of continuous variables was assessed in accordance with Kaplan-Meier univariate analysis. Differences between univariate survival curves were assessed by the Mantel-Cox test. For multivariate analysis, Cox proportional hazard regression analysis was performed with SPSS for Windows (version 6.01). Results are presented as p-values and relative risks (RR) with their 95% confidence interval (CI). We also evaluated whether the prognostic significance of cell proliferation markers was influenced by DNA-ploidy (diploidy versus aneuploidy). These analyses included the use of interaction terms.

Results

In 38 patients, local progressive disease was diagnosed unequivocally by either CT or MRI scanning and, at time of analysis, all but three had died due to tumour progression. Four patients died due to non-tumour related causes and in the remaining seven cases progressive disease could not be demonstrated after a mean follow-up period of 65 months (range 37-79). We observed that the cell proliferation markers LI, and T_{pot} could be determined adequately in 71%, and 63% of the patients, respectively (Table 1).

The results of the univariate analysis are shown in table 2 and table 3. Of the clinical variables, age at diagnosis was strongly correlated with LPFS, i.e. older patients being at a significantly higher risk of early local recurrent disease. A short pretherapeutic duration of symptoms was found to be associated with an increased risk of local progressive disease. The histological type and the extent of surgical treatment appeared to be of no significance, in our study.

Cytomorphological features like mitoses, necrosis, and endothelial proliferation were all strongly associated with LPFS. The number of mitoses per square millimetre, SPF, LI, and T_{pot}, appeared to be significantly associated to LPFS. With respect to the cell proliferation markers we found, using interaction terms, that DNA-ploidy did not influence the level of significance. DNA-ploidy appeared to be unassociated with LPFS. A high DI (>2.0), however, tended to be associated to a longer LPFS compared with that of a low DI (<2.1), i.e. 36.9 and 9.9 months, respectively. Patients with seizures as first symptom experienced a significant longer LPFS. These patients had tumours with a significantly decreased proliferation rate (mitotic figures, SPF) (Table 4). Tumours of patients with a short duration of symptoms, were characterised by a significantly increased proliferation rate (mitotic figures, LI) (Table 4). After multivariate analysis (n=49) of high plus low grade tumours, we noted that age had no additional prognostic significance to that of the presence of cells in mitoses figures ($p<0.0001$; RR 19.8 with 95% CI 5.6-69.8). With respect to LI (n=32), we could not demonstrate additional prognostic significance to that of mitotic figures ($p=0.003$; RR 10.8 with 95% CI 2.2-51.9). With respect to SPF, similar findings were noted (data not shown). We noted that the prognostic significance of mitotic figures (n=48) was superior to that of the other cytomorphological features, i.e. necrosis and endothelial proliferation ($p<0.0001$; RR 20.2 with 95% CI 5.7-71.5) (Table 5). The significance of the presence or absence of cells in mitoses in relation to LPFS is illustrated in figure 1.

Table 2. *Univariate analysis (log-rank test) on categorical variables with respect to LPFS*

	Median local progression free survival (months)	P-value
Gender:		
Male (n=34)	11.0	> 0.10
Female (n=15)	6.9	
Duration of symptoms:		
<3months (n=28)	6.0	0.01
>3months (n=18)	40.9	
Seizures as first symptom:		
Yes (n=13)	37.9	0.005
No (n=36)	7.0	
Resection:		
Not complete (n=31)	10.0	> 0.10
Complete (n=18)	10.0	
Histology:		
Astrocytoma (n=35)	6.9	> 0.10
Oligodendroglioma (n=8)	40.9	
Oligo/astrocytoma (n=6)	10.0	
Grading:		
Low grade (n=15)	49.9	0.0006
High grade (n=34)	6.9	
Necrosis:		
Yes (n=23)	2.9	<0.0001
No (n=25)	36.9	
Mitosis:		
Yes (n=27)	3.9	<0.0001
No (n=22)	40.9	
Endothelial proliferation:		
Yes (n=28)	3.9	<0.0001
No (n=20)	36.9	
Ploidy status:		
DI=1 (n=22)	10.0	> 0.10
DI≠1 (n=23)	10.0	
DNA-index:		
DI=1 (n=22)	10.0	> 0.10
1<DI>2 (n=10)	6.9	
DI=2 (n=8)	10.0	
DI>2 (n=5)	36.9	

Finally, we found that LI, age, tumour grade, and the interaction between LI and tumour grade all were significantly associated to LPFS (Table 6). For all four variables significance was noted. In low grade gliomas, prognosis was determined mainly by LI, i.e. more than 80% of the RR could be accredited to LI. In high grade gliomas, however, prognosis was determined mainly by malignancy grade, i.e. less than 20% of the RR could be accredited to LI. From these results, it was concluded, that the prognostic significance of LI was pronounced in low grade gliomas, whilst this effect was diminished in high grade gliomas.

Table 3 *Univariate analysis on continuous variables with respect to LPFS*

	Number	Mean	P-value
Age	49	48 years	<0.0001
Mitoses	48	6.2 per mm^2	<0.0001
SPF	38	0.048*	0.01**
LI	32	0.027*	0.008**
T_{pot}	26	5.6 days*	0.04**
*geometric mean	**level of significance was not influenced by DNA ploidy		

Table 4 *Symptoms and cell proliferation markers*

	SPF geometric mean	LI geometric mean	T_{pot} (days) geometric mean	Mitotic figures yes/no
Seizures as first symptom:				
Yes (n=13)	0.034	0.018	7.5	2/11
No (n=36)	0.054	0.031	5.0	25/11
P-value	***0.03***	***ns****	***ns****	***0.002***
Duration of symptoms:				
< 3months (n=28)	0.051	0.030	5.1	20/8
> 3months (n=18)	0.045	0.015	8.0	5/13
P-value	***ns****	***0.02***	***ns****	***0.009***
* p > 0.10				

Table 5 *Cox regression analysis of mitosis, necrosis, and endothelial proliferation with respect to LPFS (n=48)*

	P-value	RR
Mitoses	<0.0001	20.2 (CI:5.7-71.5)
Necrosis	ns*	na**
Endothelial proliferation	ns*	na**

*p > 0.10 **not applicable

Table 6 *Cox regression analysis of LI, age, grade and the interaction term LI x grade with respect to LPFS (n=32)*

	Mean	P-value	RR
LI	0.027*	0.013	5.5
Age	48 years	0.034	1.03
Grade	na**	0.016	9.0
LI x grade	na*	0.030	5.0

*geometric mean **not applicable

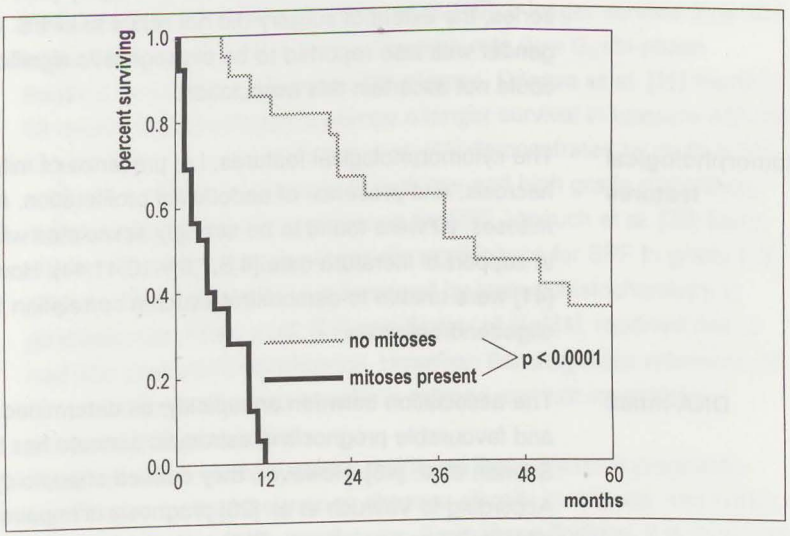

Figure 1 *Local progression free survival in the presence and the absence of mitotic figures*

Discussion

The prognostic significance of clinically, histologically and flow cytometrically derived parameters was evaluated in 49 glioma patients with a long term follow up period. Special attention was given to the prognostic significance of cell proliferation markers obtained by either FCM or by histological examination.

Success rates LI T_{pot}

In our study, we found that the proliferation markers LI and T_{pot} could be adequately obtained in 71%, and 63%, respectively. In head and neck tumours, these success rates appeared to be higher [40]. In eight glioma cases, however, we found that LI and, hence, T_{pot} could not be adequately obtained due to the absence of a distinct and reliable separation of BrdUrd labelled and BrdUrd unlabelled cells. In the majority of these cases this was related to very low labelling indices.

Clinical variables

We found, in accordance with to the literature that, among the clinical variables, age at diagnosis was strongly correlated with LPFS [4,6,7,8,9,10,41]. A short duration of symptoms, i.e. duration less than three months, appeared to be associated with an increased risk of local progressive disease, as was reported by Curran et al. [4]. Patients with seizures as an initial symptom experienced a significantly longer LPFS. These two clinical variables appeared to be significantly associated to indicators of the proliferation-rate. These findings reflect the prognostic relevance of cell proliferation markers in gliomas.
There has been considerable controversy over the concept of treating high grade gliomas with cytoreductive surgery [4,9,41,42,43]. In our series, the extent of surgery did not relate to LPFS. In some studies, gender was also reported to be of prognostic significance [7,41]. We could not ascertain this association.

Cytomorphological features

The cytomorphological features, i.e. presence of mitoses, presence of necrosis, and presence of endothelial proliferation, and the number of mitoses, all were found to be strongly associated with the LPFS, being in support of literature data [4,6,7,8,9,10,41,44]. However, Coons et al. [41] were unable to demonstrate such a correlation for mitotic figures in oligodendrogliomas.

DNA-index

The association between aneuploidy, as determined by image cytometry and favourable prognosis in astrocytic tumours has been observed by Salmon et al. [45]. However, they defined aneuploidy as a DI>1.30. According to Vavruch et al. [26] prognosis is impaired in case of DNA-diploidy as compared with DNA tetraploid cases (1.9<DI>2.1). DNA-

diploidy, determined by flow cytometry, was found to have a more favourable prognosis in other studies [27,46,47,48,49,50]. Coons et al. [11] found an increased risk of death to be associated with near-diploid tumours. However, Ganju et al. [10] did not observe a correlation between DI and prognosis in astrocytic tumours and Kross et al. [44] did not find prognostic significance for DI in oligodendrogliomas. According to these literature data it was concluded, that the prognostic significance of DNA-index (including DNA-ploidy) in gliomas, has not been demonstrated unequivocally. In our series, we did not observe an association of DNA-ploidy and LPFS. However, patients with tumours of a high DI (>2.0), in contrast to those with a low DI (<2.1), tended to have a longer LPFS.

SPF LI T_{pot}

The mean BrdUrd sampling time (ST) of three hours, in our study, is relatively short. A ST longer than T_{G2+M} has been recommended [39,40]. But T_{G2+M} is not known in gliomas. These tumours are generally characterised by very rapid growth and invariably by a short T_{G2+M}. A ST longer than T_{G2+M} leads to progression of cells through G_2+M-phase into G_1-phase, making proper corrections for the determination of LI, and RM necessary. Höyer et al. [51] stated that ST should be adjusted to tumour type and should generally be between 60% and 100% of the expected T_s. Therefore, a ST of three hours in gliomas seemed appropriate. After univariate analysis, a longer LPFS was found to be significantly associated with low SPF, low LI, and high T_{pot} values. These findings are in line with expectations and correspond with literature data. According to Ganju et al. [10], a longer survival time was related to tumours with a low proliferation-rate (low G_2+M-phase fraction), in patients with grade I-III gliomas. Danova et al. [25] found in 68 mainly high grade astrocytomas a longer survival in tumours with low LI, and high T_{pot} values. Coons et al. [11] demonstrated by multivariate analysis, in 230 astrocytomas of both low and high grade malignancy, independent prognostic significance for SPF. Vavruch et al. [26] found after multivariate analysis prognostic significance for SPF in grade II-III gliomas. With respect to LI determined by immunohistochemistry in glioblastomas, Ritter et al. [23] and Barker et al. [24], reported that LI had little prognostic significance. However, the prognostic relevance of the presence or absence of cells in mitoses was not separately analysed in these studies.

In high plus low grade tumours, we found that additional prognostic significance to the presence or absence of cells in mitoses, was noted neither for LI (nor SPF), nor for age. From these findings, it is concluded

that the reported association of both LI (and SPF) and age, with the LPFS is based mainly on differences in tumours with or without mitotic figures. The importance of the prognostic significance of the presence of cells in mitoses in gliomas follows from the fact that, after multivariate analysis, both necrosis and endothelial proliferation had no additional prognostic relevance to that of the presence or absence of cells in mitoses.

With respect to the subgroup of low grade gliomas we noted, using interaction terms, prognostic significance for LI, whilst this effect was diminished for high grade gliomas. These data are in line with the reported observations based on immunohistochemistry [22,23,24,52]. Ito et al. [52], and Hoshino et al. [22] found that LI had prognostic significance in low grade gliomas. These results indicate that in low grade gliomas, as opposed to high grade gliomas, LI, determined either by FCM or by immuno-histochemistry, holds prognostic significance.

Conclusions

I Proliferation markers LI and T_{pot} could be obtained adequately in 71% and 63% of gliomas, respectively.

II Seizures, duration of symptoms and age tended to be associated with prognosis and appeared to be related to cell proliferation markers.

III DNA-ploidy was not and the magnitude of DNA-aneuploidy might be associated with prognosis.

IV SPF, LI, and T_{pot} were associated with prognosis, but no additional prognostic value could be demonstrated when the presence or absence of cells in mitoses was taken into account.

V The prognostic significance of age was of no importance when compared with that of the presence or absence of cells in mitoses;

VI After multivariate analysis it appeared that of the cytomorphological features, the presence or absence of mitotic figures, representing the proliferation rate, was the strongest prognosticator.

VII In low grade gliomas LI was of prognostic significance.

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Chapter 7

Prognostic relevance of mitotic figures, MIB-1 immunoreactivity, S-phase fraction, and bromodeoxyuridine labelling indices in gliomas

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Summary

The prognostic significance of cell proliferation markers was evaluated in 27 glioma patients with long-term follow-up.
Cell proliferation markers were assessed by i) flow cytometry (FCM), i.e. S-phase fraction (SPF), BrdUrd-labelling index (LI_{fcm}), ii) immunohistochemistry (IHC), i.e. BrdUrd-labelling index (LI_{ihc}), MIB-1 immunoreactivity (MIB-1-LI), and iii) histological examination, i.e. the presence or absence of cells in mitoses.
After univariate analysis, we observed that a longer local (tumour) progression free survival (LPFS) was significantly associated with low SPF, low LI_{fcm}, and low MIB-1-LI. For LI_{ihc} no significant association was found. Cox regression analysis indicated LI_{fcm} ($p = 0.015$; RR 2.2) to be a more promising prognosticator than MIB-1-LI. However, in comparison with this marker, the presence or absence of mitotic figures appeared an even stronger prognosticator ($p = 0.002$; RR 10.7). By using interaction terms, we found that the prognostic significance of LI_{fcm} was especially of importance in low grade (versus high grade) gliomas ($p < 0.05$).
We concluded, that in gliomas

- cells in mitoses (M-phase) was of more prognostic significance, than was LI_{fcm} (S-phase) or MIB-1-LI (non-G_0-phase fraction),
- of the tested cell proliferation markers in gliomas, LI_{fcm} appeared to be of more prognostic significance than MIB-1-LI, SPF, and LI_{ihc}
- LI_{fcm} is likely to be an important prognosticator in low grade gliomas.

Introduction

In recent years, the prognostic significance of quantification of the proliferative potential has been investigated in neuro-oncology. A range of approaches have evolved to quantify this potential, both directly and indirectly, in vitro as well as in vivo [1]. Detailed characterisation of the monoclonal antibody Ki-67 showed specific reaction with a nuclear antigen, expressed in proliferating cells throughout all parts of the cell cycle, with exception of the G_0-phase [2]. It appeared that the monoclonal antibody MIB-1 had the same potential for formalin fixed and paraffin embedded tissue. The relevance of Ki-67 and MIB-1 positivity to cell proliferation activity has been documented in brain neoplasms [3,4,5,6]. After intravenous administration of the thymidine analogue 5-bromo-2'-deoxy-uridine (BrdUrd), the fraction of cells positive for BrdUrd uptake can be assessed by either immunohistochemistry (IHC), resulting in LI_{ihc}, or by flow cytometry (FCM), resulting in LI_{fcm}. The total fraction of S-phase cells (SPF) can be obtained by FCM. The potential prognostic value of these cell proliferation parameters has been evaluated in numerous studies [7,8,9,10,11,12,13,14].

However, it remains unclear whether these experimental cell proliferation markers have additional prognostic value apart from the proven prognostic significance of the presence of cells in mitosis, determined in histologic sections [15,16,17]. We, therefore, decided to evaluate the prognostic significance of these experimental cell proliferation markers and the presence or absence of mitotic figures.

Materials and methods

Patients Between 1990 and 1994, patients admitted to the department of Neurosurgery of the University Hospital Utrecht for treatment of a primary (supratentorial) glioma, were asked to participate in cell kinetic studies involving BrdUrd infusion. Only those patients with evaluable tumor samples for LI_{fcm} and from whom paraffin-embedded surgical specimen were available, were included in this analysis. The prognostic relevance of cell proliferation markers was analysed in 27 patients (19 males and 8 females), with a mean age of 48 years (range 21-71). All patients had a minimum Karnofsky performance status of 80%.

Grading The histological slides of all 27 cases were reviewed (GHJ), in order to assess the correct histological diagnosis and grading, and the presence or absence of mitotic figures.
Grading of astrocytomas (n=23), oligodendrogliomas (n=3) and one mixed tumor was assessed, in accordance with the criteria of the revised WHO classification [17]. Seven astrocytomas were of low grade malignancy, i.e. six grade II and one grade I. Sixteen astrocytomas of high grade malignancy were diagnosed, i.e. three grade III and 13 grade IV. In three cases, an oligodendroglioma of highest malignancy grade was diagnosed. An oligo-astrocytoma of high grade malignancy was diagnosed in one patient.

Treatment After surgical intervention, patients were referred for radiotherapy. Radiation therapy consisted of external beam irradiation without concomitant or adjuvant chemotherapy. Patients with high grade gliomas (n=20) received whole brain irradiation followed by a boost, with a margin of 2 cm, to the primary tumor site. Fraction doses varied from 2.0-2.35 Gy, resulting in total doses to the primary tumor site of 60 and 56 Gy, respectively. In nine high grade glioma patients, the total radiation dose could not be given due to rapid tumor progression. The median local progression free survival (LPFS) of these nine patients was one month. Patients with low grade gliomas (n=7) received focal irradiation with a margin of 2-3 cm. Fraction doses varied from 1.8-2.0 Gy to total doses of 54-60 Gy, respectively. In two low grade glioma patients, it was decided not to administer postoperative radiotherapy. One of these patients developed progressive disease after 19 months. The other patient had a LPFS of 75 months after surgical treatment.

BrdUrd-labelling Preoperatively, 100 mg BrdUrd was administered intravenously as a bolus, in 5-10 minutes. After a minimum period of 30 minutes, fresh tumour samples were obtained and fixed in 70% ethanol for a minimum

of 24 hours. From these tumour samples, cell suspensions were made and analyzed by dual parameter FCM, resulting in LI_{fcm} and SPF. Another section was formalin fixed and embedded in paraffin and, thereafter, LI_{ihc} and MIB-1 positivity were determined by immunohistochemistry.

Flow cytometry

BrdUrd staining was performed by the method of Schutte et al. [18]. Prior to flow cytometric analysis, total DNA was stained by propidium iodide. A FACS-analyser (Becton Dickinson) flow cytometer was used. Fifty thousand cells were analysed from each tumor sample. DNA-histograms were analysed using an iterative program [19]. Triplets were counted and used to calculate doublets counts [20] and then to recalculate G_1PF, SPF, and G_2PF, taking into account the number of doublets and the measured number of triplets.
The green signal (BrdUrd detection) was log amplified. With dual parameter FCM, analysis SPF and LI_{fcm} were assessed. Only if a distinct separation between BrdUrd labelled and unlabelled population was obtained, the measurements were used in the analysis.

Immuno-histochemistry

A three step immunohistochemical staining technique was carried out immediately after preparation of the 4 µ sections. As primary antibodies we used a murine monoclonal antibody directed against BrdUrd (Boehringer, Mannheim) and a murine monoclonal antibody MIB-1 (Immunotech. S.A. Marseille) directed against Ki-67. Sections were then washed in PBS (both for BrdUrd and MIB-1 staining) and incubated with a second biotinylated horse-anti-mouse antibody, followed by washing in PBS and incubation with peroxidase labelled streptavidin. Finally, diaminobenzidine was used as a substrate for visualisation. Sections were counterstained with haematoxylin and mounted. Controls consisted of omitting the specific primary antibody or by replacing it with an anti-CD 34 antibody. Tumour specimens were scored positive (over-expression) when tumour nuclei showed strong positivity. Labelling positivity was scored by semi-automated videoplan analysis at a magnification of 40. The first microscopic field was selected within the tumour tissue, followed by a number of adjacent fields, until a total number of 1000 nuclei were counted. The immunohistochemically derived labelling index (LI_{ihc}) is expressed as:

$$LI_{ihc} = \frac{\text{number of positive nuclei}}{\text{number of positive + number of negative nuclei}} \times 100$$

Statistical analysis

At the time of the analysis, 20 patients had died due to tumour progression. Two other patients died after surgical treatment, but were at that time without tumour progression. It was decided to evaluate the prognostic significance of the cell proliferation parameters with respect to the local progression free survival (LPFS), instead of overall survival. The continuous variables (except SPF) all showed a skewed distribution and we, therefore, decided to transform them logarithmically. Differences between levels of a categorical variable were assessed by the log-rank test. The prognostic significance of continuous variables was assessed by Wald's test. For multivariate analysis, Cox proportional hazard regression analysis was performed with SPSS for Windows (version 6.01). For multivariate analysis, results are presented as p-values and relative risks (RR) with their 95% confidence interval (CI). We evaluated the influence of tumour grading (low grade versus high grade) on the prognostic significance of LI_{fcm} by the use of interaction terms.

Results

Local progressive disease was demonstrated unequivocally by CT or MRI scanning in 22 patients. At the time of analysis, 20 patients had died due to tumour progression. Two other patients died 1 month and 11 months after surgical treatment but were at that time free from tumour progression. Three remaining patients were found to be without signs of progressive disease 37, 63, and 75 months, respectively, after surgery. After univariate analysis, we found a significant effect of SPF, LI_{fcm}, and MIB-1-LI on LPFS (Table 1).

Cox regression analysis revealed that of the FCM cell proliferation markers, i.e. LI_{fcm} and SPF, significance was noted only for LI_{fcm} (data not shown). We then assessed, by regression analysis, the prognostic significance of LI_{fcm} and MIB-1-LI together. Prognostic significance was found only for LI_{fcm}, i.e. $p = 0.015$; RR 2.2 (Table 2). However, for LI_{fcm}, no additional prognostic significance, compared with that of the presence or abscence of mitotic figures, was demonstrated. For cells in mitoses a p-value of 0.002 with a RR of 10.7 was determined by regression analysis (Table 3). Using interaction terms, we found that the prognostic relevance of LI_{fcm} was significantly increased in low grade (versus high grade) gliomas ($p < 0.05$). However, the number of low grade gliomas (n=7) in our study was too small to perform a separate and reliable statistical analysis for this group.

Table 1 *Univariate analysis of SPF, LI_{fcm}, LI_{ihc}, MIB-1-LI, and the presence or absence of mitotic figures, with respect to the LPFS*

	Number of patients	Mean	P-value
SPF	22	0.045	0.04
LI_{fcm}	27	0.027*	0.02
LI_{ihc}	24	0.019*	0.92
MIB-1-LI	27	0.036*	0.05
Mitotic figures			
yes/no	17/10	na**	0.002

*geometric mean **not applicable

Table 2 *Cox regression analysis of LI_{fcm} and MIB-1-LI positivity, with respect to the LPFS*

n=24	Mean	P-value	RR	(95% CI)
MIB-1-LI	0.036*	ns**	na***	na***
LI_{fcm}	0.027*	0.015	2.2	(1.2-4.1)

*geometric mean **not significant ***not applicable

Table 3 *Cox regression analysis of LI_{fcm} and the presence or absence of mitotic figures, with respect to the LPFS*

n=27	Mean	P-value	RR	(95% CI)
LI_{fcm}	0.027*	ns**	na***	na***
Mitotic figures				
yes/no	na***	0.0001	10.7	(2.4-48.4)

*geometric mean **not significant ***not applicable

Discussion

The prognostic relevance of cell proliferation markers was evaluated in 27 glioma patients with a long term follow-up period. Cell proliferation markers were assessed by histological examination, flow cytometry, and immunohistochemistry.

After univariate analysis, we found that a longer LPFS was associated with low SPF, low LI_{fcm}, and low MIB-1-LI values. These findings are consistent with the reported prognostic potential of SPF, LI_{fcm} [8,12,13] and MIB-1-LI or Ki-67-LI [3,4,5,6]. In our study, no significant association of LI_{ihc} with LPFS was found. Similar results concerning the poor prognostic potential of LI_{ihc} in high grade glioma patients were reported by other authors. With respect to glioblastoma multiforme only, Ritter et al. [11] and Barker et al. [14] reported that LI_{ihc} was of little prognostic significance with regard to survival probability. By Cox regression analysis, we demonstrated that LI_{fcm} was the most promising prognosticator of the experimental proliferation markers. These findings indicate that S-phase related prognosticators (LI_{fcm}) are of more significance than non-G_0-phase related prognosticators (MIB-1-LI). The prognostic significance of LI_{fcm} appeared to be of minor importance however, when compared with that of the presence or absence of mitotic figures, representing the M-phase of the cell cycle.

We found, by the use of interaction terms, that the prognostic significance of LI_{fcm} was increased in low grade gliomas. In support of our observations Hoshino et al. [9] and Ito et al. [21] demonstrated that LI_{ihc} had prognostic value in low grade gliomas.

Conclusions

I In gliomas, cells in mitoses (M-phase) was of more prognostic significance than was LI_{fcm} (S-phase) or MIB-1-LI (non-G_0-phase fraction).

II Of the tested cell proliferation markers in gliomas, LI_{fcm} appeared to be of more prognostc significance than MIB-1-LI, SPF, and LI_{ihc}.

III LI_{fcm} is likely to be an important prognosticator in low grade gliomas.

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Chapter 8

General discussion

"Te weten wat men weet, en te weten wat men niet weet, dat is kennis."

Confucius

Assessment of proliferation markers and DI

Identification of S-phase cells, labelled with the (non-radioactive) thymidine analogue 5-bromo-2'-deoxyuridine (BrdUrd), has facilitated determination of cell proliferation markers in human tumours. After intravenous infusion, BrdUrd is incorporated into DNA of cells in S-phase. A tumour sample was taken some hours after BrdUrd infusion after which it was possible to determine the progression-rate of labelled cells through the cell cycle. DNA-index (DI), G_1-phase fraction (G_1PF), S-phase fraction (SPF), G_2-phase fraction (G_2PF), BrdUrd-labelling index (LI), duration of S-phase (T_s), and potential doubling time (T_{pot}) were assessed by means of flow cytometry (FCM).

T_s T_{pot} calculation

We used the standard approach of relative movement (RM) determination to assess T_s and T_{pot} [1]. The optimal sampling time (ST), between injection of the thymidine analogue BrdUrd and biopsy, however, is debatable. T_{pot} emerged with a twofold overestimation after a short ST and it was, therefore, recommended that ST should exceed T_{G2+M} [1]. However, T_{G2+M} is, a priori, unknown. A ST longer than T_{G2+M} leads to progression of labelled cells through G_2+M-phase into G_1-phase. These labelled divided cells usually are not correctly taken into account in the T_{pot} calculation method. LI will be overestimated, if the division of these labelled cells is not corrected for. Moreover, for a correct RM calculation, the number of divided labelled cells should be divided by two and counted as being still in G_2-phase. Thus, by not correcting for labelled cells that have passed through mitosis, LI values are overestimated and values of RM, as well as of T_s, are underestimated. Causing T_{pot} values, as these are calculated by T_s/LI, to be underestimated even more. The magnitude of these miscalculations, depends on the proportion of labelled divided G_1-phase cells. In our series, however, the fraction of divided labelled cells appeared to be limited. However, in a few clinical cases, we noted after a very short ST (15-30 minutes) labelled and, hence, not divided cells to be present in G_1-phase. Tumours with a relatively low proliferative activity may also exhibit labelled (not divided) G_1-phase cells, after longer time intervals. As a consequence, correction for labelled G_1-phase in these cases may affect the calculation results in a direction opposite to that was intended.

The mean ST of 4 hours, as applied in our study on head and neck tumours, was consistent with the recommended ST of 4-6 hours [1,2]. In our series, the mean ST of three hours in brain tumours may appear to be relatively short. However, malignant brain tumours, are generally characterised by very rapid growth and may therefore have a short

T_{G2+M}. The used ST, both for head and neck tumours and for brain tumours, were in accordance with the results of Höyer et al. [3] who demonstrated in experimental tumours that ST should be between 60% and 100% of the (expected) T_s.
With the more sophisticated T_{pot} calculation methods of White et al. [4] and Ritter et al. [5], the problem of the optimal sampling time remains unsolved. The approach of T_{pot} calculation by Durand et al. [6] was suggested not to be influenced by ST duration. However, all these alternative calculation methods of T_{pot} lack clinical evaluation.

We conclude that it is essential to determine the optimal T_{pot} calculation method in order to assess its prognostic relevance.

Study in progress

We have planned to further evaluate reliability of T_{pot} calculation methods obtained both by standard approach and by alternative approaches. Serial samples, of experimental and of human head and neck tumours will be analysed for that purpose.

In brain tumours, the success rates to assess T_s and T_{pot} were lower, when compared with those in head and neck tumours. In some tumours this was caused by an impaired ability to distinct low labelled cells from unlabelled cells. These low success rates are a major drawback for the clinical application of T_s and T_{pot} as prognosticators in brain tumours.

Regional heterogeneity and intra observer variability

For DI, we noted in head and neck tumours i) a low intratumoural variability, ii) a low intraobserver variability, and iii) a high intertumoural variability. We found that the regional heterogeneity of these tumours for LI, T_s, and T_{pot} was moderate, i.e. a a coefficient of variance (CV) of 20%, 17%, and 30%, respectively. With respect to the intraobserver variability, we found that the calculated CV for T_s and T_{pot} was 17% and 18%, respectively.

We conclude, from this, that DI as well as DNA-ploidy are suitable to serve as prognosticators in head and neck tumours.

The observed regional heterogeneity and the intraobserver variability for SPF, LI, T_s, as well as for T_{pot}, however, is moderate. It must be emphasized that both regional heterogeneity and intraobserver variability are, to some degree, even underestimated since our analyses were based on either diploid or aneuploid tumour samples of a specific case. Clinical use of each of these parameters seems reliable only if the level of its prognostic significance outweighs the level of its regional

heterogeneity and the intraobserver variability markedly. The ability to analyse more than one sample from one tumour may provide more information with respect to regional heterogeneity of a specific tumour.

From the literature, we found that the interobserver variability of flow cytometric analyses of LI and RM, by the use of the standard approach of Begg et al. [1], was substantial and it was concluded that this may largely be caused by a lack of standardization [7,8,9].

We conclude that, with respect to the T_{pot} calculation method as suggested by Begg et al. [1], standardisation of LI and RM calculations are essential for a reliable assessment of the clinical relevance of cell proliferation markers in multicentre studies and to adequately compare results between various laboratories.

Study in progress

We initiated the analysis of the interobserver (interlaboratory) variability of cell proliferation markers.

DNA-ploidy and proliferation markers

In our series, we found in DNA-aneuploid laryngeal tumours, when compared with DNA-diploid laryngeal tumours, a significantly higher mean LI value. Other authors also reported that in diploid tumours the proliferative activity is underestimated [10,11,12]. This is caused by the fact that, in the flow cytometric analysis of diploid tumours, the G_1-phase fraction of tumour cell is contaminated by G_1-phase normal cells. As a consequence in diploid tumours, this will lead to an underestimation of LI values in tumour cells. Apart from this, we found T_s to be shorter in diploid laryngeal tumours, i.e. the ratio of diploid to nondiploid was 0.6. However, only a trend towards significance for this difference was demonstrated in our series. Others reported similar, and statistically significant differences in this respect in head and neck tumours at various sites [12,13,14].

We conclude that

- *distinction of DNA-diploid tumour cells from DNA-diploid normal cells may provide a more reliable estimation of LI values in diploid tumours [15,16],*
- *with respect to analyses of the prognostic relevance of cell proliferation markers in head and neck tumours, DNA-ploidy should be taken into account.*

Repopulation

According to Trott et al. [17] in 1990, repopulation caused by accelerated proliferation of surviving tumour cells during a fractionated course of radiotherapy, should be regarded as a hypothetical mechanism to explain the increase in the radioresistance of tumours when overall treatment time is increased. Theoretically, it seems indicated to treat tumours, characterised by a short T_{pot} with an accelerated fractionation scheme, in an attempt to complete the course of radiotherapy before repopulation may adversely affect treatment efficacy [1,2,17,18,19].

In our laboratory, however, accelerated proliferation could not be observed in serial samples of experimental tumours. For this purpose, experimental tumours were analysed by BrdUrd-labelling at different time intervals, both after single dose irradiation and as well as during and after fractionated irradiation. It appeared that proliferative activity (LI, T_{pot}) remained significantly decreased until tumour regrowth occurred. At that time, the parameters of the proliferative activity returned to pre-irradiation control levels (personal communication D. Rutgers).

These findings are in line with the reported results of Speke et al. [20]. They measured T_{pot} as well as effective doubling time in four experimental and one human tumour during irradiaton. From this they stressed the need for caution in applying measurements of T_{pot} for prediction of regrowth rates in individual patients' tumours.

In the literature, no conclusive data exist with regard to the efficacy of cell proliferation markers in the prediction of the outcome of a radiation scheme in (human) head and neck tumours [2,13,21,22,23]. In our series, the clinical relevance of cell proliferation markers in head and neck tumours could not be demonstrated either.

The validity of the phenomenon of repopulation should be reconsidered, if it is confirmed that repopulation cannot be predicted using cell proliferation markers, including T_{pot}.

Theoretically, however, cell cycle time (T_c) better representates the proliferative potential of a tumour. After all, T_{pot} reflects kinetics of tumour cells, taking account both of proliferating fraction (P-fraction) and of quiescent fraction (Q-fraction) of cells, whereas T_c determination is based on P-fraction only. T_c can be estimated by the use of the parameters T_s, BrdUrd-LI (LI), and Ki-67-LI, following the formulae:

$$T_{pot} = T_s/LI = T_c/GF \text{ and hence } T_c = GF \cdot T_s/LI = GF \cdot T_{pot}$$

Ki-67-LI represents the fraction of the non-G_0 cells [24], i.e. the growth fraction (GF).

It seems appropriate to evaluate whether radioresistance can be explained by analysing the prognostic relevance of T_c. Clinical relevance was reported for T_c. Both in experimental and in clinical tumours, it was demonstrated that radiation-induced reassortment was caused by an arrest of cells in G_2-phase [e.g. 25,26]. Cell cycle time (T_c), as determined by autoradiography, was shown to govern the pattern of reassortment [26]. The presence of reassortment, during the first two weeks of treatment, was found to be the most powerful predictor of local recurrent disease in human oral cavity tumours [26].

We conclude that i) we could not demonstrate clinical relevance cell proliferation markers in head and neck tumours, ii) further study of the T_c related moment of reassortment to predict radiation treatment efficacy is feasable and indicated.

Study in progress

The T_c related moment of reassortment to predict radiation treatment efficacy in head and neck tumours will be evaluated.

T-stage and proliferative activity

To date, in studies evaluating the relevance of cell proliferation markers, locally advanced tumours at various sites were included [2,13,21,22,23]. These locally advanced tumours, however, comprised various T-stages. It must be emphasised that, with regard to head and neck tumours, it is generally accepted that both T-stage and tumour site are important prognosticators. In our series, we noted in laryngeal tumours that T_{3-4} stage tumours, as opposed to T_{1-2} stage tumours, were characterised by significantly lower mean LI values, significantly higher T_{pot} values and higher T_s values ($p=0.06$). These differences appeared to be independent of N-stage. In 1973, Hermens [27] was one of the first authors to report on the same phenomenon. He found in an experimental tumour lower LI values in large tumours, when compared with those in small tumours. Schultz et al. [28] found similar results for tumours, derived from 3 separate cell lines.

We conclude that in head and neck tumours stratification for T-stage and tumour site is necessary, to clarify in future studies whether repopulation can be predicted by estimating the proliferative activity of tumour cells.

Proliferative activity and tumour oxygenation

In accordance to the data of Schultz et al. [28], it was deduced that the decreased LI values in T_{3-4}-stage tumours might be determined by hypoxia. This is in support of the observation of Rutgers et al. [29], who observed that severely hypoxic cells in central necrotic areas of an experimental tumour, were proliferating but at a lower rate than cells in the viable periphery of the tumour. These observations are in full support of our finding that in more advanced T-stages the ratio of SPF to LI was higher. Moreover, we noted that high SPF/LI values were associated with an increased risk of recurrent locoregional disease. This ratio was regarded to reflect the extent of hypoxia. Apart from this, we and others found T_s to be shorter in diploid tumours in head and neck tumours [12,13,14]. Since the group of T_{1-2}-stage tumours had a higher fraction of diploidy, this phenomenon may reflect the fact that diploid tumours, when compared with aneuploid tumours, have a more favourable physiologic environment, i.e. no hypoxia.

We conclude that further study is needed to elucidate whether radioresistance head and neck tumours can be explained to some degree by the extend of hypoxia.

If hypoxia turns out to be the governing factor determining radioresistance [30], fractionated radiotherapy and hyperthermia should be a effective treatment modality. In head and neck tumours promising results of this combined treatment modality have been reported [30]. However, a cooling effect of large blood vessels was noted.

DNA-index and prognosis

In our series, DNA-diploid tumours appeared to be more radiosensitive. Also according to the literature, patients with DNA-diploid tumours and treated by radiation therapy had a lower probability of loco-regional recurrent disease [31,32,33,34,35,36,37]. Alternatively, DNA-aneuploidy (and DNA-tetraploidy) may relate to the degree of hypoxia, since the proportion of DNA-aneuploidy is higher in the more hypoxic T_{3-4} tumours, when compared with that in the more radiosensitive T_{1-2} tumours. Moreover, T_s was reported to be shorter in (more radiosensitive) diploid tumours [12,13,14], which is in support of our observations and which may be regarded to be related to a better oxygenation status.

We conclude that the evaluation on the prognostic relevance in head and neck tumours of DNA-index (focussed on DNA-tetraploidy also), T_s, and SPF/LI needs further evaluation.

Proliferation markers and DI in brain tumours

Tumour characterization

In adults, long-term survival probability in low grade glioma patients is low [38]. We demonstrated that gliomas with histologically benign characteristics had the same proportion of malignant FCM features, as in histologically malignant brain tumours. These malignant FCM features consisted of

- a high percentage of DNA-aneuploidy,
- a high mean DI (for DI>1),
- a high proliferative capacity.

We conclude that low grade glioma is a malignant disease.

Furthermore, we found a significant lower proliferation rate (SPF, LI, T_{pot}) in diploid benign tumours, than in diploid malignant tumours. In aneuploid tumours, these differences appeared to be less pronounced.

Study in progress

The prognostic relevance of cell proliferation markers in histologically benign brain tumours of our study will be evaluated as soon as the follow-up period of these patients is sufficiently long.

Prognostic relevance

In adults, the prognostic relevance of clinically, histologically, and flow cytometrically derived parameters was assessed in high grade gliomas. The evaluation of the prognostic relevance of cell proliferation markers when specifically compared with that of the presence or absence of cells in mitoses, has not been reported in the literature. Cytomorphological features of mitoses, necrosis, and endothelial proliferation were, therefore, analysed separately. We found, after multivariate analysis, that both for necrosis and for endothelial proliferation no additional prognostic relevance to that of the presence or absence of cells in mitoses was noted. Additional prognostic significance was noted neither for cell proliferation markers determined by flow cytometry, i.e. SPF, LI, T_{pot} nor for those determined by immunohistochemistry, i.e. LI, MIB-1-LI.

We conclude that, in adults, the presence or absence of cells in mitoses, is the most important, single, prognosticator in gliomas.

In our study, the prognostic relevance of cell proliferation in low as well as in high grade gliomas was clearly demonstrated. The growth of a tumour, however, depends upon the balance between cell proliferation and cell death. The underlying mechanism of cell death, however, is one of two processes: necrosis or apoptosis. The most important difference between necrosis and apoptosis is that apoptosis is a

process which is regulated actively by genes. Further study is needed. Theoretically, however, the experimental cell proliferation markers should be expected to be of more prognostic relevance than the presence of mitotic cells. This is related to that SPF, LI, or $S+G_2+M$-phase fraction of cells should better reflect the proliferative potential than the presence or absence of cells in mitoses. Apparently, in high grade gliomas, the lack of space for expanding tumour growth is less restrictive than in low grade gliomas. In high grade tumours, with cells in mitoses, tumour cells are apparently capable of infiltrative growth on the expence of the surrounding tissue. We assume that this feature may also relate to hypoxia, since high grade gliomas are, in the majority of cases, characterised by extensive necrosis.

We conclude that, it is appropriate to investigate the phenomena of apoptosis and necrosis in gliomas.

Furthermore, the cytomorphologically obtained feature of endothelial proliferation reflects the presence or absence of abnormal blood vessels but does not reflect proliferation. With respect to its clinical relevance, it must be emphasised that quantification of microvessel density after immunohistochemically vessel staining with factor VIII may enable a better prediction of prognosis [39,40].
In our series, no prognostic relevance was noted for DNA-ploidy.

We conclude that further study is needed to elucidate whether DI has prognostic relevance in gliomas.

Finally, in our series we demonstrated that LI was of prognostic relevance in the subgroup of low grade gliomas. These data are in line with the reported observations based on immunohistochemistry [41,42].

We conclude that further study is indicated to confirm prognostic relevance of LI in low grade gliomas.

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Chapter 9

Summary

and summary in Dutch

Summary

In this thesis we report about some aspects of the clinical relevance of cell proliferation markers and DNA-index for patients with either a head and neck tumour or a brain tumour. The objective of this study was explained to 500 patients. Ultimately 278 agreed to participate. Cell proliferation markers and DNA-index were obtained by flow cytometry.

In *Chapter 2,* we analysed the interrelationships between DNA-ploidy, cell proliferation markers, T-stage, and N-stage in 64 single site head and neck tumours, i.e. the larynx. We found that locally advanced (T_{3-4}) laryngeal tumours were characterised by a significantly higher percentage of DNA-aneuploidy. This finding confirms the reported poor prognosis in DNA-aneuploid tumours. A significantly higher proliferative potential, i.e. a higher mean LI and a lower mean T_{pot} value was found in early stages (T_{1-2}), as opposed to that in locally advanced (T_{3-4}) tumours. This difference in proliferative activity was not related to N-stage. We stated that the tumour stage related decrease of the proliferation rate, might relate to an increased level of hypoxia in the larger tumours.
These findings may in part explain the reported poor prognostic relevance of cell proliferation markers in head and neck tumours and imply that the predictive value of (LI and) T_{pot} should be evaluated only after stratification for T-stage (T_{1-2} versus T_{3-4}). A higher mean LI value was found in DNA-aneuploid tumours. This emphasises the need for distinction of DNA-diploid tumour cells from DNA-diploid normal cells. We found that T_s tended to be longer in DNA-aneuploid tumours ($p=0.07$).

In *Chapter 3*, we reported on the prognostic significance of proliferation markers and DNA-index in 103 patients with head and neck squamous cell carcinomas, treated with curative intent. Treatment regimen consisted of radiotherapy alone, surgery and radiotherapy, or surgery alone. A conventionally fractionated scheme was administered in the case of radiotherapy. We demonstrated that cell proliferation markers (including T_{pot}) were unrelated to loco-regional recurrence probability. However, we noted that high values of the ratio SPF to LI, were associated with an increased rate of recurrent disease. It was postulated that this ratio might be indicative for the size of the hypoxic fraction. This may, at least in part, explain the absence of prognostic relevance of the cell proliferation markers in laryngeal tumours and imply that the predictive value of (LI and) T_{pot} should be evaluated only after stratification for T-stage (T_{1-2} versus T_{3-4}).
The reported prognostic significance for DNA-index was confirmed and

appeared to be related to the poor prognosis associated with DNA-aneuploidy and specifically DNA-tetraploidy. Furthermore, we found that the probability of loco-regional recurrent disease was significantly decreased in primarily irradiated DNA-diploid tumours.

Cell proliferation markers are generally estimated from a single biopsy. Both regional heterogeneity and intraobserver variability may interfere with the reliability of the parameter determination. In *Chapter 4*, we determined the intratumoural, intertumoural and intraobserver variability of proliferation markers and DNA-index in head and neck tumours. We found that, i) with regard to regional heterogeneity, DNA-ploidy status and DI showed less variation than did LI, and SPF, ii) the ratio of the intratumoural variability to the intertumoural variability was lowest for DI, iii) the regional heterogeneity for T_s and T_{pot} was moderate, iv) the intraobserver variability was low for DNA-ploidy status, DI, and LI, and moderate for RM. We thus concluded that, in head and neck tumours, the conditions for DI as a prognosticator are better than those for SPF, LI, G_1PF, T_s, and T_{pot}.

In *Chapter 5*, brain tumours were characterised with respect to differences in DNA-index and cell proliferative potential. We noted that malignant brain tumours, as opposed to benign brain tumours, were characterised by malignant FCM features, such as i) a significantly higher proportion of DNA-aneuploid tumours, ii) a significantly higher mean DI (for DI>1), and iii) a significantly higher proliferative activity (SPF, LI, and T_{pot}), as noted for diploid tumours only. T_s of benign and malignant tumours appeared to be relatively short, i.e. 4.5 hours, and not to differ between benign and malignant brain tumours. From this, it follows that the differences in T_{pot} were due mainly to differences in LI. Low grade gliomas were, according to the literature, found to be characterised by low (long-term) survival probability and malignant FCM features. Therefore, low grade gliomas should be considered as a malignant disease.

In *Chapter 6*, we analysed the prognostic relevance of clinically, histologically, and flow cytometrically derived parameters in 49 glioma patients after long-term follow-up. We noted that LI and T_{pot} could be adequately obtained in 71% and 63% of the tumours, respectively. The success rate of LI and T_{pot} determination was higher in head and neck tumours. In glioma however, we found that LI and, hence, T_{pot} could not be adequately obtained in a number of cases, due to very low labelling indices.

Seizures, duration of symptoms, and age appeared to be associated with cell proliferation rate and were found to be associated with prognosis. SPF, LI, and T_{pot} were also associated with prognosis. However, by regression analysis, no additional prognostic relevance, compared with that of the presence or absence of cells in the mitoses, could be demonstrated. After multivariate analysis of established prognosticators such as necrosis, endothelial proliferation, and mitoses, we found the presence or absence of cells in mitoses to be the strongest single prognosticator. Prognostic significance of LI was noted in the subgroup of low grade gliomas.

In *Chapter 7*, we evaluated in 27 glioma patients, the prognostic significance of tumour cell proliferation markers obtained by flow cytometry (FCM), i.e. SPF and LI_{fcm}, by immunohistochemistry (IHC), i.e. MIB-1-LI and LI_{ihc} and by histologic examination i.e. of the presence or absence of cells in mitoses. Long term follow-up was achieved. After multivariate analysis, we found the presence of mitotic cells, to be of more prognostic significance than LI_{fcm}, or MIB-1-LI. Of the tested cell proliferation markers, LI_{fcm} appeared to be of more prognostic importance in gliomas than MIB-1-LI, SPF, and LI_{ihc}. From this, it was concluded that LI_{fcm} may be of prognostic significance, specifically in low grade gliomas, since cells in mitoses are by definition not found in low grade gliomas.

Samenvatting

In dit proefschrift wordt gerapporteerd over een aantal onderwerpen welke betrekking hebben op de klinische relevantie van celproliferatie-markers en DNA-index bij patiënten met een hoofd/halstumor of een hersentumor. Aan 500 patiënten werd uitleg gegeven over het doel van de studie en uiteindelijk bleken 278 van hen bereid te zijn tot deelname aan deze studie. De celproliferatie-markers en DNA-index werden bepaald met flowcytometrie.

In *Hoofdstuk 2* wordt gerapporteerd over de onderlinge relatie van de DNA-ploïdie status (diploïdie versus aneuploïdie), de DNA-index (DI), de celproliferatie-markers, de T-status en de N-status van 64 larynxtumoren. Bij de "locally advanced" (T_{3-4}) tumoren bleek er sprake te zijn van een significant hoger percentage DNA-aneuploïdie. Deze bevinding komt overeen met de in de literatuur gerapporteerde slechtere prognose van DNA-aneuploide tumoren. Vervolgens werd vastgesteld dat er sprake was van een significant grotere proliferatieve activiteit (een hogere BrdUrd-labelingsindex (LI) en een kortere potentiële verdubbelingstijd (T_{pot})) bij kleine tumoren (T_{1-2}), vergeleken met de proliferatieve activiteit van grote tumoren (T_{3-4}). Dit verschil bleek niet gerelateerd te zijn met de N-status. De afname van de proliferatieve activiteit, welke dus alleen afhankelijk bleek te zijn van de grootte van de primaire tumor, werd verklaard door een grotere mate van hypoxie in grotere tumoren. Op grond hiervan concludeerden wij dat deze bevindingen, althans voor een deel, een verklaring kunnen zijn voor het feit dat er, tot heden, geen duidelijke prognostische betekenis kon worden aangetoond voor de celproliferatie-markers van hoofd/halstumoren. De consequentie van deze bevindingen is dan ook dat, in toekomstige studies, de prognostische betekenis van LI en T_{pot} geëvalueerd moeten worden nadat gestratificeerd is voor de T-status (T_{1-2} versus T_{3-4}). Voorts bleek dat de LI van DNA-aneuploïde tumoren significant hoger was dan de LI van DNA-diploïde tumoren. Dit alles benadrukt de noodzaak om DNA-diploïde tumorcellen te onderscheiden van DNA-diploïde normale cellen, zodat de LI van DNA-diploïde tumoren op meer betrouwbare wijze kan worden bepaald. Tenslotte stelden wij vast dat de duur van de S-phase (T_s) van de DNA-aneuploïde tumoren, langer leek te zijn dan die van de DNA-diploïde tumoren (p=0.07).

In *Hoofdstuk 3* wordt gerapporteerd over de prognostische betekenis van de celproliferatie-markers en de DNA-index bij 103 patiënten met een plaveiselcelcarcinoom in het hoofd/hals gebied en behandeld met curatieve opzet. De patiënten werden behandeld met òf alleen radiotherapie, òf alleen chirurgie, òf chirurgie en radiotherapie. Wij toonden in onze studie aan, dat er geen relatie was tussen de celproliferatie-markers (inclusief T_{pot}) enerzijds en de kans op een locoregionaal recidief anderzijds. Bij hoge waardes van de ratio van de fractie S-fase cellen (SPF) en de LI werd echter gevonden dat er een grotere kans was op een locoregionaal recidief. Deze ratio werd als indicatief beschouwd voor de grootte van de hypoxische fractie van de tumor. Anders gezegd: de proliferatieve activiteit van tumorcellen wordt verlaagd door hypoxie. Hierdoor wordt, althans gedeeltelijk, verklaard waarom er tot heden geen prognostische betekenis kon worden aangetoond voor celproliferatie-markers van hoofd/halstumoren. Vervolgens bleek dat de prognostische betekenis van de DNA-index bevestigd kon worden. Met name DNA-tetraploïdie bleek geassocieerd te zijn met een slechte prognose. Tenslottte werd vastgesteld, dat de kans op een locoregionaal recidief significant lager was bij de (primair bestraalde) DNA-diploïde tumoren, vergeleken met DNA-aneuploïde tumoren.

Celproliferatie-markers worden in het algemeen bepaald nadat slechts één tumorbiopt is verkregen. De klinische relevantie van deze markers kan beïnvloed worden door zowel de regionale heterogeniteit binnen één tumor, als door de "intraobserver" variabiliteit. In *Hoofdstuk 4* werden, bij hoofd/halstumoren, de intratumorale, de intertumorale en de "intraobserver" variabiliteit van celproliferatie-markers en de DNA-index geëvalueerd. Vastgesteld werd dat i) de regionale heterogeniteit van zowel de DNA-ploïdie-status alsmede de DI veel kleiner was, dan de regionale heterogeniteit van de LI, SPF, T_s en T_{pot}, ii) de ratio van de intratumorale variabiliteit en de intertumorale variabiliteit het laagst was voor de DI, iii) de regionale heterogeniteit van T_s en T_{pot} respectievelijk 17% en 30% was, iv) de "intraobserver" variabiliteit gering was voor de DNA-ploïdie-status, de DI en de LI en dat de "intraobserver" variabiliteit van de "relative movement" (RM) van de met BrdUrd gelabelde (S-fase) cellen significant verschillend was. Vanwege de betere representativiteit en reproduceerbaarheid van de DI bepaling werd geconcludeerd dat de DI (en de DNA-ploïdie-status), vergeleken met de SPF, de LI, de G_1PF, de T_s en de T_{pot} beter geschikt lijkt te zijn als prognostische factor.

Hersentumoren werden, in *Hoofdstuk 5*, gekarakteriseerd door verschillen in DNA-index en proliferatieve activiteit. Wij stelden, met behulp van flow cytometrie (FCM), vast dat kwaadaardige hersentumoren, in vergelijking met goedaardige hersentumoren, gekarakteriseerd worden door de volgende FCM kenmerken van maligniteit i) een significant hoger percentage DNA-aneuploïde tumoren, ii) een significant hoger gemiddelde waarde van de DI (voor DI>1), iii) een significant hogere proliferatieve activiteit (SPF, LI en T_{pot}), hetgeen, vanwege het kleine aantal aneuploïde goedaardige tumoren, alleen voor de DNA-diploïde tumoren kon worden aangetoond. De proliferatieve activiteit van DNA-aneuploïde kwaadaardige en goedaardige bleek niet significant verschillend te zijn. De T_s was van relatief korte duur (4.5 uur) en bleek hetzelfde te zijn bij goedaardige en kwaadaardige tumoren. Op grond hiervan werd geconcludeerd, dat de verschillen van de gemiddelde T_{pot} waardes tussen de twee groepen hersentumoren voornamelijk veroorzaakt worden door verschillen in LI. Histologisch maligne kenmerken zoals mitosen, necrose en endotheelproliferatie ontbreken bij laaggradige gliomen. Op grond van literatuur gegevens werd vastgesteld dat dergelijke patiënten slechts een geringe kans hebben op een langdurige overleving. Met flowcytometrie werd aangetoond dat laaggradige gliomen, vergeleken met kwaadaardige hersentumoren, in dezelfde mate (FCM) kenmerken van maligniteit vertoonden. De conclusie was dan ook dat een laaggradig glioom beschouwd moet worden als een maligne ziekte met een grote kans op een uiteindelijk fatale afloop.

In *Hoofdstuk 6* wordt de prognostische betekenis geanalyseerd van klinisch, histologisch en flowcytometrisch bepaalde parameters bij 49 patiënten met een glioom, na langdurige follow-up. De LI en de T_{pot} konden worden bepaald bij respectievelijk 71% en 63% van de patiënten. De kans op succes bij de bepalingen van LI en T_{pot} was hoger bij de hoofd/halstumoren. De LI, en dus ook de T_{pot}, kon bij een aantal gliomen niet worden bepaald vanwege zeer lage BrdUrd-labelingsindices (LI).
Zowel de aan- of afwezigheid van epilepsie, de duur van de klachten als eerste symptomen van de ziekte als de leeftijd van de patiënt bleken geassocieerd te zijn met zowel de celproliferatie als met de prognose. De SPF, de LI en de T_{pot} bleken ook gerelateerd te zijn met de prognose. Echter, na regressie-analyse, kon geen extra prognostische betekenis worden vastgesteld voor de celproliferatie-markers en de leeftijd, naast de invloed van de aan- of afwezigheid van mitosen. Na

multivariate analyse van erkende prognostische factoren, zoals necrose, endotheelproliferatie en mitosen, werd vastgesteld dat de aan- of afwezigheid van cellen met mitosen de sterkste enkelvoudige prognostische factor was. In de subgroep van laaggradige gliomen, evenwel, werd de prognostische betekenis van de LI duidelijk aangetoond.

In *Hoofdstuk 7* werd bij 27 patiënten met een glioom de prognostische betekenis geëvalueerd van diverse celproliferatie-markers. De SPF en de LI_{fcm}, werden bepaald met flowcytometrie (FCM), de MIB-1-LI, (waarmee alle prolifererende cellen oftewel de groeifractie worden aangekleurd), en de LI_{ihc} werden bepaald met immunohistochemie (IHC), en de aan- of afwezigheid van cellen in mitosen werd bepaald na histologisch onderzoek. Langdurige follow-up werd bereikt. Na multivariate analyse bleek dat de aanwezigheid van mitotische cellen van grotere prognostische betekenis was dan de LI_{fcm} of de MIB-1-LI. Van de FCM en IHC celproliferatie-markers bleek, dat de LI_{fcm} een grotere prognostische betekenis had dan de MIB-1-LI, de SPF en de LI_{ihc}. Op grond van de resultaten van deze studie werd geconcludeerd dat de LI_{fcm} van prognostische betekenis zou kunnen zijn bij laaggradige gliomen omdat cellen met mitosen hier per definitie niet worden gezien.

Dankwoord

"Er zou weinig van mij overblijven indien ik alles moest afstaan, wat ik aan anderen te danken heb."

Goethe

Dankwoord

Ook deze promotie werd voorafgegaan door jarenlange noeste arbeid. Ik heb dit echter met veel plezier gedaan. Maar tegelijkertijd realiseer ik mij dat dit allemaal niet mogelijk was geweest zonder de hulp van een groot aantal mensen. Graag wil ik van de gelegenheid gebruik maken om een ieder die een bijdrage heeft geleverd aan de totstandkoming van dit proefschrift te bedanken. In eerste instantie verdienen alle *patienten*, die hun toestemming hebben gegeven om mee te werken aan dit onderzoek, een woord van dank. Hartelijk dank, dus. Een aantal personen wil ik graag (in chronologische volgorde) met naam en toenaam noemen.

Samen met *Derk Rutgers* werd, na langdurige discussies, de onderhavige studieopzet uitgedacht, op papier gezet en uiteindelijk gestart in 1989. Het motto was steeds "een goed begin is het halve werk". Derk bedankt!

De *medewerkers van de apotheek* van het AZU wil ik graag bedanken omdat zij, na een telefoontje mijnerzijds, het BrdUrd steeds tijdig, om half vier des namiddags dus, hadden geprepareerd. Vele malen (circa 300 keer) dank!

De *verpleegkundigen en arts-assistenten van de afdelingen Neurochirurgie, Neurologie, KNO, en Mondheelkunde* ben ik zeer dankbaar voor hun inzet. Met name de arts-assistenten van de KNO verdienen een extra woord van dank omdat zij, ten behoeve van het onderzoek, vele malen vroegtijdig (in de avonduren dus) een infuus hebben ingebracht. Vervolgens werd het "zakje met BrdUrd" in de nachtelijke uren aangesloten door de vepleegkundigen. Door deze extra inspanningen was het mogelijk om de patient met het BrdUrd en voorzien van het formulier te laten vertrekken in de richting van de OK. Hartelijk dank!

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Eerst *Marion Klooster* en later *Trudie Wieman* en *Dorien Rooth* zorgden er voor dat biopten van de OK naar het Radiobiologisch laboratorium van onze afdeling werden vervoerd. Hartelijk dank voor jullie inspanningen!

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Cees Imhof, samen hebben wij urenlang geprobeerd om duidelijke, fraaie en verantwoorde tabellen en figuren te maken. Hartelijk dank daarvoor (en voor het appelgebak)!

Derk, samen met jouw en met gebruikmaking van alle moderne "faxiliteiten" werd het mogelijk om de concepten van de zes studies vele malen heen en terug te faxen en ze daarna op (proef)schrift te stellen. Hartelijk dank voor jouw zeer grote aandeel in dit geheel!

Ook *Ingrid van Dijk*, secretaresse op de afdeling Radiotherapie van het AZU, verdient een (en waarschijnlijk wel veel meer dan één) woord van dank voor al haar inspanningen!

I would like to thank *Sally* from London (UK) for her assistance in correcting and, thereby, improving the quality of the (English) text. From here to you, many happy BrdU!

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Mijn paranymphen, *Vera de Ru* en *Wopke van der Lei*. Jullie zijn een leuk, humoristisch, enthousiast, dynamisch etcetera duo. Hiermee hebben jullie het paranymphenschap duidelijk gestalte gegeven! Bedankt!

Tot slot dank aan alle trouwe supporters van het thuisfront. *Irene, Bram, Simone, Frederieke*, dank voor het geduld dat jullie in deze periode hebben opgebracht!

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 8 november 1951 te Drachten. Het HBS-B diploma werd behaald in 1969 (Ichthus College, Drachten). In september 1969 begon hij met de studie Geneeskunde aan de Rijksuniversiteit te Groningen. Deze studie werd met het behalen van het artsexamen, afgerond in oktober 1975. Hij volgde de opleiding tot huisarts van april 1976 tot april 1977. Hierna besloot de schrijver dezes om geen huisarts te worden. Van mei 1977 tot augustus 1979 werkte hij als arts-assistent geneeskundige in opleiding op de afdeling Interne Geneeskunde van het Diaconessenhuis te Leeuwarden (opleider Dr. M. Leemhuis). Hierna besloot de schrijver dezes om geen internist te worden. Van 1 augustus 1979 tot 1 augustus 1983 werd hij in het Academisch Ziekenhuis te Leiden opgeleid tot radiotherapeut (opleider Prof. P. Thomas). Op 1 augustus 1983 volgde inschrijving in het Specialisten Register. Vanaf 1 augustus 1983 is hij als radiotherapeut werkzaam op de afdeling radiotherapie van het Academisch Ziekenhuis te Utrecht.

Stellingen

behorend bij het proefschrift

"Prognosis in brain and head & neck tumours.
Clinical relevance of cell proliferation markers and DNA-index".

1 De ratio van de fractie S-fase cellen (SPF) en de BrdUrd-labelingsindex (LI) is indicatief voor de hypoxische fractie van een tumor.
dit proefschrift

2 Voorspelling van versnelde proliferatie met behulp van de potentiële verdubbelingstijd is niet van klinische betekenis bij hoofd/halstumoren.
dit proefschrift

3 In toekomstige studies naar de waarde van de celproliferatie, ter voorspelling van de effectiviteit van bestraling van hoofd/halstumoren, dient gestratificeerd te worden voor de T-status van de tumor.
dit proefschrift

4 Bij gliomen is de prognostische betekenis van celproliferatie-markers groot; dit geldt met name voor de parameter welke betrekking heeft op de M-fase van de celcyclus.
dit proefschrift

5 Het laaggradig glioom bij de volwassene is een ziekte met een grote kans op een uiteindelijk fatale afloop; zowel de patiëntinformatie als het beleid dienen hierop te worden afgestemd.
dit proefschrift

6 Bij het laaggradige glioom is de BrdUrd-labelingsindex (LI) van grote prognostische betekenis.
dit proefschrift

7 Waarschijnlijk is de duur van de celcyclus, in tegenstelling tot de potentiële verdubbelingstijd, wel van prognostische betekenis bij hoofd/halstumoren.

8 De mate van significantie van wetenschappelijk onderzoek wordt aangeduid met de p-waarde, de klinische relevantie evenwel wordt bepaald door het relatieve risico.

9 Two forms of the "Consensus Syndrome" are recognized, i) the milder form: "Disputes are resolved not by the scientific process of identifying and repairing methodological problems but by a form of political process: the majority vote of a panel of selected authorities", ii) the more severe form: "The process extends from politics to religion".

A. Feinstein. Fraud, distortion, delusion, and consensus: the problems of human and natural deception in epidemiological studies. Am.J.Med. 84:475-478 (1988).

10 Naar analogie van het beleid bij andere ernstige besmettelijke ziektes, dient bij verdenking op seropositiviteit van het AIDS-virus nader onderzoek te worden verricht en dient de uitslag aan de betrokkene te worden meegedeeld.

11 Rugpijn veroorzaakt door kanker wordt te vaak te laat ontdekt.

12 De weerstand van patiënten tegen deelname aan studies zal aanmerkelijk verminderen, indien resultaten van klinisch wetenschappelijk onderzoek op meer genuanceerde wijze in de publiciteit worden gebracht.

13 Het gezegde "Geen woorden maar daden" dient, gezien het veranderde tijdsbeeld, te worden vervangen door "Eerst overleg dan daden".

14 Het bestaande budgetteringsbeleid van ziekenhuizen heeft tot gevolg dat de wachttijden voor diagnostiek en behandeling in het begin van een boekhoudkundig jaar korter zijn.

15 In het kader van maatregelen, die beogen de achterstandspositie van vrouwen en minderheden op te heffen, verdient het aanbeveling in het schaakspel de rol van koning en dame te wisselen en de zwartspeler de beginzet te gunnen.

16 Het rendement van stellingen, die niet verdedigd kunnen of mogen worden, is twijfelachtig.

Utrecht, 11 februari 1997

Hendrik Struikmans