Virchows Arch (1997) 431:173–179

ORIGINAL ARTICLE

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DNA Aneuploidy, S-phase fraction, nuclear p53 positivity, and survival in non-small-cell lung carcinoma

Received: 18 February 1997 / Accepted: 25 March 1997

Abstract Inactivation of the *p53* gene plays a key role in tumour biology, probably through a disturbed cell cycle control and an increased genetic instability in p53-inactivated tumours. To learn more about the relationship between p53 alterations, proliferation and genetic instability (DNA aneuploidy) in lung cancer patients, specimens of 220 surgically resected lung carcinomas with clinical follow-up information were examined by immunohistochemistry (p53; CM1) and flow cytometry. Nuclear *p53* positivity – found in 49.5% of the tumours – was associated with both high S-phase fraction (SPF) and DNA ploidy aberrations. SPF was higher in p53-positive tumours (15.9 ± 10.2) than in p53-negative tumours (10.3 ± 8.7; $P = 0.03$). The rate of p53 positivity was higher in 101 DNA-aneuploid and DNA-multiploid tumours (55%) than in 27 diploid and peridiploid carcinomas (33%; $P = 0.0512$). These results are consistent with an in vivo role of *p53* inactivation for increased proliferative activity and development of genomic instability in lung cancer. There was no association between SPF and prognosis. Although prognosis was worse in DNA-aneuploid and multiploid tumours than in diploid, peridiploid and tetraploid carcinomas ($P = 0.029$), DNA ploidy was not an independent predictor of poor prognosis in multivariate analysis. These data show that DNA-flow cytometry has little prognostic value for patients with resected non-small-cell lung carcinoma.

Key words Non-small-cell lung cancer · DNA aneuploidy · S-phase fraction *p53* Nuclear overexpression · Prognosis

Abbreviations DI DNA-index, SPF S-phase fraction, CV coefficient of variation, NSCLC non-small-cell lung carcinoma

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Introduction

The prognosis of patients with lung cancer is generally poor [5, 23, 31]. Some 30% are still operable at the time of diagnosis, and only a minority of them will be definitely cured by surgery. Sixty percent of the operated patients with non-small cell lung cancer (NSCLC) die within 5 years after operation despite thorough preoperative case selection [10, 31]. Improved prediction of the individual prognosis of patients with NSCLC could help to improve selection of patients who will benefit from surgery and from adjuvant treatment.

The proliferative activity of tumour cells, as measured by flow cytometry (FCM), mitosis count, or thymidine incorporation, has been suggested as a potential prognostic factor in various tumours, including lung cancer [19, 40]. It has also been suggested that a disturbed cellular DNA-content (DNA aneuploidy) resulting from increased genomic instability is associated with poor prognosis in lung carcinomas and other malignancies [40]. Both tumour cell proliferation and DNA aneuploidy may be linked to the inactivation of the *p53* gene, a key tumour suppressor gene involved in the control of both cell cycle and genomic stability [17, 44]. In a previous study we have shown that an altered *p53* expression is associated with poor prognosis in node-negative NSCLC [3].

The present study was undertaken to evaluate the realtionship between *p53* alterations, tumour cell proliferation (S-phase fraction) and disturbed genomic stability (as evidenced by DNA aneuploidy) and also to study the prognostic significance of S-phase fraction and DNA aneuploidy in NSCLC.

Materials and methods

The tumour material consisted of formalin-fixed, paraffin-embedded tissue samples from 220 consecutive NSCLC patients treated by resection with curative intent between 1980 and 1990 at the University Hospital of Basel. All tumours were larger than 1 cm in diameter. All lung resection specimens were grossly examined according to a standardized procedure. The lungs were fixed in expanded state with 4% buffered formalin (pH 7.4) and then cut into slices 0.5–1.0 cm thick. All available lymph nodes were examined histologically. The pTN stage was recorded according to the recommendations of the International Union Against Cancer (UICC) [11]. The carcinomas were classified following the International Histological Classification of Tumours [49]. Poorly differentiated squamous cell carcinomas, solid adenocarcinomas and large cell carcinomas were rated as having high-grade, all other carcinomas as having low-grade malignancy. Overall survival data from 210 cases were obtained from the Basel Cancer Registry (Director: J.T.). There were 67 surviving patients to be monitored at the time scheduled for their last clinical follow-up examination; 143 patients had died. These 143 patients included 7 who had died within the first 2 months after operation and were not included in survival analysis.

Flow cytometry (FCM) was performed for DNA-measurement in 214 cases on 50-µm sections of formalin-fixed, paraffin-embedded tumour tissue. The tissue block with the highest proportion of non-necrotic tumour tissue was selected. A mild pepsin digestion was used for cell dissociation, as previously described [29]. Pelleted cells were then mixed up with 250 µl C100T (2.1 g citric acid, 0.5 ml Tween 20/100 ml destilled water), shaken for 5 min and then left overnight at room temperature. Nuclei were stained with 4.5-diamino-2-phenyl-indole (DAPI). Dissociated nuclei were analysed with a PAS II flow cytometer (Partec). For each specimen 10,000 to 20,000 events were collected. The Multicycle Program (Phoenix Flow Systems, San Diego, Calif.) was used for data analysis. The lowest G0/G1-peak was considered to be diploid and given a DNA index (DI) of 1.00. The DI of other G0/G1 peaks was calculated as the ratio of their G0/G1 peak channel number to the diploid G0/G1 peak channel number of the histogram. Tumours with a nondiploid G0/G1 peak with a DI < 1.2 were considered peridiploid. Tumours were defined as DNA-diploid if only one G0/G1 peak was present and the G2M peaks were < 15%. If the G2/M peak was ≥ 15% the presence of a tetraploid tumour cell population (DI 1.9–2.1) was suggested and the tumour was called DNA-tetraploid. Tumours with G0/G1 peaks not in the peridiploid or tetraploid range (DI > 1.2 < 1.9 or DI > 2.1) were considered aneuploid. Multiploid tumours were defined as having more than one nondiploid G0/G1 peak. The coefficient of variation (CV) was calculated for each DNA-peak. Only tumours with a diploid CV≤ 6% were included in this study. The S-phase fraction (SPF) was calculated separately for each DNA-peak. For statistical analysis, diploid S-phase was used in DNA-diploid tumours, average S-phase in peridiploid tumours, and aneuploid S-phase in aneuploid tumours. No S-phase calculation was done in multiploid tumours.

The 220 cases included 204 that had previously been examined for nuclear *p53* positivity [3]. The 4-µm sections for immunohistochemical studies were taken from the same tissue block as the sections for FCM. The rabbit polyclonal antibody CM-1 recognizing both wild-type and mutant p53 protein (1:4000/MEDAG, Hamburg, Germany) was used. Immunostaining was performed as described elsewhere [21]. A tumour was considered *p53*-positive if an unequivocal nuclear positivity was seen in at least 1% of cells [3].

A Mann-Whitney U-test was applied to examine the relationship between SPF and histological parameters, ploidy and *p53* status. Contingency table analysis was applied to evaluate the relationships between ploidy, *p53* status, and histological variables. The Kaplan-Meier method with a log-rank test was used for survival analysis. Cox regression analysis was used to test for independent prognostic significance.

Results

The 220 tumours examined were made up of 129 (58.6%) squamous cell carcinomas, 64 (29.1%) adenocarcinomas, and 27 (12.3%) large cell carcinomas. Seventy-three squamous cell carcinomas were poorly differentiated, and 56 were well or moderately differentiated. There were 36 tubular or tubulo-papillary, 13 bronchoalveolar, and 15 solid adenocarcinomas, thus 105 (47.7%) of the 220 carcinomas were rated as having low-grade and 115 (52.3%) as having high-grade malignancy.

Tumour staging resulted in 50 (22.7%) pT1, 138 (62.7%) pT_2, 28 (12.7%) pT_3, and 4 (1.8%) pT_4 tumours. Lymph node metastases were seen in 106 (48.2%) patients, while 114 (52.7%) patients were classed as pN0. Metastases were more frequent in $pT_{2–4}$ (90/170) than in pT_1 tumours (16/50, $P = 0.01$). There was no significant association between histological type or grade and presence of lymph node metastases (data not shown).

For DNA Ploidy the median coefficient of variation (CV) was 5.3 (range 2.9–12.0) in all 214 tumours analysed by FCM. DNA ploidy was determined in the 145 (67.7%) cases with a diploid CV≤ 6%. Twenty-two of these tumours were multiploid. The frequency distribution of the DI in the remaining 123 tumours showed that the DI was typically either between 1.4 and 1.7 or in the diploid/peridiploid range (DI<1.2) (Fig. 1). Tumours with a DI between 1.4 and 1.7 were most frequently seen ($n = 62$), followed by tumours with a DI between 1.0 and 1.2 ($n = 31$). Ten of the tumours of the latter group were

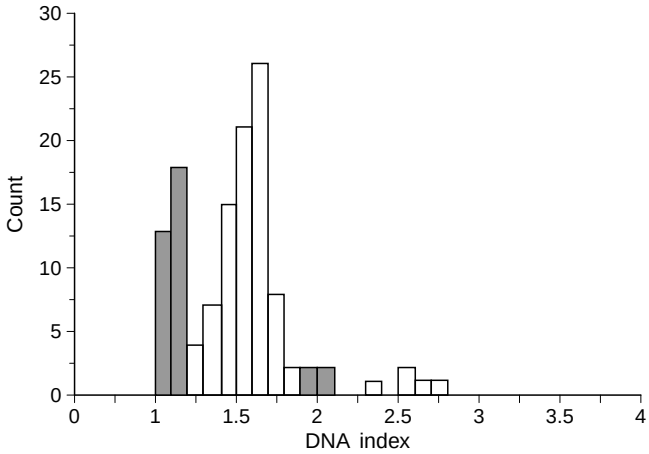

Fig. 1 DNA indices in 123 cases of non-small-cell lung carcinoma. The histogram shows the frequency distribution of DNA indices in non-multiploid lung carcinomas. Diploid, peridiploid, and tetraploid tumours were defined as tumours with minor ploidy aberrations. These are shown with *dark shading*

Table 1 DNA ploidy and tumour phenotype (*NSCLC non-small-cell lung carcinoma*, *SQCL* squamous cell carcinoma, *ADCL* adenocarcinoma, *LCCL* large-cell carcinoma)

Parameter	*n*	Diploid	Peridiploid	Tetraploid	Aneuploid	Multiploid
All NSCLC	145	10	21	4	88	22
Histological type						
SQCL	84	5	14	2	52	11
ADCL	36	5	5	2	22	2
LCCL	25	0	2	0	14	9
Grade[a]						
Low	64	7	11	3	30	13
High	81	3	10	1	58	9
pT stage[a]						
pT1	34	5	6	1	18	4
pT2	89	4	13	3	53	16
pT3	18	1	2	0	13	2
pT4	4	0	0	0	4	0
Nodal status[a]						
pN0	71	7	11	2	38	13
pN1	54	2	8	2	36	6
pN2	20	1	2	0	14	3

[a] Differences not significant

Table 2 S-phase fraction (*SPF*) and tumour phenotype

	n	SPF Median±SD
All NSCLC	127	12.9±10.5
Histological type[a]		
SQCL	74	16.6±11.5
ADCL	38	7.5±6.7
LCCL	15	10.4±5.8
Grade[b]		
Low	57	8.1±11.1
High	70	14.9±9.6
pT stage[c]		
pT1	32	9.7±9.1
pT2	76	13.6±10.7
pT3	15	10.3±12.9
pT4	4	18.5±4.6
Nodal status[d]		
pN0	63	10.5±10.5
pN1	46	17.4±9.8
pN2	18	14.2±12.0
DNA ploidy type[e]		
Minor ploidy aberration	18	7.0±5.5
Major ploidy aberration	78	16.5±10.5

[a] SQCL vs ADCL; $P<0.0001$; ADCL vs LCCL $P=0.066$; SQCL vs LCCL not significant
[b] $P=0.001$
[c] Differences not significant
[d] $P=0.03$ for N0 vs N1 and N2
[e] $P=0.0006$

Table 3 *p53* Overexpression and DNA ploidy

	n	p53-positive (number of cases)
DNA ploidy[a]		
Diploid	9	3
Peridiploid	18	6
Peritetraploid	4	3
Aneuploid	82	48
Multiploid	19	7

[a] $P=0.0512$ for DNA diploid/peridiploid vs DNA aneuploid/multiploid (Chi-square test)

diploid and 21 were peridiploid. Both tetraploid ($n = 4$) and hypertetraploid tumours ($n = 5$) were rare. There was no significant difference in histological type, grade, stage, or nodal status between tumours of different ploidy status (Table 1).

The S-phase fraction (SPF) was evaluable in 127 cases and ranged from 0.4% to 56.8% with a median value of 12.9%. Ten percent of the tumours had an SPF higher than 28.7%. The relationship of SPF with histological variables and DNA ploidy is given in Table 2. SPF was strongly associated with tumour histology, being highest in squamous cell carcinomas, lowest in adenocarcinomas, and intermediate in large cell carcinomas. The difference in SPF reached significance for squamous cell carcinomas and adenocarcinomas ($P < 0.0001$), while SPF of large cell carcinomas did not differ significantly from that of other histological tumour types. SPF was significantly higher in high-grade than in low-grade malignancy ($P = 0.001$). Although SPF was lowest in pT1 tumours (9.7 ± 9.1), there was no significant association between SPF and pT stage. SPF was significantly higher in node positive than in node negative tumours, however ($P = 0.03$). A strong association was also found between a high S-phase and a disturbed cellular DNA-content ($P = 0.0006$).

Almost half (101, or 49.5%) of the 204 NSCLC examined for *p53* stained positive. *p53*-Positive staining predominated in peripheral areas in some tumours. Such *p53* positivity was associated with stage, grade, and histological tumour type. These results were expected, since the present set of patients represents a subset of a series of patients for whom we have previously reported similar results [3]. There was a tendency towards a higher frequency of *p53* positivity in DNA aneuploid/multiploid tumours (55 of 101; 55%) than in diploid/peridiploid carcinomas (9 of 27; 33%; $P = 0.0512$; Table 3). *p53* Posivity was significantly related to a high SPF. SPF was higher in *p53*-positive (15.9 ± 10.2) than in *p53*-negative tumours (10.3 ± 8.7; $P = 0.010$).

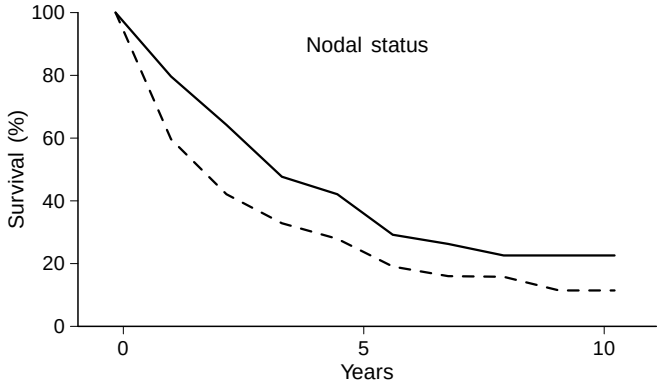

Fig. 2 Nodal status and survival. Significant differences (P = 0.0007) are seen between 116 N0 (——) and 94 N1 (– –) tumours

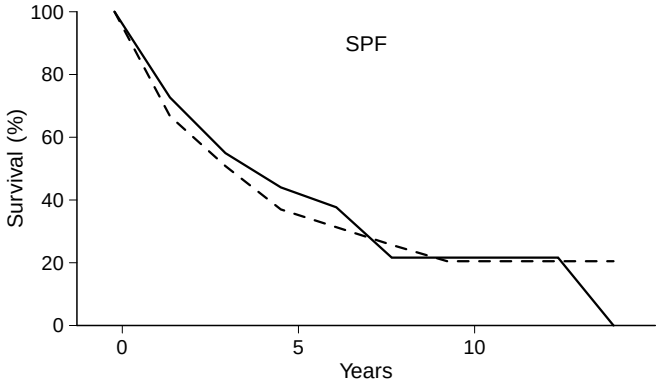

Fig. 3 S-phase fraction (SPF) and survival. No significant difference (P = 0.4) was seen between 59 tumours with SPF median value ≤ 12.9% (——) and 57 tumours with SPF > 12.9% (– –)

Nodal status (P = 0.0007, Fig. 2), pT stage (P = 0.0001) and grade (P = 0.001) were strong prognostic factors in this set of patients. The prognostic significance found for *p53* positivity in node-negative (P = 0.04) but not in node-positive (P = 0.67) patients was expected because this series of patients overlaps with a previously published patient set in whom we reported a prognostic relevance of *p53* positivity for node-negative lung carcinomas [3]. SPF was not related to prognosis in this set of patients. This held true not only when all patients were examined (Fig. 3) but also in separate analyses of subgroups of identical nodal status, stage, grade or histological tumour type (data not shown). The analysis of the prognostic value of DNA ploidy information was hampered by the low number of diploid or peridiploid tumours. Therefore, diploid, peridiploid, and tetraploid tumours (tumours with minor aberrations) were grouped together for survival analysis and compared with aneuploid and multiploid tumours (tumours with major aberrations). This analysis revealed a significantly better prognosis for tumours with minor ploidy aberrations than for aneuploid or multiploid tumours (P = 0.029; Fig. 4). Multivariate analysis examining the prognostic significance of nodal status, stage and ploidy simultaneously revealed no independent prognostic significance of DNA ploidy, however.

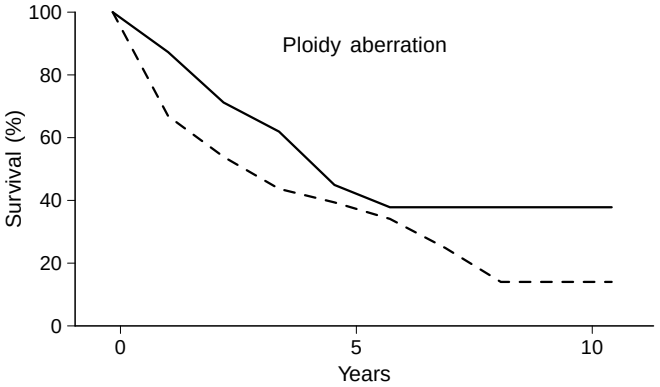

Fig. 4 Ploidy and survival. Survival was significantly longer (P = 0.029) for the 23 tumours with minor ploidy aberrations (diploid, peridiploid, tetraploid) (——) than for the 74 tumours with major DNA ploidy aberrations (aneuploid, multiploid) (– –)

Discussion

The results of this study show that a disturbed cellular DNA-content is (weakly) associated with poor prognosis in lung cancer patients. The finding of a high SPF and an increased prevalence of DNA aneuploidy in tumours with nuclear p53-positivity is consistent with an in vivo role of *p53* inactivation for a disturbed cell cycle control and increased genomic instability in lung carcinomas.

According to FCM on formalin-fixed tumour specimens, a high fraction of tumours with a major aberration in their DNA-content was found (> 90%). This proportion is in the range of previous studies in which fresh tissue was used for flow analysis [18, 37, 48] but higher than has been found in most studies performed on formalin-fixed material [4, 25, 27, 34, 50]. This is probably due to our stringent criteria for FCM interpretation analysing only tumours with a CV≤ 6.0. Such CVs are in the range that can usually be obtained from fresh tissues. Given the high number of DNA aneuploid cases in this study, the lack of a significant association with tumour phenotype is not surprising. While there was a tendency towards a higher rate of DNA-aneuploidy in high-grade and high-stage tumours than in low-grade and low-stage tumours, this difference did not reach statistical significance. This weak relationship is consistent with the controversial results in the literature, some authors having found an association between DNA aneuploidy and tumour phenotype [4, 14, 25, 47, 50] while others have been unable to confirm these results [27, 28, 37].

It has been suggested that progression of malignant tumours is accompanied by sequential changes in the cellular DNA-content. Development of DNA aneuploidy is believed to occur through tetraploidization of previously diploid or peridiploid tumours and a subsequent loss of chromosomal material [9, 39]. In the light of this hypothesis, the frequency distribution of DNA-indices found in this study argues for a high degree of genetic instability in lung cancer. There are only a few tumours with a DI in the DNA-tetraploid (DI 1.9–2.1) or hypotetraploid (DI 1.7–1.9) range, suggesting that these tumours

are genetically highly unstable after tetraploidization, resulting in a rapid loss of a significant amount of genomic material. The low number of tumours with a diploid or peridiplopid DI and the high frequency of DNA aneuploid tumours with a comparatively low DI (1.4–1.7) suggest that lung carcinomas are at a genetically advanced stage at the time of diagnosis, which could be one of the reasons for the malignant behaviour and poor prognosis of these tumours.

The strong association found between SPF and DNA aneuploidy, high tumour grade, and lymph node metastases found in this set of patients is consistent with the results of other studies [8, 46]. Interestingly, SPF was dramatically higher in squamous cell carcinomas than in adenocarcinomas ($P < 0.0001$). While some authors have previously failed to find proliferation differences between histological tumour types [35], our results are in line with studies comparing the proliferating cell nuclear antigen (PCNA) between different histological tumour types [1, 36]. Additional studies are needed to evaluate whether the high SPF in squamous cell carcinoma is related to the expression of specific growth receptor proteins, such as epidermal growth factor receptor (EGFR). EGFR is frequently expressed in squamous cell carcinomas and was found to be related to rapid tumour cell proliferation in bladder, head and neck, and other tumours [16, 30, 38].

The *p53* gene is a tumour suppressor gene and involved in the development and progression of many neoplasms [12]. Inactivation of *p53* occurs, frequently, through deletion of one allele with concomitant mutation of the other allele. Since many mutations of the *p53* gene result in a prolonged half-life of the protein, *p53* mutations can be detected by immunohistochemistry [7], and we have recently shown that nuclear *p53* positivity is associated with poor prognosis in node-negative NSCLC [3]. While the exact function of *p53* is still not clear, it has been shown that the protein can act as a cell cycle checkpoint and that it is involved in the regulation of apoptosis control, DNA repair, and genomic stability [17]. A major aim of this study was therefore to evaluate the relationship of *p53* aberrations and parameters for increased tumour cell proliferation (high SPF) or increased genomic instability (DNA aneuploidy). The higher frequency of *p53* overexpression in DNA-aneuploid/multiploid tumours than in diploid/peridiploid carcinomas ($P = 0.0512$) is consistant with an increased genomic instability in *p53*-altered lung tumours. The finding of a significantly higher SPF in p53-positive than in *p53*-negative tumours fits in well with the hypothesis of a disturbed cell cycle control in *p53*-inactivated tumours. This finding is in contrast to the results of Morvke et al., who could not find a relationship between *p53* expression and SPF by FCM [22]. However, a positive correlation between *p53* alterations and proliferation parameters have recently been reported for various tumours, including bladder and prostate cancer [21, 43].

Survival analysis showed that tumour stage, grade and nodal status were strongly associated with prognosis, as seen in several earlier studies [5, 10, 23], and provides indirect evidence of the validity of our survival data although overall survival data were used. To evaluate the prognostic significance of DNA ploidy alterations, tumours were categorized into those with minor and those with major ploidy aberrations. Diploid, peridiploid and tetraploid tumours were considered to have minor DNA-ploidy aberrations and aneuploid and multiploid tumours, to have major ploidy aberrations, since these tumours had presumably undergone tetraploidization and a subsequent DNA loss. Using this definition, prognosis was significantly better for tumours with minor aberrations than for those with major aberrations. This result must be interpreted with caution, since earlier studies had yielded conflicting results on the prognostic relevance of ploidy aberrations in lung cancer [2, 15, 20, 25–27, 32–34, 37, 42, 45, 47, 48] and the prognostic relevance of ploidy aberrations was not independent of nodal involvement and pT stage in this study. However, if our results are confirmed, DNA-ploidy determination could have some practical relevance in the preoperative evaluation of lung cancer patients. Information on ploidy can be obtained from small biopsies or cytological specimens before nodal involvement, and the pT stage can be determined with certainty. In contrast to ploidy, SPF was not associated with prognosis in this study. This is in agreement with the observations of some investigators, but conflicts with the results of others who have shown a correlation between survival and SPF in lung cancer [6, 13, 24, 41].

In summary, the results of this study show that nuclear *p53* positivity is linked to a high proliferation rate and DNA aneuploidy in NSCLC. This result is consistant with a role of *p53* inactivation for increased proliferative activity and genomic instability in lung cancer. Major ploidy aberrations, but not a high SPF, were related to poor patient prognosis, but this was not idependent of tumour stage or nodal status. We conclude that flow cytometrical evaluation of ploidy and S-phase on resected NSCLC specimens has little practical relevance.

Acknowledgements The authors are much obliged to Mrs. E. Schultheiss for excellent technical assistance, and to Mr. B. MacGandy for critical reading of the manuscript. This work was supported by the Krebsliga beider Basel.

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