

# Gene Amplification and Overexpression of CDK4 in Sporadic Breast Carcinomas Is Associated with High Tumor Cell Proliferation

Han-Xiang An,\* Matthias W. Beckmann,\*  
Guido Reifenberger,<sup>†</sup> Hans G. Bender,\* and  
Dieter Niederacher\*

From the Department of Obstetrics and Gynecology,\* and Center for Biological and Medical Research (BMFZ), Heinrich-Heine-University, Düsseldorf, Germany and the Department of Neuropathology,<sup>†</sup> University of Bonn, Germany

**Amplification of the cyclin-dependent kinase 4 (*CDK4*) gene, located at 12q13-q14, has been found as an alternative genetic alteration to *CDKN2A* inactivation in various human tumors including malignant gliomas and sarcomas. In the present study, we have evaluated the frequency of the *CDK4* gene amplification in sporadic breast cancer by applying a non-radioactive quantitative differential polymerase chain reaction based on fluorescent DNA technology. Fluorescent-labeled polymerase chain reaction products were analyzed with an automated DNA sequencer. Amplification of *CDK4* gene was detected in 15 (15.8%) of 95 breast cancers. All tumors with *CDK4* gene amplification showed high CDK4 protein expression determined by immunohistochemistry. Furthermore, the mean Ki-67 labeling index in tumors with *CDK4* gene amplification was significantly higher than in those without *CDK4* gene amplification. No significant associations were observed between *CDK4* gene amplification and any specific histopathological parameter. The findings of this study provide the first evidence of *CDK4* gene amplification in breast cancer and suggest that *CDK4* gene amplification appears to be of importance in the pathogenesis of a subset of sporadic breast cancer. (*Am J Pathol* 1999, 154:113–118)**

Critical transitions in the different phases of the cell cycle are regulated by sequential activation of cyclins and their catalytic subunits, the cyclin-dependent kinases (CDKs). Disruption of the cell cycle machinery might enhance genomic instability, contribute to uncontrolled cell growth, and lead to the development of cancer.<sup>1–3</sup> When activated by cyclin D1, CDK4 is able to phosphorylate the retinoblastoma gene product (pRB) and can promote cell cycle progression through G1-phase into S-phase.<sup>3,4</sup> The activation of CDK4, however, can be constrained by

binding the p16 protein, a cyclin-dependent kinase inhibitor encoded by the *CDKN2A* gene.<sup>3,5</sup> Alterations of these individual components have been implicated in the pathogenesis of many tumor types. The involvement of *CDK4* gene in tumorigenesis has been suggested by the findings that suppression of CDK4 can lead to terminal differentiation of erythroleukemia cells, whereas overexpression of CDK4 can induce uncontrolled cell growth and eventual malignant transformation.<sup>5</sup> Furthermore, amplification and consequent overexpression of the *CDK4* gene, located in the 12q13-q14 region, have been found in various cancers including different types of sarcomas and glioblastomas.<sup>6–8</sup> A somatic point mutation (R24C) of *CDK4* gene was identified in human melanomas, causing a tumor-specific antigen and disrupting the interaction between CDK4 and its inhibitor p16.<sup>9</sup>

Alterations of cell cycle control genes, such as amplification of *cyclin D1*<sup>10,11</sup> and inactivation of *RB* and *CDKN2A*,<sup>12–14</sup> have been well documented in human breast cancer. However, there has been no report on amplification and overexpression of *CDK4* in breast cancer so far, although amplification of 12q13 has been detected by cytogenetic analyses in breast cancer.<sup>15,16</sup> Moreover, increased expression of CDK4 was found to be common in carcinogen-induced rat mammary tumors.<sup>17</sup>

To delineate the role of the *CDK4* gene in the genesis of breast cancer, we investigated *CDK4* gene amplification in breast cancer by fluorescent differential polymerase chain reaction (PCR) followed by fragment analysis on an automated DNA sequencer. This approach enables rapid, nonradioactive quantitative analysis of gene amplification on small tumor samples.<sup>18</sup> The biological relevance of *CDK4* gene amplification was examined by immunohistochemical staining for expression of CDK4 protein in breast cancer. *CDK4* gene amplification and expression were correlated to relevant clinical and tumor characteristics.

---

Supported in part by Grant Be 1215/6-3 from the Deutsche Forschungsgemeinschaft, Bonn, Germany.

Han-Xiang An's current address is Division of Cytogenetics (H400), German Cancer Research Center, INF 280, 69120 Heidelberg, Germany.

Accepted for publication October 4, 1998.

Address reprint requests to Dr. D. Niederacher, Molekulargenetisches Labor, Frauenklinik, Heinrich-Heine Universität, Moorenstrasse 5, D-40225 Düsseldorf, Germany. E-mail: niederac@uni-duesseldorf.de.

## Materials and Methods

### Tumor Samples and DNA Extraction

Snap-frozen samples were obtained from 95 patients treated by surgery for primary breast cancer (80 ductal and 15 lobular breast carcinomas) at the Department of Obstetrics and Gynecology, Heinrich-Heine-University Düsseldorf, Germany. None of the breast carcinoma patients had a positive family history (at least two cases of breast or ovarian cancer, one case below the age of 60 years). None of the patients had distant metastases at the time of primary surgery. In case of axillary dissection (at least 10 lymph nodes), the number of lymph node metastases was determined. Tumors were classified according to the TNM classification (Union Internationale Contre le Cancer). The histological grade was determined according to the criteria of Elston and Ellis.<sup>19</sup> Histological evaluation of frozen tumor sections assured that all specimens studied contained at least 60% tumor cells. High molecular weight DNA was prepared from tumor tissues and blood lymphocytes as described previously.<sup>20</sup> DNA from 20 malignant gliomas with *CDK4* gene copy number determined previously by Southern blot analysis<sup>8</sup> were used as controls to establish the differential PCR assay.

### Fluorescent Differential PCR

The gene dosage of the *CDK4* gene was analysed by differential PCR with fluorescein-labeled primers.<sup>18</sup> Two different reference loci were used: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) on 12p13 and adenine phosphoribosyltransferase (*APRT*) on 16q23. Primer sequences for *CDK4* and the control loci were as follows: 5'-CATGTAGACCAGGACCTAAGG (sense) and 5'-AACTGGCGCATCAGATCCTAG (antisense) for *CDK4* resulting in a 206-bp PCR product, 5'-F-AACGTGT-CAGTGGTGGACCTG (sense) and 5'-AGTGGGT-GTCGCTGTTGAAGT (antisense) for *GAPDH* generating a 160-bp PCR product, and 5'-TGGGAAAGCTGTTTACT-GCG (sense) and 5'-CAGGGAACACATTCCTTTGC (antisense) for *APRT* generating a 134-bp PCR product. One primer of each primer pair was labeled with fluorescein at the 5'-end. PCR amplification was carried out in a 50- $\mu$ l volume containing 50 ng of genomic DNA, 1 $\times$  PCR buffer (10 mmol/L Tris/HCl, pH 9.0, 50 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 0.001% gelatine), 150  $\mu$ mol/L dNTPs, 0.3  $\mu$ mol/L primers for *CDK4* and *GAPDH* or *APRT*, and 2.0 units Taq DNA polymerase (Pharmacia, Freiburg, Germany). The reaction consisted for 25 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute followed by a final extension for 8 minutes at 72°C. In each PCR experiment, DNA extracted from 1) placenta and peripheral blood leukocytes as reference tissue with normal *CDK4* gene copy number and 2) two primary gliomas with known *CDK4* gene amplification were included as controls. The fluorescein-labeled PCR products were separated with an automated fluorescent DNA sequencer (A. L. F.<sup>TM</sup>, Pharmacia) on 6% denaturing polyacrylamide

gels. Quantitative analysis of the peak areas obtained for *CDK4* and *GAPDH* or *APRT* was performed with the Fragment Manager<sup>TM</sup> (FM1.1) software (Pharmacia), and *CDK4* gene dose was calculated relative to control blood as described.<sup>18</sup> Only increases in the *CDK4/GAPDH* and *CDK4/APRT* quotients of more than three times relative to control blood and normal placenta were considered as evidence of *CDK4* gene amplification.

### Southern Blot Analysis

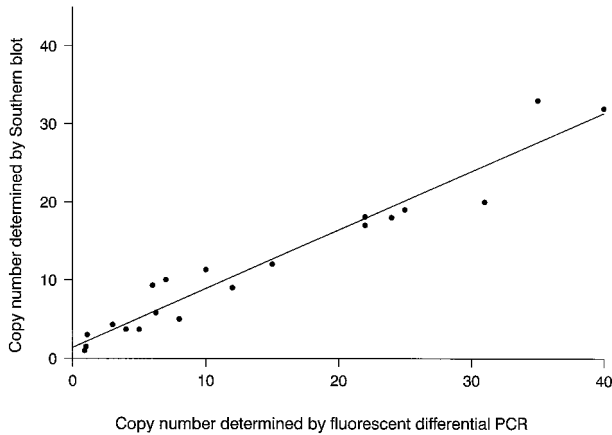
DNA from tumors and normal leukocytes (2.5  $\mu$ g) were digested with the restriction enzyme *TaqI*, separated on 0.8% agarose gels, and blotted. The membranes were sequentially hybridized with probes for *CDK4* and the control locus *ERBB3*, as described.<sup>8</sup>

### Immunohistochemistry

Immunohistochemical stainings for *CDK4* protein, the proliferation-associated nuclear antigen Ki-67, ER, and PgR were performed on paraffin-embedded formalin-fixed sections using the indirect avidin-biotin peroxidase method as described.<sup>21</sup> To enhance immunoreactivity for Ki-67, sections were pretreated by microwave heating in 10 mmol/L citrate buffer (pH 6.0) for 3  $\times$  10 minutes. Sections to be stained for *CDK4* protein were treated in 0.1% trypsin for 5 minutes at 37°C. Histological sections were incubated for 30 minutes in methanol with 1% hydrogen peroxide. The sections were incubated with goat anti-*CDK4* polyclonal antibody (C-22, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), MIB-1 mouse anti-Ki-67 monoclonal antibody (Dianova, Hamburg, Germany), mouse anti-ER monoclonal antibody, or mouse anti-PgR monoclonal antibody (Sigma, Deisenhofen, Germany) for 16 hours at 4°C. This was subsequently followed by biotinylated secondary antibodies and by avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. As negative controls, irrelevant rabbit/mouse monoclonal IgG were used instead of the primary antibodies. ER and PgR immunoreactivities were analysed and scored (according to recommendations of Remmele and Stegner) as described previously.<sup>20</sup> Immunoreactivity for *CDK4* and Ki-67 was quantified by counting positive and negative tumor cells. A minimal number of 1000 cells was counted for each case. For *CDK4* expression, a semiquantitative scoring was applied as described.<sup>22</sup> The assigned score first reflects the staining intensity A (0, negative; 1, weak; 2, moderate; 3, high) and second the percentage of positive cells B (0, no positive cells; 1, <25% positive cells; 2, 25 to 50% positive cells; 3, >50% positive cells). The sum of A + B attained a maximum score of 6. A score more than 3 was considered as positive.

### Statistical Analysis

Associations of *CDK4* gene amplification and other factors were calculated by Fisher's exact test or Student's unpaired *t*-test.



**Figure 1.** Correlation between Southern blot analysis and fluorescent differential PCR. Copy number of *CDK4* in 20 human malignant gliomas was determined by fluorescent differential PCR (PCR conditions see Material and Methods) and compared with results from previous Southern blot analysis.<sup>8</sup> The correlation coefficient was 0.86, the correlation rate was highly significant ( $P < 0.01$ ) according to Student's *t*-test.

## Results

### Validation of Fluorescent Differential PCR

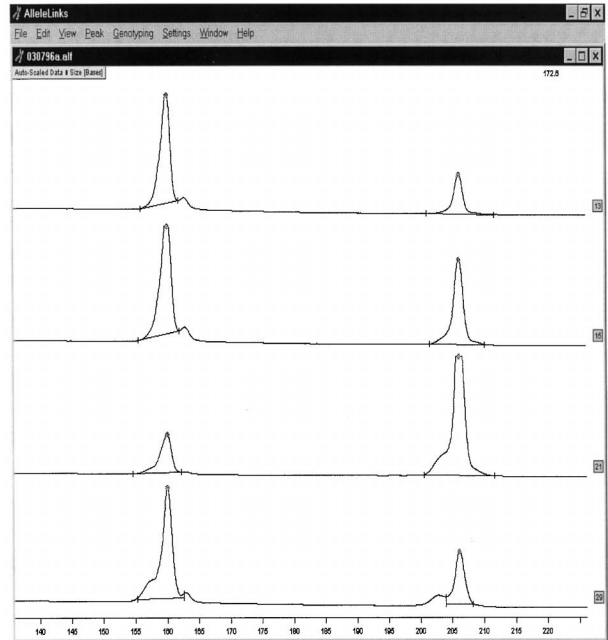
The fluorescent differential PCR method was established and optimized in terms of amplimers, enzyme concentration, magnesium concentration, and cycle number to establish conditions for exponential amplification of *CDK4* and *GAPDH* or *APRT* specific sequences. To validate the sensitivity of differential PCR combined with detection of fluorescent-labeled PCR products by an automated DNA sequencer, *CDK4* gene amplification of 20 malignant gliomas was determined by fluorescent differential PCR and compared with results from previous Southern blot analysis.<sup>8</sup> Results of gene copy numbers showed a linear relationship with a correlation coefficient of 0.86 between detection of *CDK4* by fluorescent differential PCR and by Southern blot (Figure 1).

### CDK4 Amplification in Breast Cancer

Ninety-five breast cancers were analyzed for *CDK4* gene amplification by the fluorescent differential PCR. Among the 95 tumors analyzed, 15 (15.8%) were found to demonstrate *CDK4* amplification ranging from 3- to 10-fold (12 tumors corresponded to a three to fivefold amplification, three tumors presented 5- to 10-fold amplification). Representative examples of *CDK4* amplification are shown in Figure 2.

### Expression of CDK4 Protein in Breast Cancer

In order to determine if *CDK4* gene amplification also resulted in overexpression of the gene and increased levels of the gene product, immunohistochemical analysis of the tumor tissue specimens including the 15 breast cancers with *CDK4* gene amplification was performed by using an antibody directed against CDK4 protein. Moderate and intense nuclear and/or cytoplasmic stainings



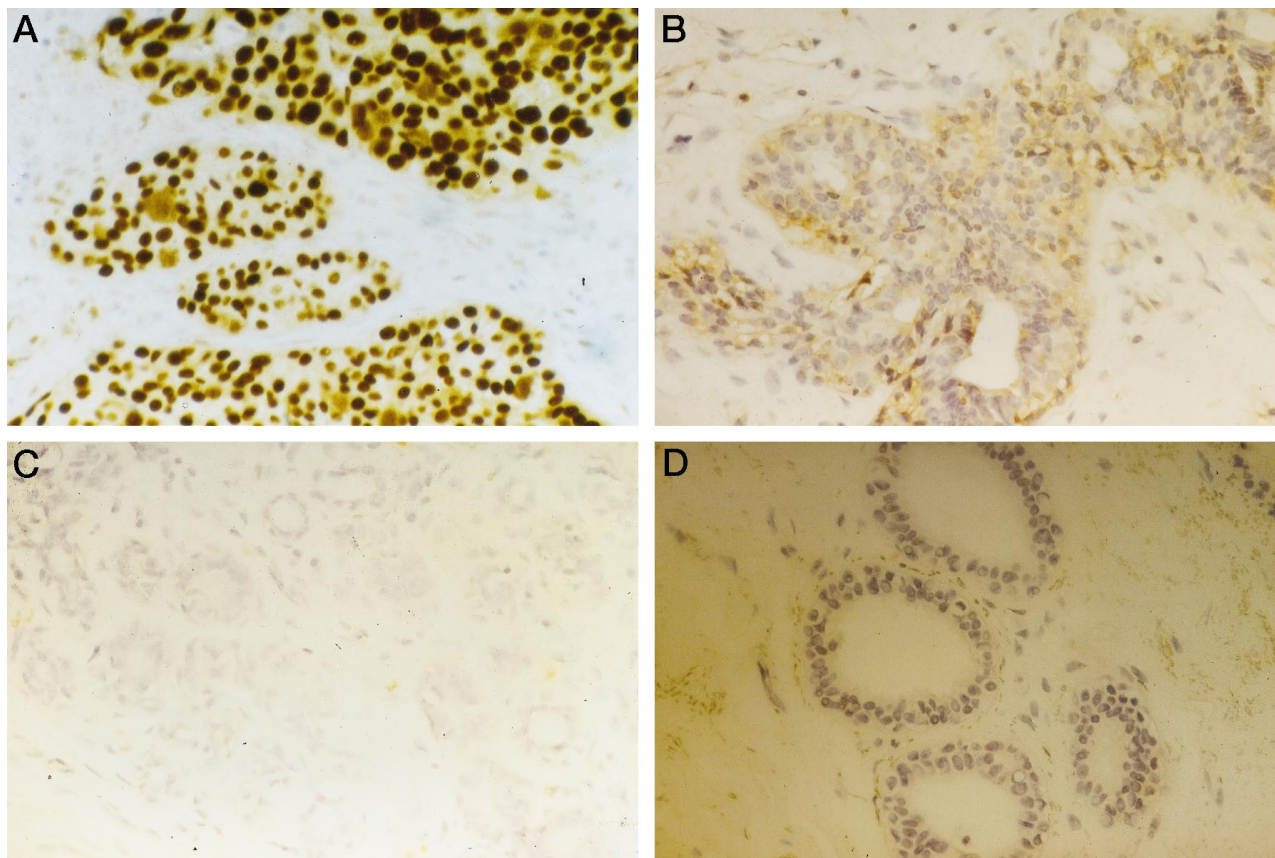
Lane	Peak	Peak area	CDK4/GAPDH	CDK4 amplification
13	1	4701.7	0.38	1.0
	2	1786.4		
15	1	4821.6	1.32	3.3
	2	6368.5		
21	1	1921.8	4.02	9.8
	2	7689.8		
29	1	5099.2	0.40	1.0
	2	2003.7		

**Figure 2.** Analysis of *CDK4* amplification by fluorescent differential PCR. Electrophoretogram of PCR-products of *CDK4* (206 bp) and the control gene *GAPDH* (160 bp) in representative cases. The data were obtained with an automated fluorescent DNA sequencer (A. L. F., Pharmacia) and analyzed with the Fragment Manager™ software. The abscissa of the electrophoretogram shows the fragment size in bp. Lane 13, tumor DNA without *CDK4* amplification; lane 15, tumor DNA with *CDK4* amplification (threefold); lane 21, tumor DNA with *CDK4* amplification (10-fold); lane 29, placenta DNA as normal control.

were observed in breast cancer cells, and distribution of positive cells was heterogeneous in a cancer nest and between cancer nests (Figure 3, A and B). All 15 tumors with *CDK4* gene amplification showed overexpression of the CDK4 protein. Overexpression of the CDK4 protein was also observed in three tumors without gene amplification. The other breast cancer samples were negative for CDK4 protein, although scattered CDK4-positive tumor cells were found in some instances (Figure 3C).

### Relationships between CDK4 Gene Amplification and Clinicopathological Parameters

The association between *CDK4* gene amplification and clinicopathological characteristics was determined. No significant associations were found between *CDK4* gene amplification and patient's age, tumor size, and lymph-node status. Although statistically not significant, *CDK4* gene amplification was observed more frequently in tumors of higher histological grade (Table 1). The mean



**Figure 3.** Immunohistochemical demonstration of CDK4 protein expression in breast carcinomas. Representative examples of invasive ductal adenocarcinomas with CDK4 amplification showing strong nuclear staining (A) or moderate cytoplasmic staining (B). Negative staining results in an invasive ductal breast carcinoma without *CDK4* amplification (C) and in normal mammary epithelial cells (D). Original magnification,  $\times 400$ .

Ki-67 labeling index was significantly higher in breast cancers with *CDK4* gene amplification and overexpression than in tumors with normal copy number of *CDK4* gene ( $P < 0.05$ ; Table 2).

### Discussion

Oncogene amplification is a common mechanism of proto-oncogene activation and contributes to neoplastic cell transformation and tumor progression. Detection of oncogene amplification using fluorescent differential PCR as well as application of automated fluorescent DNA technology for LOH analysis have been described by various groups.<sup>18,23,24</sup> In this study, differential PCR and automated fluorescent DNA technology were applied for quantitative determination of *CDK4* amplification in breast cancers. This approach offers several advantages compared with other detection and staining methods: 1) Separation and quantitative determination of PCR products can be performed automatically without the delay inherent in autoradiography, requiring approximately 3 hours only. 2) The enhanced sensitivity of the fluorescent detection method requires 25 PCR cycles only to achieve detectable results and allows to analyze PCR products in the exponential phase of the PCR reaction. Furthermore, linearity of fluorescence detection covers a much wider

**Table 1.** Correlation between *CDK4* Amplification and Clinicopathological Characteristics of 95 Breast Cancer Patients

Characteristics	<i>n</i> = 95	<i>CDK4</i> amplification	<i>P</i> value
Age (years)			
<50	25	4	$P > 0.05$
$\geq 50$	70	11	
Tumor size			
pT1	50	6	$P > 0.05$
pT2	33	6	
pT3/4	12	3	
Lymph node status			
N-	47	6	$P > 0.05$
N+	48	9	
Grade			
G1	12	1	$P > 0.05$
G2	58	8	
G3	25	6	
ER			
Negative	43	7	$P > 0.05$
Positive	52	8	
PgR			
Negative	39	6	$P > 0.05$
Positive	56	9	

**Table 2.** Correlation between *CDK4* Amplification and Nuclear Antigen Ki-67 Labeling Index of 95 Breast Cancers

<i>CDK4</i>	<i>n</i>	Index of Ki-67 (%) mean ± standard
Overexpression with gene amplification	15	21.3 ± 4.9*
Overexpression without gene amplification	3	14.8
No amplification	77	4.7 ± 3.2

\*Significant difference compared with tumors without *CDK4* gene amplification ( $P < 0.05$ , *t*-test).

range than scanning of autoradiograms or ethidium bromide or silver-stained gels, resulting in an improved quality of data. Therefore, this approach is particularly suitable for the routine clinical analysis of genetic alterations in large series of samples.

By cytogenetic analysis, translocations and amplifications on 12q13 have been detected in karyotypes of breast carcinomas.<sup>15,16</sup> Furthermore, increased expression of CDK4 occurs frequently in carcinogen-induced rat mammary tumors.<sup>17</sup> These findings have raised the hypothesis that amplification of *CDK4* might be one of the genetic alterations in breast cancer, which contribute to disruption of cell cycle control in the pathogenesis of breast cancer. In the present study, we provide the first evidence of amplification of the *CDK4* gene in breast carcinomas. The frequency of *CDK4* amplification in breast carcinomas (15.8%) determined here is similar to that observed in malignant gliomas (13 to 15%) but lower than the frequencies reported for human sarcomas (27 to 50%). Two previous studies on expression of CDK4 protein in breast cancer reported that immunoreactivity of CDK4 protein was found in between 80 and 95% of breast carcinomas analyzed by immunohistochemical staining.<sup>25,26</sup> In contrast, overexpression of CDK4 protein was detected in 19% of our series of breast cancers. This discrepancy should be attributed to the difference in applied methods, the criteria for scoring of immunoreactivity, and characteristics between the various primary carcinoma tissues used for different studies. All breast tumors of our series with *CDK4* gene amplification showed overexpression of CDK4 protein determined by immunostaining. These findings are in line with recent studies on other cancers and cancer cell lines revealing increased levels of CDK4 protein in association with gene amplification.<sup>27,28</sup> In addition, overexpression of CDK4 without gene amplification in three breast cancers may represent increased transcription or post-translational modification of the protein in cancer cells. The recent report of Zhang et al<sup>29</sup> has demonstrated that overexpression of CDK4 protein is accompanied by a significant increase in the number of proliferating cells in colon adenomas as shown by BrdU incorporation and immunostaining of proliferating cell nuclear antigen (PCNA). Ito et al<sup>25</sup> have found that overexpression of CDK4 determined by immunostaining was significantly more heterogeneous in breast tumors of larger sizes and/or at higher stages. In agreement with these observations, a signifi-

cant relationship between *CDK4* amplification and Ki-67 labeling index has been found in breast cancers in this study. The results presented here as well as the finding of Ito et al thus indicate an important role of *CDK4* amplification and overexpression in human breast carcinomas.

Alterations in the cell cycle regulatory pathway involving p16, pRB, cyclin D1, and CDK4 have been implicated in tumorigenesis. Inactivation of *RB* and *CDKN2A* or activation of *cyclin D1* or *CDK4* have been demonstrated in various types of human cancers.<sup>1-3,5</sup> Furthermore, it has been hypothesized that alterations of any of the components in the growth-regulatory pathway (p16, pRB, cyclin D1, and CDK4) may have a similar effect leading to disturbance of the cell cycle regulatory system in favor of cell proliferation.<sup>2,30</sup> This hypothesis is supported by the following observations: 1) *CDK4* amplification and alteration of *CDKN2A* are not found concomitant in human gliomas and sarcomas<sup>27,31,32</sup>; 2) loss of expression of p16 is restricted to tumors with wild-type *RB* in lung cancers and glioblastomas<sup>33,34</sup>; and 3) amplification of *cyclin D1* is associated inversely with mutation of *RB* in esophageal cancers.<sup>35</sup> Previous studies in breast cancer have reported that amplification of *cyclin D1*<sup>10,11,35</sup> and inactivation of *RB*<sup>12,13</sup> are present in a significant fraction of breast carcinomas. Hypermethylation of the 5'-CpG island of the *CDKN2A* gene associated with loss of *CDKN2A* transcription is also frequently found in primary breast cancers,<sup>14</sup> although homozygous deletions and point mutations of this gene are rare in breast cancers.<sup>36</sup> As presented here, *CDK4* gene amplification may be an additional genetic alteration in breast cancer. Additional functional studies of CDK4 protein kinase activity and the interactions of cell cycle regulatory proteins in breast cancer with *CDK4* amplification and overexpression may clarify and extend the importance of the role of this regulatory pathway in the pathogenesis of breast cancers.

### Acknowledgments

The authors thank the staff from the operating room and the technicians from the Laboratory of the Pathomorphology, Frauenklinik, Heinrich-Heine Universität, Düsseldorf, Germany, for the recruitment of tumors and their persistent support. Thanks to Mrs. Dorotheé Larbig for expert technical assistance.

### References

- Sherr CJ: Mammalian G1 cyclins. *Cell* 1993, 73:1059-1065
- Hartwell LH, Kastan MB: Cell cycle control and cancer. *Science* 1994, 266:1821-1828
- Hunter T, Pines J: Cyclins and cancer: II. Cyclin D and CDK inhibitors come of age. *Cell* 1994, 79:573-582
- Matsushime H, Ewen ME, Strom DK, Kato JY, Hanks SK, Roussel MF, Sherr CJ: Identification and properties of an atypical catalytic subunit (p34<sup>PSK-J3/cdk4</sup>) for mammalian D type G1 cyclins. *Cell* 1992, 71:323-334
- Xiong Y, Zhang H, Beach D: Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. *Genes Dev* 1993, 7:1572-1583
- Khatib ZA, Matsushime H, Valentine M, Shapiro DN, Sherr CJ, Look

- AT: Coamplification of the CDK4 gene with MDM2 and GLI in human sarcomas. *Cancer Res* 1993, 53:5535-5541
7. Collins VP: Gene amplification in human gliomas. *Glia* 1995, 15:289-296
  8. Reifemberger G, Reifemberger J, Ichimura K, Meltzer PS, Collins VP: Amplification of multiple genes from chromosomal region 12q13-14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS, and MDM2. *Cancer Res* 1994, 54:4299-4303
  9. Wölfel T, Hauer M, Schneider J, Serrano M, Wölfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Buschenfelde KH, Beach DA: p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* 1995, 269:1281-1284
  10. Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CK, Musgrove EA, Sutherland RL: Expression and amplification of cyclin genes in human breast cancer. *Oncogene* 1993, 8:2127-2133
  11. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G: Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res* 1994, 54:1812-1817
  12. Lee EY, To H, Shew JY, Bookstein R, Scully P, Lee WH: Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science* 1988, 241:214-221
  13. Lungberg C, Skoog L, Cavenee WK, Nordenskjöld M: Loss of heterozygosity in human ductal breast cancer indicates a recessive mutation on chromosome 13. *Proc Natl Acad Sci USA* 1987, 84:2372-2376
  14. Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D, Baylin SB: Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res* 1995, 55:4525-4530
  15. Geleick D, Müller H, Matter A, Thorhorst J, Regenass U: Cytogenetics of breast cancer. *Cancer Genet Cytogenet* 1990, 46:217-229
  16. Courjal F, Cuny M, Simony-Lafontaine J, Louason G, Speiser P, Zeillinger R, Rodriguez C, Theillet C: Mapping of DNA amplifications at 15 chromosomal localizations in 1875 breast tumors: definition of phenotypic groups. *Cancer Res* 1997, 57:4360-4367
  17. Sgambato A, Han EK, Zhang YJ, Moon RC, Santella RM, Weinstein IB: Deregulated expression of cyclin D1 and other cell cycle-related genes in carcinogen-induced rat mammary tumors. *Carcinogenesis* 1995, 16:2193-2198
  18. An HX, Niederacher D, Beckmann MW, Göhring UJ, Scharl A, Picard F, van Roeyen C, Schnürch H-G, Bender HG: ERBB2 gene amplification detected by fluorescent differential polymerase chain reaction in paraffin-embedded breast carcinoma tissues. *Int J Cancer* 1995, 64:291-297
  19. Elston CW, Ellis IO: Pathological prognostic factors in breast cancer: I. the value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991, 19:403-410
  20. Beckmann MW, Niederacher D, Massenkeil G, Tutschek B, Beckmann A, Schenko G, Schnürch H-G, Bender HG: Expression analyses of epidermal growth factor receptor and HER-2/neu: no advantage of prediction of recurrence or survival in breast cancer patients. *Oncology* 1996, 53:441-447
  21. Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981, 29:577-580
  22. Volm M, Koomägi R, Rittgen W: Clinical implications of cyclins, cyclin-dependent kinase, RB and E2F1 in squamous-cell lung carcinoma. *Int J Cancer* 1998, 79:294-299
  23. Cawkwell L, Lewis FA, Quirke P: Frequency of allele loss of DCC, p53, RB1, WT1, NF1, NM23 and APC/MCC in colorectal cancer assayed by fluorescent multiplex polymerase chain reaction. *Br J Cancer* 1993, 70:813-818
  24. Niederacher D, Picard F, van Roeyen C, An HX, Bender HG, Beckmann MW: Pattern of allelic loss on chromosome 17 in sporadic breast carcinomas detected by fluorescent labelled microsatellite analysis. *Genes Chromosome Cancer* 1997, 18:181-192
  25. Ito Y, Kobayashi T, Takeda T, Nakano Y, Tamaki Y, Komoike Y, Wakasugi E, Shin E, Takatsuka Y, Kikkawa N, Matsuura N, Monden M: Expression of p16 and cyclin-dependent kinase 4 proteins in primary breast carcinomas. *Oncology* 1997, 54:508-515
  26. Sasano H, Frost AR, Saitoh R, Taniyama Y, Nagura H, Matsunaga G, Takehana K, Kimura M, Silverberg SG: Immunolocalisation of cyclin D and E and cyclin dependent kinase (cdk) 2 and 4 in human breast carcinoma. *Anticancer Res* 1997, 17:3685-3690
  27. He J, Allen JR, Collins VP, Allalunis-Turner MJ, Godbout R, Day RS 3rd, James CD: CDK4 amplification is an alternative mechanism to p16 gene homozygous deletion in glioma cell lines. *Cancer Res* 1994, 54:5804-5807
  28. Rollbrocker B, Waha A, Louis DN, Wiestler OD, von Deimling A: Amplification of the cyclin-dependent kinase 4 (CDK4) gene is associated with high cdk4 protein levels in glioblastoma multiforme. *Acta Neuropathol* 1996, 92:70-74
  29. Zhang T, Nanney LB, Luongo C, Lamps L, Heppner KJ, DuBois RN, Beauchamp RD: Concurrent overexpression of cyclin D1 and cyclin-dependent kinase 4 (Cdk4) in intestinal adenomas from multiple intestinal neoplasia (Min) mice and human familial adenomatous polyposis patients. *Cancer Res* 1997, 57:169-175
  30. Serrano M, Hannon GJ, Beach DA: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D1/CDK4. *Nature* 1993, 366:704-707
  31. Maelandsmo GM, Florenes VA, Hovig E, Oyjord T, Engebraaten O, Holm R, Borresen AL, Fodstad O: Involvement of the pRb/p16/cdk4/cyclin D1 pathway in the tumorigenesis of sporadic malignant melanomas. *Br J Cancer* 1996, 73:909-916
  32. Schmidt EE, Ichimura K, Reifemberger G, Collins VP: CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. *Cancer Res* 1994, 54:6321-6324
  33. Otterson GA, Kratzke RA, Coxon A, Kim YW, Kaye FJ: Absence of p16INK4 protein is restricted to the subset of lung cancer lines that retains wildtype RB. *Oncogene* 1994, 9:3375-3378
  34. Ueki K, Ono Y, Henson JW, Efrid JT, von Deimling A, Louis DN: CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. *Cancer Res* 1996, 56:150-153
  35. Jiang W, Zhang YJ, Kahn SM, Hollstein MC, Santella RM, Lu SH, Harris CC, Montesano R, Weinstein IB: Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. *Proc Natl Acad Sci USA* 1993, 90:9026-9030
  36. Xu L, Sgroi D, Sterner CJ, Beauchamp RL, Pinney DM, Keel S, Ueki K, Rutter JL, Buckler AJ, Louis DN, Gusella JF, Ramesh V: Mutational analysis of CDKN2 (MTS1/p16ink4) in human breast carcinomas. *Cancer Res* 1994, 54:5262-5264