A Transgenic Mouse Model with Cyclin D1 Overexpression Results in Cell Cycle, Epidermal Growth Factor Receptor, and p53 Abnormalities

Annegret Mueller, Robert Odze, Timothy D. Jenkins, Ali Shahsesfai, Hiroshi Nakagawa, Takuya Inomoto, and Anil K. Rustgi

Gastrointestinal Unit [A. M., T. D. J., H. N., T. I., A. K. R.], Hematology-Oncology Unit [A. K. K.], Massachusetts General Hospital, and Department of Pathology, Brigham and Women’s Hospital [R. O., A. S.], Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT

The cyclin D1 oncogene is critical in the progression of the cell cycle through the G1 phase. It is frequently overexpressed in squamous cell carcinomas originating from the head/neck and esophagus. Yet, the functional consequences of aberrant cyclin D1 overexpression are not entirely understood apart from increased cell proliferation. To address this question, we have developed a transgenic mouse model in which the EBV ED-L2 promoter targets cyclin D1 to the stratified squamous epithelium in a tissue-specific fashion to the tongue and esophagus, thereby resulting in a dysplastic phenotype. We now demonstrate that the dysplastic phenotype is associated with increased cell proliferation based on proliferating cell nuclear antigen overexpression and abnormalities in cyclin-dependent kinase 4, epidermal growth factor receptor, and p53. In aggregate, these studies suggest that alterations in certain oncogenes and tumor suppressor genes occur early during head/neck and esophageal carcinogenesis.

INTRODUCTION

Cyclin D1 is a key protein that contributes to cell cycle progression through the G1 phase. It associates with cdk4, preferentially cdk4 or cdk6, and PCNA. A consequence of the association between cyclin D1 and cdk4 or cdk6 is the phosphorylation of the retinoblastoma tumor suppressor gene product (pRb, Ref. 2). Hyperphosphorylation of pRb leads to its dissociation from transcriptional factors, such as E2F, that transcriptionally regulate growth-promoting genes. Although pRb remains a key target of cyclin D1/cdk4 or cdk6, other proteins have been postulated to be putative targets, for example the myb-like protein (3).

Cyclin D1 overexpression is associated with a number of malignancies. One prominent pattern is that of cyclin D1 overexpression due to gene amplification in squamous cell cancers, originating from the head/neck (4–11) and esophagus (12–14). Alternatively, chromosomal translocation is responsible for cyclin D1 overexpression in parathyroid adenomas (15), centrocytic lymphomas (16), and some breast cancers, although gene amplification is a major factor in breast cancers (17). Insights into how cyclin D1 contributes to malignant transformation have been gained through in vitro and in vivo approaches. Overexpression of cyclin D1 in cultured cells leads to a more rapid traversal through the G1 phase and entry into the S phase (18, 19). The oncogenic properties of cyclin D1 have been demonstrated in vitro by its ability to cooperate with ras (20) and its ability to complement a defective adenovirus E1a (21) in cultured cells.

Transgenic mouse models have involved the targeting of cyclin D1 to breast tissue, resulting in mammary hyperplasia and carcinomas in lactating mice (22). More recently, we have developed a transgenic mouse model in which the EBV ED-L2 promoter when fused to the human cyclin D1 cDNA results in dysplasia restricted to the oral cavity and esophagus, both sharing a stratified squamous epithelium (23). This tissue specificity is attributable to the interaction of squamous epithelial nuclear transcriptional factors with the EBV ED-L2 promoter (24).

One of the inherent advantages of the dysplastic phenotype resulting from the targeting of cyclin D1 to the tongue and esophagus is the molecular dissection of the dysplastic tissues to assess whether other genetic alterations are acquired as a function of cyclin D1 overexpression. Thus, a multistage model of oral-esophageal carcinogenesis can be developed with the correlation of key genetic alterations with dysplasia. We show that cyclin D1 overexpression is associated with increased cell cycle proliferation as inferred from PCNA overexpression, enhanced cdk4 overexpression, and increased EGFR overexpression, as well as p53 abnormalities.

MATERIALS AND METHODS

Generation of Cyclin D1 Transgenic Mice. We employed the EBV ED-L2 promoter and fused it to the human cyclin D1 cDNA to create a transgene from which founder lines were generated and maintained in the FVB/N genetic background. The cyclin D1 transgene is expressed in a tissue-specific fashion with targeting in the tongue, esophagus, and forestomach (23).

The cyclin D1 transgenic mice and age-matched wild-type mice were sacrificed at 1, 9, and 20 months for tissue processing and examination. There were 15 cyclin D1 transgenic mice from two different founder lines analyzed each at 9 and 20 months; these time points demonstrate dysplasia in the tongue and the esophagus. Five transgenic mice from two different founder lines with normal tongue and esophageal histology were analyzed at age 1 month. Because the wild-type mice did not show any tongue or esophageal histological changes at the three different time points, a total of five wild-type mice (ages 1, 9, and 20 months) were considered in aggregate as one group for statistical analysis.

Immunohistochemistry. All comparisons were made between age-matched (1, 9, and 20 months) wild-type and cyclin D1 transgenic mice tongue and esophageal tissue sections. Tissues from the tongue and esophagus were extracted, fixed in 10% formalin, embedded in paraffin, and processed for H&E staining. Immunohistochemical staining of the tongue and esophagus was performed in the following manner with modifications as outlined for individual antibodies. Tongue (encompassing dorsal and ventral surfaces) and esophageal (proximal, mid, and distal) tissue sections were cut 3–5 μm thick, and slides were dried at room temperature for 2 days. Then, the sections were baked at 60°C in a microwave oven for 20 min, deparaffinized in xylene, and rehydrated in alcohol and water. For EGFR and cyclin D1, slides were further treated with 3% H2O2 in absolute methanol for 5 min to block endogenous peroxidase activity. All slides were then microwave treated (800 W) in 10 mM sodium citrate (pH 6.0) at 93°C for 30 min. The slides were then cooled for 15 min and transferred to PBS.

After blocking with 1.5% goat serum (blocking step for EGFR, cyclin D1, and PCNA) for 20 min, the sections were incubated with primary antibodies overnight at 4°C. The antibodies used were as follows: cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; EGFR, epidermal growth factor receptor.

Received 4/14/97; accepted 10/15/97.

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 This work was supported by the ADHF/AGA Fiterman and Funderburg Awards (to A. K. R.), an American Cancer Society Jr. Faculty Research Award JFRA-649 (to A. K. R.), NIH Grants DK40561 and DK53377 (to A. K. R.), and Department of Energy DE-FG-29-91ER61228 (to H. N. and A. K. R.). A. M. was supported by the Deutsche Forschungsgemeinschaft (1304-1-1). R. O. was supported by the Stanley L. Robbins Research Fund from the Department of Pathology at Brigham and Women’s Hospital. T. J. was supported by the Glaxo Wellcome Institute for Digestive Health Award.

2 To whom requests for reprints should be addressed, at Gastrointestinal and Hematology-Oncology Units, Jackson 904, Massachusetts General Hospital, 50 Blossom Street, Boston, MA 02114. Phone: (617)724-3740. Fax: (617)726-3673. E-mail: rustgi@helix.mgh.harvard.edu.

The abbreviations used are: cdk, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; EGFR, epidermal growth factor receptor.
Table 1 Antibodies for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody clonality</th>
<th>Dilution</th>
<th>Incubation time (h)</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-PCNA (PC 10)</td>
<td>Monoclonal</td>
<td>1:600</td>
<td>1</td>
<td>Pharmingen (San Diego, CA)</td>
</tr>
<tr>
<td>anti-cyclin D1 (#19)</td>
<td>Polyclonal (affinity purified)</td>
<td>1:1500</td>
<td>12</td>
<td>Gift of E. Harlow</td>
</tr>
<tr>
<td>anti-cdk4 (sc-260)</td>
<td>Polyclonal (affinity purified)</td>
<td>1:800</td>
<td>1</td>
<td>Santa Cruz (Santa Cruz, CA)</td>
</tr>
<tr>
<td>anti-EGFR (sc-003)</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>1</td>
<td>Santa Cruz (Santa Cruz, CA)</td>
</tr>
<tr>
<td>anti-p53 (#1801)</td>
<td>Monoclonal</td>
<td>1:1500</td>
<td>1</td>
<td>BioGenex (San Ramon, CA)</td>
</tr>
</tbody>
</table>

and cdk4 or with 1.5% horse serum (blocking step for PCNA and p53) in PBS for 15 min, the slides were incubated with the primary antibody at room temperature, except for cyclin D1 at 4°C (Table 1). After washing with PBS, slides were incubated for 30 min with a 1:200 dilution of the appropriate biotinylated secondary antibody, washed with PBS, and then incubated for 40 min with the avidin and biotinylated peroxidase complex (Vectastain Elite ABC kit; Vector Laboratory, Burlingame, CA) at room temperature. After washing with PBS, the colorimetric reaction was performed with 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.1% H2O2 in 0.5 M Tris-HCl buffer (pH 7.6). The sections were counterstained with hematoxylin, dehydrated, and mounted in Permount (Fisher Scientific, Pittsburgh, PA).

Human tissue sections served as positive controls for the primary antibodies: tonsil (PCNA), and breast cancer (EGFR, cyclin D1, cdk4, and p53). Normal goat or horse serum was used as the primary antibody to serve as negative controls.

For determination of PCNA, cyclin D1, and cdk4 nuclear staining, the average percentage positively was determined by counting the number of positively stained cells of 500 cells at X400. EGFR cytoplasmic and membranous staining was assessed in a semiquantitative fashion: grade 0, no staining; grade 1+, less than 25% of cells stained positive; grade 2+, 25-49%; grade 3+, 50-74%; and grade 4+, greater than or equal to 75% (25, 26).

Scoring of p53 staining was based upon the percentage of positive nuclei with respect to dysplastic cells and expressed in the following categories: none, no nuclei stained; rare, <30% of viable nuclei stained positive; moderate, 30-60% of viable nuclei stained positive; and strong, >60% of viable nuclei stained positive (27). Of note, the p53 antibody recognized a broad spectrum of mutant p53 proteins as well as wild-type p53. All immunohistochemical changes were assessed and scored independently in a blinded fashion by three different observers (A. M., R. O., and A. K. R.).

Statistical Analysis. Statistical comparisons between different age groups of mice were performed with the use of the one-way ANOVA test and Duncan’s multiple range test. P < 0.05 was considered statistically significant.

RESULTS

We have previously demonstrated that an EBV ED-L2 promoter-directed cyclin D1 transgene leads to a oral-esophageal tissue-specific dysplastic phenotype in mice (23), an important precursor to cancer. This is initially evident by 6–9 months in the form of nuclear atypia and progresses to greater dysplasia by 16–20 months (Fig. 1). Such changes are not evident in age-matched wild-type mice in the same FVB/N background or in young (1-month) transgenic mice, the latter likely reflecting the lack of selection of cyclin D1-overexpressing cells. Overall, this model provides a unique opportunity to determine not only whether cell cycle partners of cyclin D1 are aberrantly expressed but also to determine whether genetic alterations frequently described in human oral-esophageal carcinogenesis are associated with dysplasia. In this context, EGFR overexpression and p53 mutations have been described in human oral and esophageal dysplasia and squamous cancers.

PCNA and cdk4 Expression Are Increased in the Tongue and Esophageal Tissues of the Cyclin D1 Transgenic Mice. The increased cyclin D1 expression was evident in the 9- and 20-month-old transgenic mice tongue and esophageal tissues in comparison to age-matched wild-type and 1-month-old transgenic mice, as apparent...
in the immunohistochemical staining in Fig. 2 and the quantitative scoring in Fig. 3. The vast majority of cyclin D1 staining is nuclear, although rare cytoplasmic staining is noted. We then determined whether the cyclin D1 overexpression resulted in corresponding increases in PCNA and cdk4 expression. The 9- and 20-month-old transgenic mice demonstrated an increase in nuclear PCNA expression (Fig. 4) in the tongue and esophageal basal and intermediate layers in comparison to the 1-month-old transgenic mice and all the age-matched wild-type mice. Nearly 60% of cells counted in the tongue epithelium were positive for PCNA staining in the 9-month-old transgenic mice, which increased to approximately 70% of cells in the 20-month-old transgenic mice, representing statistically significant increases when compared to the 1-month-old transgenic mice and all of the age-matched wild-type mice (Fig. 5). Similar numbers of positive cells for PCNA staining were observed in the esophageal epithelia of the 9- and 20-month-old transgenic mice (Fig. 5), also statistically significant. Additionally, there was increased cdk4 expression in the tongue and esophageal epithelial layers in the same age groups (9- and 20-month-old) of transgenic mice when compared to the 1-month-old transgenic mice and all of the age-matched wild-type mice (Fig. 6). Again, the number of cells staining positive for cdk4 in the basal and intermediate layers of the 9- and 20-month-old transgenic mice was greater than those in the 1-month old transgenic and all the age-matched wild-type mice (Fig. 7) in a statistically signifi-
TRANSGENIC MOUSE MODEL WITH CYCLIN D1 OVEREXPRESSION

Fig. 3. Quantitative scoring of cyclin D1 immunohistochemical staining in the tongue and esophagus. Wild-type mice; 1-, 9-, and 20-month old groups are transgenic mice. ++, age-matched wild-type mice compared to all transgenic mice groups; P < 0.05 for tongue and esophagus. *, 1-month old transgenic mice compared to 9-month-old transgenic mice and 1-month-old transgenic mice compared to 20-month old transgenic mice: P < 0.05 for tongue and esophagus. **, 9-month-old transgenic mice compared to 20-month-old transgenic mice: P < 0.05 for tongue and esophagus. ****, comparisons as noted above.

Fig. 5. Quantitative scoring of PCNA immunohistochemical staining in the tongue and esophagus. Wild-type mice; 1-, 9-, and 20-month old groups are transgenic mice. ++, age-matched wild-type mice compared to all transgenic mice groups; P < 0.05 for tongue and esophagus. *, 1-month-old transgenic mice compared to 9-month-old transgenic mice and 1-month-old transgenic mice compared to 20-month old transgenic mice: P < 0.05 for tongue and esophagus. **, 9-month-old transgenic mice compared to 20-month-old transgenic mice: P < 0.05 for tongue and P > 0.05 for esophagus. ***, comparisons as noted above.

cant fashion. Because cdk4 is one of the major catalytic subunit partners of cyclin D1, to our knowledge, this is the first in vivo demonstration of parallel increases in cyclin D1 and cdk4 expression in the setting of dysplasia.

Genetic Abnormalities Are Acquired in Cyclin D1 Transgenic Mice. Given the high frequency of EGFR oncogene and p53 tumor suppressor abnormalities in human head/neck (28–34) and esophageal (35–40) squamous cell cancers, we determined whether such changes may be present in the cyclin D1 transgenic mouse dysplastic oral-esophageal tissues.

In the wild-type and the 1-month-old cyclin D1 transgenic mice, focally weak EGFR cytoplasmic staining was detectable only in the basal layer consistent with grade 1+ staining. However, all 9-month and 20-month cyclin D1 transgenic mice revealed an increase in the intensity and degree of cytoplasmic and membranous EGFR staining in the basal and intermediate layers. This was
uniformly grade 2+ in the 9-month old mice and grade 3+ in the 20-month-old mice (Fig. 8). Thus, EGFR overexpression was associated with dysplasia induced by the targeting of cyclin D1 to the tongue and esophageal epithelia.

Regardless of the age of the wild-type mice, p53 staining was not detected in the wild-type mouse tongue and esophageal layers, consistent with the notion that p53 mutations did not arise in these mice. This was also the case for the 1- and 9-month-old cyclin D1 transgenic mice. In contrast, all 20-month-old cyclin D1 transgenic mice showed rare p53 staining (5–10% of cells) in the dysplastic tongue epithelium but not in the dysplastic esophageal epithelium (data not shown). It is possible that the greater degree of cell proliferation observed in the dysplastic tongue epithelium might be responsible for the increased p53 staining, which may represent de novo p53 mutations.

DISCUSSION

Cyclin D1 associates with cdk s (cdk4 or cdk6) and PCNA in the G1 phase of the cell cycle. The cyclin D1/cdk4 or cdk6 complex phosphorylates the retinoblastoma tumor suppressor gene product (2). The hyperphosphorylation of pRb leads to its dissociation from key transcriptional factors, such as E2F, which allows for these factors to activate growth-promoting genes. It is within the realm of possibility...
that other substrates are targets for the cyclin D1/cdk complex, as has been postulated recently with the myb-like transcriptional factor (3). Cyclin D1 overexpression has been associated with a number of cancers, especially those of squamous epithelial origin from the head/neck and esophagus. In an attempt to understand the molecular mechanisms that underlie the contribution of cyclin D1 to malignant transformation in this tissue and cell type, we have recently developed a transgenic mouse model where cyclin D1 is targeted to these epithelia using an EBV promoter (23).

A number of environmental and genetic factors are common to head/neck and esophageal squamous cell cancers. Among the frequently associated genetic factors are oncogene activation, cyclin D1 and EGFR) and tumor suppressor gene inactivation, (p53 and p16 mutations) (41–45). Thus, we have used the cyclin D1 model to investigate whether other genetic alterations, either directly or indirectly as a consequence of cyclin D1 overexpression, can be detected in the dysplastic phenotype by using immunohistochemical staining approaches.

One of the well-established aspects of cyclin D1 overexpression in cultured cells is a shortened $G_1$ phase, resulting in a more rapid entry into S phase and increased cell proliferation. We found that cyclin D1 overexpression resulted in increased cell proliferation in the tongue and esophageal epithelia of 9- and 20-month-old transgenic mice, as inferred from increased PCNA staining in comparison to age-matched wild-type mice and the young (1-month) transgenic mice. This may potentially be a consequence of the increased cdk4 and cyclin D1 overexpression observed in the same age groups of transgenic mice.

As noted, the cyclin D1 and cdk4 complex phosphorylates key cellular targets. It is likely in this setting of increased cell proliferation that other genetic alterations are acquired. Key abnormalities that have been associated in head and neck as well as esophageal squamous cell carcinomas are EGFR overexpression and p53 mutations. EGFR overexpression and p53 mutations have also been observed in head and neck squamous epithelial dysplasia (46, 47). Interestingly enough, p53 mutations have been found to correlate with EGFR overexpression in human esophageal squamous carcinomas (48), and p53 mutations correlate with cyclin D1 amplification in head and neck squamous cell carcinomas (49).

The link between EGFR and cyclin D1 in our transgenic mouse model may be direct or indirect. It is possible that the EGFR promoter is a potential target of cyclin D1, resulting in increased EGFR transcription. Conceivably, cyclin D1 may activate other transcriptional factors that modulate EGFR transcription. Alternatively, increased EGFR expression may be the consequence of increased cell proliferation. Nonetheless, the finding of increased EGFR expression in dysplasia is important and implicates it as an early event in head and neck as well as esophageal carcinogenesis.

p53 is a well-described tumor suppressor gene, the encoded protein of which has several effects related to cell cycle regulation, DNA replication and repair, transcriptional regulation of genes, and apoptosis. p53 serves as a cell cycle checkpoint at the $G_1$/S transition, in part through the transcriptional activation of p21, a general cdk inhibitor (50). The dysplastic phenotype in the cyclin D1 transgenic mice tongue epithelia was uniformly associated with rare p53 staining, consistent with the notion, although not definitive, of p53 mutation and stabilization of the p53 protein. This finding was restricted, interestingly, to the tongue epithelium of the 20-month-old transgenic mice, perhaps reflecting the increased expression of cyclin D1, cdk4, and PCNA in this particular age group’s tongue tissues. A possible, albeit speculative, link among these latter changes is that mutant p53 has been shown to activate the PCNA (51) and EGFR (52) promoters. Thus, although cyclin D1 overexpression may be an initiating event, mutant p53 may further augment cell proliferation, at least in the tongue, through the ongoing activation of key genes, especially EGFR. However, this is not an entirely complete explanation because EGFR overexpression preceded the p53 staining temporally and was also evident in the esophagus. Nonetheless, this may be a contributing factor in the dysplastic tongue squamous epithelium.

In aggregate, our cyclin D1 transgenic mouse model of oral-esophageal dysplasia permits the unique development of a multistage model of carcinogenesis (49, 53–55) in which cyclin D1 overexpression results in a dysplastic phenotype associated with EGFR and p53
genetic alterations. Although not tested yet, other genetic changes may be associated with dysplasia in this transgenic mouse model, such as in other tumor suppressor genes, pl6 and pRb, or other oncogenes and key enzymes (56) implicated in oral-esophageal carcinogenesis. Mechanistically, p16 mutation, deletion, or hypermethylation is likely redundant with cyclin D1 overexpression in the cyclin D1 pathway. Furthermore, actual pRb inactivation is infrequent in human head and neck cancers (57) and has not been formally investigated in esophageal cancers, although loss of heterozygosity of the locus has been implicated (58). The association of key genetic alterations with the oral-esophageal dysplastic phenotype in the cyclin D1 transgenic mouse model also lays the foundation for the application of diagnostic strategies in the early premalignant lesions and the use of tissue-specific therapeutic approaches.

REFERENCES


A Transgenic Mouse Model with Cyclin D1 Overexpression Results in Cell Cycle, Epidermal Growth Factor Receptor, and p53 Abnormalities


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/24/5542

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.