[CANCER RESEARCH 55, 975–978, March 1, 1995]

Advances in Brief

Overexpression of Cyclin D1 Correlates with Recurrence in a Group of Forty-seven Operable Squamous Cell Carcinomas of the Head and Neck

Rob Michalides,[1] Nicoletta van Veelen, Augustinus Hart, Barbara Loftus, Ellen Wientjens, and Alfons Balm

Departments of Tumor Biology [R. M., N. v. V., E. W.], Radiotherapy [A. H.], Pathology [B. L.], and Otolaryngology/Head and Neck Surgery [A. B.], the Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, the Netherlands

Abstract

We evaluated the prognostic significance of overexpression of cyclin D1 in 47 patients with surgically resected squamous cell carcinomas of the head and neck. Overexpression of cyclin D1 was detected immunohistochemically using an affinity-purified polyclonal antibody directed against the carboxyl-terminal part of the cyclin D1 protein, applied to formalin-fixed, paraffin-embedded tissue sections. Overexpression of cyclin D1 was found in 30 of 47 head and neck squamous cell carcinoma (HNSCC) cases and was associated with a more rapid and frequent recurrence of disease ($P = 0.027$). There was a 5-year disease-free interval of 47% for HNSCC patients with a strong overexpression of cyclin D1 and of 80% for cyclin D1-negative HNSCC patients. Overexpression of cyclin D1 was also associated with a shortened overall survival of these patients ($P = 0.0095$), with a 5-year survival of 60% for the cyclin D1 strongly positive cases and of 83% for cyclin D1-negative cases. Overexpression of cyclin D1 appears to indicate poor prognosis in operable HNSCC.

Introduction

Amplification of the 11q13 region has been observed in a variety of human carcinomas, including HNSCC,[2] and carcinomas of breast, esophagus, lung, bladder, and liver (1). The 11q13 amplicons found in different tumors are unusually large, harboring various putative oncogenes, of which cyclin D1 is most consistently amplified and overexpressed (2). Overexpression of cyclin D1 may also result from chromosomal translocations, as is observed in parathyroid adenomas and in centrocytic lymphomas (3, 4). More direct evidence that overexpression of cyclin D1 may play a role in tumorigenesis was provided by the introduction of cyclin D1 into murine 3T3 cells (5) and by overexpression of cyclin D1 in target sites of cyclin D1 transgenic mice (6, 7).

Amplification of cyclin D1 appeared to be correlated with poor prognosis in breast carcinomas (8, 9) and with lymph node involvement in HNSCC (10–13). It is therefore of potential interest as a marker for tumor progression in these tumor types, particularly since HNSCCs may vary largely in their aggressiveness and metastatic behavior despite their common histological pattern and clinical stage. Thus far, no direct correlations between 11q13 amplification and tumor recurrence have as yet been reported in this group of tumors.

The aim of this study was to investigate whether overexpression of cyclin D1 is associated with an increased likelihood of tumor recurrence in HNSCC. For that purpose, we investigated a retrospective series of 47 HNSCC patients who had been curatively treated by surgery, with or without postoperative radiotherapy. Our results indicate that overexpression of cyclin D1 as measured by immunohistochemistry is significantly associated with tumor recurrence in this series of 47 operable HNSCCs.

Received 12/20/94; accepted 1/20/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed.

[2] The abbreviation used is: HNSCC, head and neck squamous cell carcinoma.

Materials and Methods

Patients and Tumor Material. We studied, retrospectively, 47 HNSCC patients treated at our Institute. All patients (36 male, 11 female; average age, 59 years, ranging from 32 to 84 years) were treated with curative intent and had received no prior therapy. The clinical characteristics are summarized in Table 1. The median follow-up period for the group was 76 months, for those still alive, it was 108 months ($N = 47$), and for those who died of intercurrent disease, it was 65 months. Tumors were fixed in formalin and embedded in paraffin, and 4-μm sections were attached onto silane-coated slides. A microwave retrieval technique was applied (14).

Antibodies. An antiserum directed against cyclin D1 was generated by injection of a β-galactosidase cyclin D1 fusion protein, using the carboxyl-terminal part of cyclin D1 (amino acids 217–296; corresponding with the *Stu*I-*Dde*I fragment of cyclin D1) into rabbits. Preimmune sera of these rabbits did not react with the fusion protein. Antibodies directed against the cyclin D1 part of the fusion protein were affinity purified on a glutathione *S*-transferase-cyclin D1 fusion protein using the whole size cyclin D1 protein (corresponding with the *Nco*I-*Hind*III fragment of cyclin D1) coupled covalently to activated CH-Sepharose 4B (Pharmacia Biotech Europe). With this procedure, antibodies reactive with the β-galactosidase and bacterial (contaminating) proteins were removed.

For immunohistochemical staining we used the affinity purified polyclonal antibody, B31S, at a 1:80 dilution using PBS/1% BSA. The tissue sections were incubated with the primary antibody for 16 h at 4°C and with the peroxidase-labeled conjugate for 30 min at room temperature. A two-stage streptavidin-biotin-peroxidase technique was used (Dako Duet Kit; DAKO, Glostrup, Denmark). Negative controls consisted of omission of the antiserum from the primary incubation.

The immunohistological staining was semiquantitatively scored by two of us who were not aware of the clinical details. Scores were ranked as: (−), negative; (+/−), 0–5% of the tumor cells were positive; (+), 5–50% of the tumor cells were positive; (++), >50% of the tumor cells were positive. Only nuclear staining was observed.

Statistical Analysis. Details of the scores of immunohistochemical staining and clinicopathological variables are presented in Table 1. Survival and disease-free interval curves were calculated using the method of Kaplan and Meier (15). The statistical analyses of the differences between curves were performed using the proportional hazard model of Cox (16) with markers coded as 1–4 as continuous variables, with time to recurrence (disease-free interval) and time to death as end points. For association with location, the global log rank test was used. BMDP (Statistical Software, Inc.) was used for all statistical analyses.

Results and Discussion

Immunodetection of Overexpression of Cyclin D1 and p53. The affinity-purified rabbit polyclonal antibody B31S detected a M_r 33,000 protein (Fig. 1, *Lane 1*) from [^{35}S]methionine-labeled UM-SCC2 cells overexpressing cyclin D1 (2). This band comigrates with [^{35}S]methionine-labeled cyclin D1 protein (Fig. 1, *Lane 4*) and was absent after preincubation of the antiserum with 2.5 μg of a glutathione *S*-transferase cyclin D1 fusion protein prepared *in vitro* (Fig. 1, *Lane 2*). The B31S antiserum detected nuclear staining in tumor cells overexpressing cyclin D1 only; no staining was observed in adjacent normal tissues (Fig. 2). We also did not detect any staining in cell

Table 1 *Overexpression of cyclin D1 in 47 operable HNSCC patients*

Characteristics	Positive	Negative	*P*
n	30	17	
Age (mean ± SD)	59.8 ± 12.0	57.3 ± 13.0	0.52[a]
Sex			
Male	23	13	0.99[b]
Female	7	4	
Primary site			
Hypopharynx	19	5	0.044[c]
Oropharynx	3	6	
Larynx	7	5	
Neck + unknown primary	1	1	
TNM[d]			
T_1	7	7	0.25[e]
T_2	8	6	
T_3	7	1	
T_4	7	2	
T_x	1	1	
			0.82[e]
N_0	12	7	
N_1	11	7	
N_2	5	3	
N_3	1	1	
Primary treatment			
Surgery	8	6	0.53[b]
Surgery + RT[d]	22	11	
Recurrence			
Loco-regional	5	2	
Distant	6	1	

[a] *t* test with Welch correction for unequal variances.
[b] χ^2 test.
[c] χ^2 test: exact *P*.
[d] TNM, tumor (T)-nodes (N)-metastasis; RT, radiotherapy.
[e] χ^2 test for trend

lines that do not express cyclin D1, such as H9 which only expresses cyclin variants D2 and D3 or Saos-2 cells, which do not contain any of the D cyclins.[3] Using this antiserum, we obtained in breast tumor specimens the same staining patterns as those described previously by Gillett *et al.* (17) using fixation in methacarn.[3]

Prognostic Significance of Overexpression of Cyclin D1. The primary aim of this investigation was to determine whether overexpression of cyclin D1 could serve to identify a proportionally distinctive group of HNSCC patients. For this study, we used a selected group of tumors still amenable to surgical treatment. This is reflected in the composition of this group (Table 1), which shows an overrepresentation of the lower T and N stages. This is also evident from the overall survival data of this group, with a 5-year overall survival rate of 70%, *versus* an average of 25% for a random group of HNSCC patients (18). Despite the selected nature of this group of HNSCC patients, we still detected a significant association of tumor site and survival (Table 2), but not of tumor size or nodal status alone.

We observed strong staining (++) for cyclin D1 in 10 of the 47 HNSCC (21%) and a (+) staining in a further 20 cases (43%). Examples of the staining patterns are shown in Fig. 2. After microwave pretreatment, the B31S antiserum detected a clear nuclear staining in tumors with an overexpression of cyclin D1. Normal tissue did not stain. The staining was absent when the antiserum was preabsorbed with an excess of *in vitro* synthesized cyclin D1 protein. The staining intensity correlated with the degree of cyclin D1 amplification.[3] As shown in Fig. 3*A*, the Kaplan-Meier disease-free interval curve demonstrated that when all patients were considered, tumors recurred much more rapidly and more frequently when the primary tumors overexpressed cyclin D1 as compared with primary tumors lacking such an overexpression ($P = 0.027$). This is especially the case when more than 50% of the tumor cells were positive (++) for cyclin D1. Overall survival also appeared to be significantly associated with overexpression of cyclin D1 ($P = 0.0095$; see Fig. 3*B*). Our findings indicate a correlation between overexpression of cyclin D1 and recurrence of HNSCC treated with curative intent and confirm earlier reported associations between amplification of cyclin D1 and a more aggressive phenotype of HNSCC (10–13). The reported frequency of amplification of the 11q13 region encompassing cyclin D1 varies in HNSCC tumors from 30 to 60% (10–13). This variation may be largely influenced by the relatively small groups of tumors studied and by the sensitivity of the methods used to determine amplification of cyclin D1. Moreover, overexpression of cyclin D1 results not only from amplification but also from other deregulations of cyclin D1 gene expression (2). Gillet *et al.* (17) and we[3] have shown that overexpression of cyclin D1 in breast tumors as detected by immunohistochemistry occurred almost twice as frequently as cyclin D1 amplification. This may explain the higher frequency of 64% of the HNSCC tumors positive for cyclin D1 overexpression in our series.

Overexpression of cyclin D1 appeared not to be associated with tumor stage. This discrepancy is probably due to the selection of

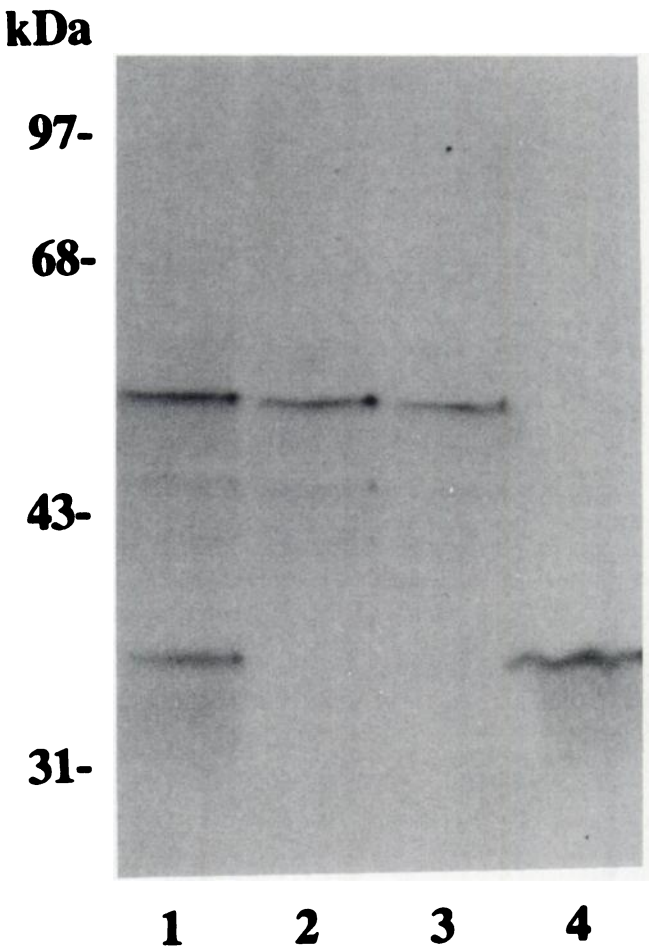

Fig. 1. Affinity-purified B31S antiserum detects cyclin D1. Equal amounts of exponentially growing UMSCC2 cells were labeled for 30 min with ^{35}S-protein labeling mix, and aliquots of cell lysates were subjected to immunoprecipitation using 5 μl of affinity-purified B31S antiserum (*Lanes 1* and *2*) or Sepharose-protein A beads as control (*Lane 3*). For *Lane 2*, the antiserum had been preincubated with 2.5 μg glutathione S-transferase cyclin D1 protein for 1 h, at 37°C. In *Lane 4*, 10^4 cpm of *in vitro* [^{35}S]methionine labeled cyclin D1 protein was applied to the gel. *kDa*, molecular weight in thousands.

[3] R. Michalides, Ph. Hageman, H. van Tinteren, L. Houben, E. Wientjens, R. Klompmaker, and H. Peterse. A clinico-pathological study on overexpression of cyclin D1 and of p53 in a series of 248 patients with operable breast cancer, submitted for publication.

Table 2 *P* values[a] for association with survival and disease-free interval in 47 HNSCC cases

	Survival	DFI[b]
Cyclin D1	0.0095	0.027
Location	0.0071	0.033
T	0.73	0.25
N	0.62	0.56
Stage	0.61	0.41

[a] *P* values according to the global log rank test for location.
[b] DFI, disease-free interval; T, tumor-stage; N, nodal stage.

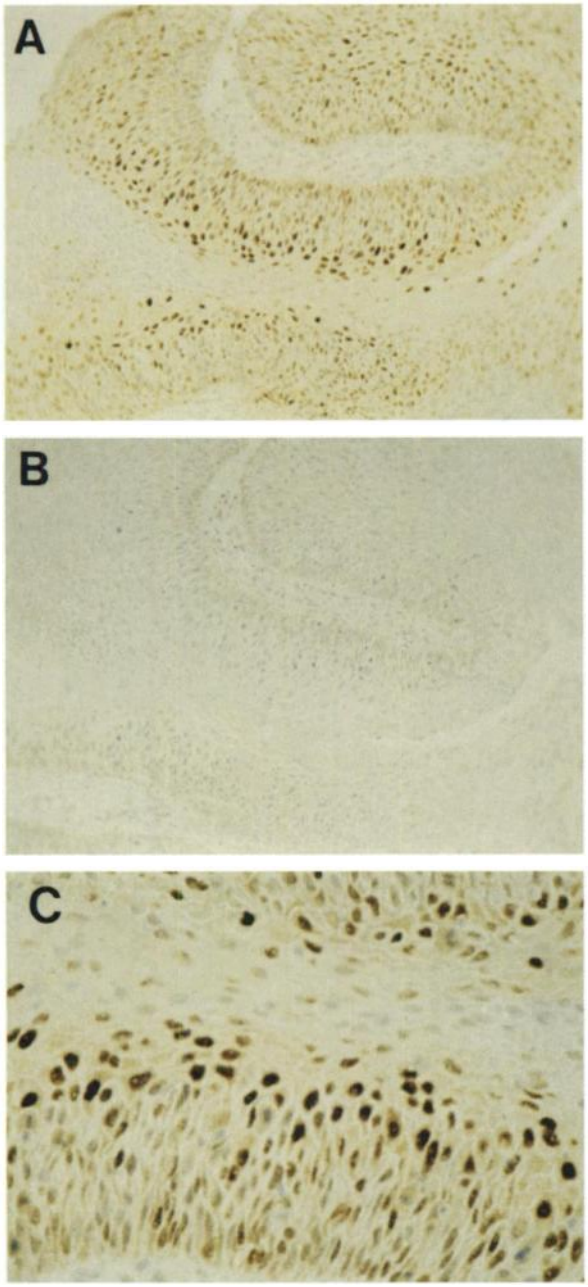

Fig. 2. Immunohistochemical staining of cyclin D1 in HNSCC tumors. *A*, strong positive (++) cyclin D1 staining of a primary oropharynx carcinoma, tumor T 88-5232. Notice the specific nuclear staining of the tumor cells and the absence of staining in stromal cells. (×100); *B*, negative staining of the same tumor using an antibody directed against p53; *C*, strong positive (++) cyclin D1 staining of oropharynx carcinoma, T 88-8428. ×250. Notice the strict nuclear staining.

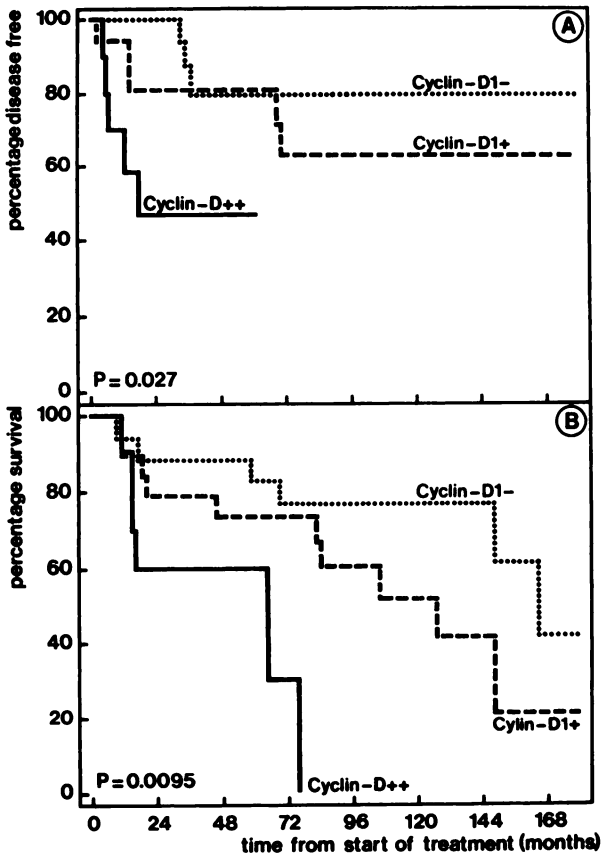

Fig. 3. Kaplan-Meier disease-free interval (*A*) and survival (*B*) curves of 47 operable HNSCC patients stratified for overexpression of cyclin D1.

operable tumors and therefore of tumors with a relatively favorable prognosis in this study. The lack of any association between overexpression of cyclin D1 and tumor size and stage state suggests that overexpression of cyclin D1 could well serve as an independent prognostic marker. These results justify an immunohistochemical study on a larger scale including other treatment modalities provided that cyclin D1 overexpression in biopsy material will be informative of the tumor. The association between overexpression of cyclin D1 and tumor recurrence in this group of operable HNSCC patients is of practical clinical relevance since it identifies a group of patients at increased risk of tumor recurrence who may benefit from more intensive treatment.

Acknowledgments

We thank R. Klompmaker, P. Wisman, and Dr. Ph. Hageman for advice and technical assistance; Dr. R. Bernards for the kind gift of *in vitro* [^{35}S]methionine-labeled cyclin D1 protein; Drs. W. Mooi and F. Hilgers for critical reading of the manuscript; and Drs. F. Hilgers and R. Gregor for sampling of the tumors.

References

1. Lammie, G., and Peters, G. Chromosome 11q13 abnormalities in human cancer. Cancer Cells, *3:* 413–417, 1991.
2. Schuuring, E., Verhoeven, E., Mooi, W. J., and Michalides, R. J. A. M. Identification and cloning of two overexpressed genes, *U21B31/PRAD1* and *EMS1*, within the amplified chromosome 11q13 region in human carcinomas. Oncogene, *7:* 355–361, 1992.
3. Rosenberg, C. L., Kim, H. G., Shows, T. B., Kronenberg, H. M., and Arnold, A. Rearrangement and overexpression of *D11S287E*, a candidate oncogene on chromosome 11q13 in benign parathyroid tumors. Oncogene, *6:*449–453, 1991.
4. Motokura, T., Bloom, T., Kim, H. G., Jüppner, H., Ruderman, J. M., Kronenberg, H. M., and Arnold, A. A novel cyclin encoded by a *bcl1*-linked candidate oncogene Nature (Lond.), *350:*512–515, 1991.

5. Hinds, P. W., Dowdy, S. F., Ng Eaton, E., Arnold, A., and Weinberg, R. A. Function of a human cyclin gene as an oncogene. Proc. Natl. Acad. Sci. USA, *91:*709–713, 1994.
6. Wang, T. C., Cardiff, R. D., Zukerberg, L., Lees, E., Arnold, A., and Schmidt, E. V. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature (Lond.), *369:*669–671, 1994.
7. Bodrug, S. E., Warner, B. J., Bath, M. L., Lindemans, G. J., Harris, A. W., and Adams, J. M. Cyclin D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the *myc* gene. EMBO J., *13:*2124–2130, 1994.
8. Schuuring, E., Verhoeven, E., Van Tinteren, H., Peterse, J. L., Nunnink, B., Thunnissen, F. B. J. M., Devilee, P., Cornelisse, C. J., Van de Vijver, M. J., Mooi, W. J., and Michalides, R. J. A. M. Amplification of genes within the chromosome 11q13 region is indicative of poor prognosis in patients with operable breast cancer. Cancer Res., *52:*5229–5234, 1992.
9. Borg, A., Sigurdsson, H., Clark, G. M., Fernö, M., Fuqua, S. A. W., Olsson, H., Killander, D., and McGurie, W. L. Association of *int-2/hst-1* coamplification in primary breast cancer with hormone-dependent phenotype and poor prognosis. Br. J. Cancer, *63:*136–142, 1991.
10. Callender, T., El-Nagger, A. K., Lee, M. S., Frankenthaler, R., Luna, M. A., and Batsakis, J. G. *PRAD-1* (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. Cancer (Phila.), *74:*152–158, 1994.
11. Field, J. K. Oncogenes and tumour-suppressor genes in squamous cell carcinoma of the head and neck. Eur. J. Cancer, *28:*67–76, 1992.
12. Parise O., Jr., Janot, F., Guerry, R., Fortin, A., Luboinski, B., Tursz, T., Schwaab, G., and Busson, P. Chromosome 11q13 gene amplifications in head and neck squamous cell carcinomas: relation with lymph node invasion. Int. J. Oncol., *5:*309–313, 1994.
13. Muller, D., Millon, R., Lidereau, R., Engelmann, A., Bronner, G., Flesh, H., Eber, M., Methlin, G., and Abecassis, J. Frequent amplification of 11q13 DNA markers is associated with lymph node involvement in human head and neck squamous cell carcinomas. Eur. J. Cancer, *2:*113–120, 1994.
14. Greenwell, A., Foley, J. F., and Maronpot, R. R. An enhancement method for immunohistochemical staining of proliferating cell nuclear antigen in archival rodent tissues. Cancer Lett., *59:*251–256, 1991.
15. Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:*457–481, 1958.
16. Mantel, N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother. Rep., *50:*163–170, 1966.
17. Gillett, C., Fantl, V., Smith, R., Fisher, C., Bartek, J., Dickson, C., Barnes, D., and Peters, G. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res., *54:*1812–1817, 1994.
18. Tobias, J. S. Cancer of the head and neck. Br. Med. J., *308:*961–966, 1994.

Overexpression of Cyclin D1 Correlates with Recurrence in a Group of Forty-seven Operable Squamous Cell Carcinomas of the Head and Neck

Rob Michalides, Nicoletta van Veelen, Augustinus Hart, et al.

Cancer Res 1995;55:975-978.