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Prognostic Significance of Intratumour Microvessel Density and Haemoglobin Level in Carcinoma of the Uterine Cervix

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The prognostic significance of intratumour microvessel density (IMD) and haemoglobin (Hb) level was studied in 152 (FIGO stage IB–IIIB) cervical cancer patients before radiotherapy. Patients' age and tumour stage, grade and degree of keratinization were also studied. IMD measurement expressed as the mean vessel count per 1 mm^2 was performed on formalin-fixed, paraffin-embedded tumour biopsies stained with anti-factor VIII antibody (DAKO Ltd.) using immunohistochemistry and the vascular hot-spot technique. The median age of patients was 55 years (29–80). Median values for IMD and Hb level were: 142.5 (range 56.3–476.6) vessels/mm^2 and 129 (range 81–160) g/l, respectively. The median time of follow-up was 26 months, with a range of 2–145 months. Tumour stage ($p = 0.7009$), grade ($p = 0.6660$) and degree of keratinization (0.2669) were not significant in the Kaplan–Meier univariate analysis. However, patients' age > 50 years ($p = 0.0079$), high vascularity (IMD > 190.0 vessels/mm^2, minimal cut-off point, $p = 0.0503$) and Hb concentration > 116 g/l ($p = 0.0213$) were favourable prognostic factors for cervical cancer treated with radiotherapy. In the Cox multivariate analysis only higher vascularity (IMD > 190/mm^2 and Hb concentration > 116 g/l were favourable prognostic factors in terms of patients' survival. However, when a Cox analysis was done separately for keratinizing and non-keratinizing tumours, it was found that higher vascularity was significant only for keratinizing, and higher Hb level only for non-keratinizing cancers.

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Many tumour biological parameters are associated with tumour control; therefore, some prognostic factors are heterogeneous even in the same tumour type. The best example of this is cervical carcinoma. Many factors have been considered as possible indicators of prognosis for patients with carcinoma of the cervix. These clinical parameters include clinical stage and volume, lymph node involvement, histological grade and patients' age, while biological parameters include tumour proliferation rate, intrinsic radiosensitivity, vascular density, Hb level and apoptotic index. From these, a consensus has emerged that clinical stage and tumour volume are of key importance, whereas tumour differentiation, age, tumour proliferation, vascular density, Hb concentration and apoptosis are sometimes conflicting. In some studies it was shown that a highly proliferative rate of cervical tumours presents a significant prognostic factor (1, 2), but other authors obtained the opposite results (3). On the basis of clonogenic tests, a correlation was found between cell intrinsic radiosensitivity and tumour control. Patients with radioresistant tumours (surviving fractions of 2 Gy–SF2 above the median value) who received radiotherapy had a survival rate significantly lower than 3 years and poorer local control than those with sensitive tumours (SF2 below the median) (4). However, no one was able to confirm these data. Furthermore, the results concerning hypoxia are discordant, and this may not be caused only by the different methods used.

Up until now, advanced squamous cell carcinoma of the uterine cervix is usually treated with radiation alone. Local or regional recurrence is frequently the reason for treatment failure caused by local advancement of the tumour. Some studies have suggested that the outcome of radiotherapy in cervical carcinoma depends on the oxygen tension (pO_2) in the tumour tissue (5). Therefore, tumour hypoxia may be an important factor in predicting relapse following radiotherapy, as hypoxia is a common feature of many human tumours.

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Tumours cannot grow beyond approximately 1–2 mg in size without neovascularization (6). During tumour growth, the capillary network develops in architectural and functional abnormalities that result in insufficient blood supply and the gradual development of a pathological microenvironment (7). The microenvironment of most tumours is characterized by low glucose concentrations, high lactate concentrations, low extracellular pH and low oxygen tensions. Malignant cells produce an angiogenetic factor, a vascular endothelial growth factor (VEGF), which may increase metastatic ability and is up-regulated in the presence of hypoxia. Many methods have been used to study the degree of hypoxia in this type of tumour. For example, vascular density (i.e. the proportion of blood vessels in the stroma) (8), inter-capillary distance (ICD) (8, 9), intratumour microvessel density (IMD) counts performed on histological cross-sections from a tumour stained with anti-factor VIII (10, 11) and percentage of endothelial cells after Trichrome staining (12). The nitroheterocyclic hypoxia marker EF5, detected by fluorescent monoclonal antibodies to the drug adducts (13), or the VEGF mRNA level (14, 15) is also applied. Other indirect methods of measuring oxygen level include positron emission tomography (PET) (16), or vascular density by colour Doppler ultrasound (17). Eppendorf pO_2 histograph is considered to be a reliable pO_2 reading in human tumours in vivo (7, 18, 19). However, as was shown recently, different methods operate in different ways and may provide substantially different results (20). For example, EF5 binding provides detailed spatial information on the distribution of hypoxia in viable tumour tissue. There was no EF5 binding in necrotic tumour tissue, because cells in such tissue were unable to metabolize the drug. In contrast, the results from the needle electrode appeared to represent an average tissue pO_2 (along the track) and did not distinguish between extreme hypoxia and either macroscopic or microscopic necrosis. Current work on hypoxia incorporates several techniques to obtain a comprehensive two-dimensional mapping of the relationships between tumour vasculature, oxygen transport and development of hypoxia (21). Siracka et al. (12) and Revesz et al. (22) found higher vascularity to be predictive of patients' survival, and Schlenger et al. (23) and Cooper et al. (10, 11) have shown that high tumour angiogenesis is associated with poorer survival. Therefore, the aims of this study were to assess the vascular density and Hb level in advanced squamous cell carcinoma (SCC) of the cervix, and to test the correlation of these parameters with patient survival after radiotherapy.

MATERIAL AND METHODS

Patients

The study included 152 patients with FIGO stage IB–IIIB SCC of the cervix treated with radiotherapy at the Centre of Oncology in Krakow from 1987 to 1999. Tumour staging was done using the International Federation of Gynecology and Obstetrics (FIGO) system. Nine patients had stage IB carcinoma, 120 patients had stage IIA & B, and 26 patients had stage III (III A & B) carcinoma. Tumour grades 1 (well differentiated), 2 (moderately differentiated) and 3 (poorly differentiated) were assessed by a pathologist according to the generally accepted pathological criteria (24). Thirty squamous cell cancers were assessed as grade 1, 66 as grade 2, and 23 as grade 3. In this series, there were 80 keratinizing and 36 non-keratinizing tumours. For 36 tumours this assessment was not available. The study had full ethical approval and patients gave prior informed consent.

Irradiation

Patients with SCC of the cervix at stages IB–IIA had three intracavitary applications of 13.9 Gy each, to point A using ^{137}Cs irradiation (Selectron LDR) at 4–5-day intervals. After a 2 to 3 weeks' break, external beam irradiation was administered, consisting of 40 or 50 Gy (in 20 or 25 fractions over 4 to 5 weeks, fraction dose 2.0 Gy). In the higher stages, IIB–IIIB, the reverse schedule of radiotherapy was given, external beam being applied prior to curietherapy. After a 3 weeks' break, curietherapy was administered as described above. Mean overall treatment time was 86 days (median 90 days), ranging from 50 to 120 days.

Vascular density

Random cervical tumour punch biopsies were taken before the radiotherapy. IMD determination was performed on formalin-fixed, paraffin-embedded tumour biopsies stained with anti-factor VIII antibody (DAKO Ltd.) using immunohistochemistry and the vascular hot-spot technique, i.e. the number of vessels in the areas of highest neovascularization. Each slide was first scanned at low power to identify the area with the highest vessel count. Then, 7–13 high-power ($\times$ 500; 40 objective, 12.5 ocular) fields (0.145 mm^2) were counted for each patient. A small area corresponding to a higher magnification will improve the detail of the image, allowing identification of more single endothelial cell sprouts. We counted highlighted endothelial cells or cell clusters clearly separated from adjacent microvessels, tumour cells and other connective tissue elements regarded as distinct countable microvessels. Therefore, a lumen of vessel is not required, nor is the presence of red blood cells. Single cell sprouts as well as larger vessels are included in the counts. The mean vessel count (IMD) per 1 mm^2 of tumour volume was used in the analysis.

Haemoglobin concentration

For assessment of the Hb concentration, we used the colorimetric haemoglobincyanide method. Hb was expressed in grams per litre.

Statistical analysis

The probability of survival and failure was calculated using the Kaplan–Meier method (25). The log-rank test was used to assess the relationship between overall survival and the assessed parameters. In the univariate analysis—50 years—116 g/l, and 190 microvessels/mm^2 were used as the cut-off points for age, Hb level and IMD, respectively. These values were chosen as a minimum p-value method (26). Altman et al. (26) advocate against using the minimal p-procedure and suggest correction for each p-value obtained in the range of 0.001–0.1 according to the approximated formula:

$$p_{cor} = -3.13\ p_{min}\ (1 + 1.65 \log_e\ p_{min}) \text{ for } \varepsilon = 5\%$$

After correction, none of the significant p-values obtained appeared to be significant. As we do not fully agree with Altman et al.'s arguments (we do not think that correction of the p-value is necessary), we decided to give both the corrected and uncorrected p-values in the figures, but to use consistently uncorrected p-values in our paper. In the Cox multivariate model, all categorized parameters (significant cut-off points, uncorrected) were entered. Additionally, Cox analysis was performed for two subgroups of cancers that differed in keratinizing status.

RESULTS

Considerable variability in the results obtained for all the parameters examined was found in our series of patients. Vascular density assessed in 92 patients ranged from 56.3 to 473.6/mm^2, with a median value of 142.5. The values for haemoglobin assessed in 152 patients ranged from 81 to 160 g/l and the median value was equal to 127 g/l. These values were not correlated with patients' age, tumour stage, or keratinization (Table 1). However, Hb was correlated with tumour grade. Patients with grade 3 (poorly differentiated) tumours had significantly lower Hb levels than those with grade 2 and grade 1 tumours (Table 1). IMD was correlated with tumour grade, but not significantly. IMD increased from 136.8 in grade 1 to 177 vessels/mm^2 in grade 3 (Table 1). A similar increase in IMD was observed in older patients (age > 50 years, 166.0 ± SE 12.3) as compared to younger patients (153.1 ± SE 11.4).

At the 8-year follow-up, 56 (36.8%) patients were still alive, 10 (6.6%) alive with disease, 71 (46.7%) died of cancer or metastases, 6 (3.9%) died of intercurrent disease and 9 (5.9%) were lost to follow-up (Table 2; these patients were treated as dead in the uni- and multivariate analyses). One hundred and ten (72.4%) were locally controlled and 27.6% were not controlled. The median time of follow-up was 26 months, ranging from 2 to 145 months. The median age of the patients was 55 years (29–80). We found 30 tumours in grade 1, 66 tumours in grade 2 and 23 tumours in grade 3. Median values for age, Hb and IMD used as cut-off points in the univariate analysis were not significant. Therefore, other significant cut-off points—50 years, 190 vessel/mm^2 and 116 g/l—were chosen for the analysed parameters. Younger patients (below 50 years of age) had significantly poorer survival time than older patients (p = 0.0079) (Fig. 1). The median survival time for younger patients (⩽ 50 years) was 16 months compared with 34 months for older patients. Clinical stage was not significant in this group of patients. Stage I patients survived non-significantly better than stage II and III patients (p = 0.7009). The median survival time for stage I, II and III patients was 56, 26 and 23 months, respectively. Patients with non-keratinizing tumours had better survival rates (non-significant) than those with keratinizing tumours (p = 0.2669). A higher haemoglobin level appeared to be predictive of patients' survival. Those with Hb levels higher than 116 g/l had a significantly better rate of survival (p = 0.0213, Fig. 2). Median survival time for the first group was 33.5 months and for the second

Table 1

Mean values of haemoglobin level and intratumour vascular density (IMD) according to patients' age and histopathological variables

Parameter	Hb (g/l) Mean ± SE	IMD (/mm^2) Mean ± SE
Stage		
I	(9)* 133 ± 0.3	(6) 139.9 ± 31.0
II	(119) 127 ± 0.1	(75) 166.6 ± 10.1
III	(24) 123 ± 0.4	(11) 134.0 ± 15.4
Grade		
1	(30) 135 ± 0.3	(20) 136.8 ± 16.6
2	(65) 128 ± 0.2**	(33) 168.3 ± 12.9
3	(23) 123 ± 0.2***	(13) 177.0 ± 33.8
Age		
⩽ 50 years	(55) 121 ± 0.2	(36) 153.1 ± 11.4
> 50 years	(97) 130 ± 0.2****	(56) 166.0 ± 12.3
Keratinizing	(36) 125 ± 0.3	(21) 137.8 ± 16.0
Non-keratinizing carcinoma	(80) 130 ± 0.2	(43) 175.0 ± 13.7

* Number of patients is given in parentheses.
** Difference between grades 1 and 2, p = 0.0295.
*** Difference between grades 1 and 3, p = 0.0090; **** p = 0.0025.

Table 2

Eight-year follow-up of cervical cancer patients treated with radiotherapy

Endpoint	n	(%)
Alive with no evidence of disease	56	36.8
Alive with disease	10	6.6
Died from cancer or metastases	71	46.7
Died from intercurrent disease	6	3.9
Lost to follow-up	9	5.9

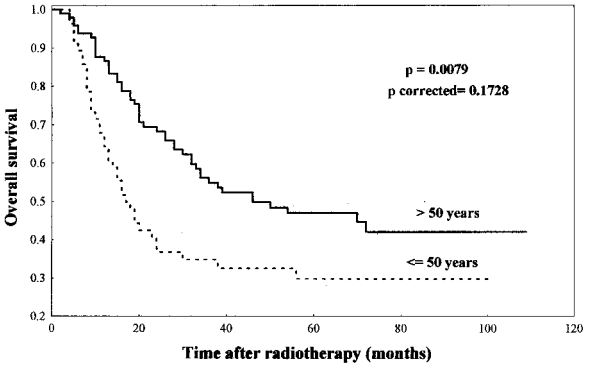

Fig. 1. The influence of age factor on survival of cervical cancer patients after radiotherapy. Patients were divided into those below and above 50 years of age (minimal p-value).

13.5 months. Higher vascularity (IMD > 190.0 vessels/mm^2) was also associated with a significantly higher incidence of survival (Fig. 3, p = 0.0504). Median survival time for patients with better vascularized tumours was 38 months, whereas for those with poorly vascularized tumours, survival time was 24 months. IMD and Hb levels were higher in patients with locally controlled tumours. However, the difference between locally controlled and non-controlled tumours was statistically significant for Hb only (130 vs. 119 g/l, p = 0.0005). The univariate analysis showed that patients' age > 50 years, high IMD, and a high Hb level were favourable prognostic factors for cervical cancers treated with radiotherapy. From all examined parameters enrolled in the multivariate Cox analysis, only IMD > 190 vessels/mm^2 and Hb > 116 g/l appeared to be prognostic factors for patients' survival (Table 3). When Cox's analysis was performed separately for keratinizing and non-keratinizing cervical tumours, it appeared that IMD > 190 vessels/mm^2 was significant only for keratinizing (p = 0.0318) and not for non-keratinizing cancers (0.4304). However, Hb > 116 g/l was significant for non-keratinizing (0.0093) but not for keratinizing tumours (0.2975).

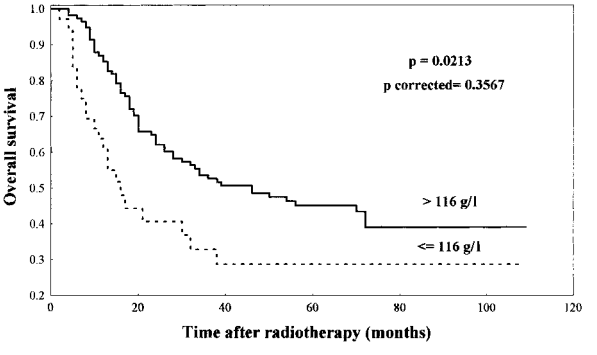

Fig. 2. The influence of haemoglobin (Hb) level on cervical cancer patients' survival after radiotherapy. Patients were stratified into those with an Hb level lower or higher than 116 g/l (minimal p-value).

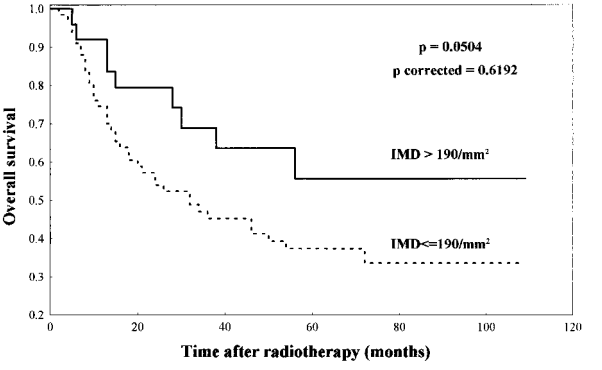

Fig. 3. The influence of intratumour vascular density (IMD) on patients' survival. Patients having higher vascularized tumours (IMD > 190 vessel/mm^2) survived significantly better than those with low vascularized tumours (minimal p-value).

DISCUSSION

Tumour stage and size are indirectly linked. Therefore in most studies, a tumour size of > 6 cm is associated with a poorer prognosis (4, 27). In our series of patients, clinical stage was not significant. Stage I patients survived non-significantly better than stage II and III patients. This could be due to the fact that 6 cases in stage I disease were bulky cancers which can be larger than small stage III cancers. The lack of significance of tumour stage in our study could be the effect of our patients being selected by availability of tumour material for biopsy instead of being taken consecutively. This result confirms our earlier data (1) and is in agreement with other authors' results (3, 10, 28), although it contradicts the results of other studies (27). Pretreatment haemoglobin level was not correlated with tumour stage, which is in agreement with other studies (9). However, in the study by Tsang et al. (3), patients with

Table 3

Cox multivariate analysis of cumulative overall survival of cervical cancer patients treated with radiotherapy (final model)

Variable	RR[a]	p-value
Age		
≤50 years	1.69 (0.88–3.23)	0.1172
>50 years	1.00	
Stage	1.04 (0.48–2.25)	0.9241
Grade	0.84 (0.53–1.32)	0.4371
Keratinizing	1.33 (0.63–2.81)	0.4551
Non-keratinizing carcinoma	1.00	
Hb concentration		
≤116 g/l	2.26 (1.06–4.76)	0.0339
>116 g/l	1.00	
IMD		
≤190 vessels/mm^2	2.52 (1.05–6.25)	0.0387
>190 vessels/mm^2	1.00	

[a] Relative risk with 95% confidence limits.
Abbreviation: IMD = intratumour vascular density.

smaller tumours showed a significantly higher level of haemoglobin. In our analysis, higher haemoglobin levels appeared to be predictive of patients' survival, particularly for patients with non-keratinizing tumours, in which penetration of oxygen was probably better than in keratinizing tumours. This shows not only the prognostic but also the predictive value of Hb assessment. Patients with Hb levels higher than 116 g/l had a significantly better survival time (33.5 months) than those with Hb < 116 g/l (13.5 months), which confirms the results of Tsang et al. (3) and Grogan et al. (29). Bush et al. (30) also found that maintaining patient haemoglobin levels above 125 g/l significantly improves pelvic control rates compared with the rates in patients with lower haemoglobin levels. More recently, Grogan et al. (29) hypothesized that the negative prognostic impact of low haemoglobin levels at presentation can be overcome by administering blood transfusions. Because there was an additional gain observed at each level of haemoglobin up to 120 g/l, it is proposed that levels be maintained up to at least ≥ 120 g/l during radiotherapy. The same level of Hb concentration (116 g/l) appeared to be significant in our study. However, in other types of tumours (head and neck cancers), neither correlation between the volumes of the measured lymph node metastasis nor the pretreatment haemoglobin concentration was shown (31).

Higher vascularity IMD > 190 vessels/mm^2 was predictive of patients' survival. Those with higher vascularity had a significantly better survival time. It was interesting to show that vascular density is predictive in terms of patients' survival only in keratinizing cancers. This could suggest that lower oxygen penetration and higher hypoxia in these tumours might stimulate neovascularization manifested by a higher vessel number. Our results are contradictory to those of Cooper et al. (10) and Schlenger et al. (23), who have shown that high tumour angiogenesis is associated with a poorer survival time. In the study by Cooper et al. (10), patients with lower vascularity fared better than those with high vascularity. The reason for this difference is unclear because we used the same method of analysis (anti-factor VIII staining and the hot-spot technique). The authors counted only three random fields at a lower magnification ($\times 300$) than that used in our study. The difference cannot be explained simply by the sensitivity of the staining, since the same type of staining was used. Many small tumour vessels are atypical, resembling venules or sinusoids rather than capillaries, and it is uncertain whether the sensitivity of the staining method is as high in tumours as in normal tissue, and whether it is independent of the histological grade of the tumour. With some methods, not all the blood vessels may have been recognized and vascularity may have been underestimated (32). The difference between our results and those of Schlenger et al. (23) may be due to the different methods used and the fact that 22 out of 42 patients were treated with primary surgery. The Schlenger group determined the distance between random points within the tumour and the closest microvessel in histological sections immunohistochemically stained for Factor VIII-related antigen. Our data are, however, in concordance with the earlier published data of Awwad et al. (9), Siracka et al. (12), Revesz et al. (22) and Davidson et al. (8). Awwad et al. (9) studied intercapillary distances in pretreatment biopsies of SCC of the cervix. They reported that the mean intercapillary distance and the proportion of distances exceeding 300 μm were smaller in patients with local tumour control than in patients with local recurrence. Siracka et al. (12) carried out a morphometric analysis after Trichrome staining and identified the blood vessels. Our data are also in agreement with the Hockel et al. (18) and Lyng et al. (27) studies, in which hypoxia was measured by polarographic methods. Low pO_2 tumours ($pO_2 < 10$ mmHg) responded less favourably to radiotherapy and patients with hypoxic tumours had shorter survival times, mainly because of locoregional failure with or without distant metastases. It was confirmed that tumours with high vascular density showed higher pO_2 values than those with low vascular density (19). This is why our results suggest that tumour vascularity is not only a prognostic, but also a predictive factor. The Eppendorf pO_2 histograph method is considered to be the gold standard for measuring tumour oxygenation (33). However, polarographic measurements of pO_2 by Eppendorf microelectrodes are more likely to detect transient hypoxia involving many cell layers. Therefore, recent data on application of Eppendorf needles are disappointing, suggesting overestimation of hypoxia (20). Perhaps, the measurement of vascular density in vivo by Doppler ultrasound will be more promising, particularly for predicting lymph node metastases in cervical carcinoma (17).

Many clinicians now believe that anaemia at presentation may not be a treatment-related factor but may be solely or partially a tumour-related manifestation of more aggressive cervical carcinoma (18, 23). In our analysis we found only 16 (10%) metastases out of 152 tumours, so we cannot confirm the relationship between hypoxia and aggressiveness of the tumour. The existence of hypoxic cells as a result of the inadequate microvasculature and large intercapillary distances has long been postulated to increase the radioresistance of tumour cell populations. Complete absence of oxygen at the time of irradiation can increase the cellular resistance by a dose-modifying factor of 3. Hypoxia influences tumours in paradoxically opposing ways: it is lethal to cells but prolonged exposure can also have a protective effect, via the selection of cells with reduced apoptotic ability (34). The latter can lead to therapy-resistant hypoxic areas that are likely to act as the foci of tumour regrowth. Such a scenario in cervical cancer is probably due to the occurrence of p53 mutations and human papillomaviruse (HPV) infection. Inactivation of

the p53 function is important in the pathogenesis of cervical carcinoma (35). HPVs are heavily implicated in the etiology of this tumour (35, 36). Protein E2 of oncogenic HPV inhibits the expression of oncoprotein *E6* and *E7* genes and suppresses the proliferation of cervical cancer cells. Loss of the functional *E2* gene may provide selective advantages in the development of cervical cancer. The oncoproteins *E6* and *E7* from these viruses bind to p53 and RB1 protein products, inactivating these genes, and allowing progression of tumour cells in cycle. Infection by HPV can mimic loss of p53 function resulting from either deletion or mutation. Mutant p53 proteins that have altered conformation oligomerize with wild-type p53 and cause it to assume a mutant phenotype (35). Lack of p53 protein in highly proliferating tumours prevents cell cycle arrest in sublethally damaged cells and increases radiosensitivity, which might not necessarily be identical with inherent radiosensitivity. This could lend support to our study showing that fast-proliferating cervical cancer might be more radioresponsive than slowly proliferating cancer (1). Giaccia (37) favour the hypothesis that HPV cells in the early development of carcinoma will die by apoptosis if hypoxia is able to uncouple wild-type p53 from E6-mediated degradation. Hypoxia induces apoptosis in oncogenically transformed cells, and further genetic alterations such as loss of the p53 tumour-suppressor gene or overexpression of the apoptosis-inhibitor protein Bcl-2, and substantially reduces hypoxia-induced cell death (34). These results provide evidence for the role of hypoxia-mediated selection of cells with diminished apoptotic potential in progression of human tumours and can in part explain why cervical tumours with low pO_2 values are more difficult to cure. If this hypothesis proves to be correct, it may explain why the presence of hypoxia correlates with a poor outcome of cervical cancers independent of treatment modality.

As the majority of cervical carcinomas pose a high-risk type HPV and approximately half of these tumours possess oxygen-depleted regions, hypoxia could select oncogenically transformed cervical epithelial cells that have lost their apoptotic ability and respond poorly to radiotherapy. Developing tumours derived from oncogenically transformed cells that possess wild-type p53 have substantially higher apoptotic potential than tumours without wild-type p53 (34). Unfortunately, neither expression of the p53 gene nor HPV infection was studied in our series of patients.

All the data presented in this paper point to the prognostic importance of pretreatment measurements of oxygenation in cervical cancer patients and suggest that further improvements in therapy might be gained by selection of alternative treatment strategies (predictive significance) such as neoadjuvant chemotherapy or radiation sensitizers, blood transfusions or the use of vasoactive drugs in patients with a low level of Hb and low vascular density. In the early 1980s it was shown by Carde & Laval (38) that cisplatin is twice as effective when cells are irradiated in hypoxia. This may confirm, the recently published results of concomitant radiochemotherapy in cervical cancer (39).

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