PRAD-1/Cyclin D1 Gene Amplification Correlates with Messenger RNA
Overexpression and Tumor Progression in Human Laryngeal Carcinomas

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ABSTRACT

PRAD-1 is a putative oncogene localized on chromosome 11q13 which encodes cyclin D1, a novel cyclin involved in cell cycle regulation. Amplification of this gene has recently been reported in several human tumors including breast and head and neck carcinomas. In this study we have analyzed the presence of PRAD-1/cyclin D1 gene amplification and mRNA overexpression in a series of 46 matched normal mucosas and squamous cell carcinomas of the larynx. PRAD-1/cyclin D1 was found to be amplified 2- to 12-fold in 17 carcinomas (37%). DNA amplification correlated with advanced local invasion (P = 0.0015), presence of lymph node metastases (2- to 12-fold in 17 carcinomas (37%). DNA amplification correlated with overexpression in a series of 46 matched normal mucosas and squamous cell carcinomas of the larynx. PRAD-1/cyclin D1 was found to be amplified 2- to 12-fold in 17 carcinomas (37%). DNA amplification correlated with advanced local invasion (P = 0.0015), presence of lymph node metastases (P = 0.0078), and stage IV of the tumors (P = 0.0021). mRNA overexpression was found in 15 of the 43 (35%) cases examined and it was also significantly associated with advanced local invasion (P = 0.0028) and stage IV carcinomas (P = 0.0032). A significant association was observed between gene amplification and mRNA overexpression (P < 0.0001) with only 3 discordant cases (2 amplifications with no overexpression and 1 overexpressed carcinoma with no gene amplification). Furthermore, the degree of DNA amplification correlated with the levels of mRNA expression (r = 0.6; P = 0.024). These findings suggest that the PRAD-1/cyclin D1 gene may be an important target of 11q13 amplifications in laryngeal carcinomas and the activation of this gene may be involved in the progression of these tumors. Its association with advanced-stage tumors indicates that PRAD-1/cyclin D1 gene amplification and overexpression may be of prognostic significance.

INTRODUCTION

SCC of the larynx is an aggressive malignant neoplasm relatively frequent in certain countries (1). Epidemiological studies have identified personal habits (tobacco and alcohol), environmental, and viral, as major factors in the pathogenesis of laryngeal and other head and neck carcinomas (1). Malignant conversion of laryngeal mucosa is a progressive process in which several premalignant lesions have been morphologically recognized (2). The molecular mechanisms implicated in the malignant transformation and progression of these neoplasms are not well known.

Cytogenetic analysis of head and neck SCC have identified frequent chromosome abnormalities at 11q13, including clonal rearrangements and presence of homogeneously stained regions (3, 4). Several molecular studies have shown amplification of genes located on this band (int-2, hst-1, and bcl-1 locus) in 30–50% of the SCC of head and neck (5–8). Similarly, amplification of genes located on the 11q13 region may be involved in the development of other solid tumors such as lung (9), esophageal (10, 11), breast (5, 12–14), urinary bladder (15), and hepatocellular carcinomas (16). However, in spite of their amplifications, no RNA expression of int-2, hst-1, or bcl-1 locus have been consistently demonstrated in fresh tumors or cell lines, suggesting that none of them are significant targets of the 11q13 amplification (6, 17).

PRAD-1 is a gene localized on chromosome 11q13 which was originally isolated as a gene clonally rearranged and overexpressed in parathyroid adenomas (18). This gene also seems to be the bcl-1-related gene activated by the t(11;14) translocation in some B-cell lymphomas/leukemias (19). PRAD-1 encodes a novel cyclin called cyclin D1 which may play an important role in the control of the cell cycle at the G1-S transition, probably by interacting with the retinoblastoma gene product (20, 21). Recently, transfection studies have demonstrated that PRAD-1/cyclin D1 may function as a cooperating oncogene in the malignant transformation of cells (22). PRAD-1/cyclin D1 gene may also be a target of 11q13 amplifications in solid tumors. Amplification and overexpression of this gene have been observed in breast and squamous cell carcinomas (17, 23–26). However, overexpression in squamous cell carcinomas has been mainly investigated in tumor cell lines (17, 23–25) and, therefore, the role of this gene in the progression of these tumors is unknown.

In this study we have analyzed PRAD-1/cyclin D1 gene amplification and mRNA expression in a large series of human laryngeal carcinomas, and the results were correlated with the clinical and pathological characteristics of the patients.

MATERIALS AND METHODS

Patients and Tissues. A total of 46 human SCC of the larynx were obtained from 46 male patients (median age, 61 years, range, 39 to 92) who underwent surgery in our hospital from January 1992 to June 1993. Samples from tumor areas and nonneoplastic adjacent mucosas were snap-frozen in isopentane precooled in liquid nitrogen and stored at –80°C until studied. The remaining specimen was fixed in formalin and routinely processed.

The anatomical location of the tumors was glottic in 12 (26%), supraglottic in 16 (35%), pyriform sinus in 2 (4%), and tranglottic in 16 (35%) (Table 1). Tumors were classified as keratinizing in 28 cases (61%) and nonkeratinizing in 18 (39%). The grade of differentiation was established according to Broders’ criteria. Grades 1 and 2 were considered as low-grade carcinomas (15 cases, 32%) and Grades 3 and 4 as high-grade carcinomas (31 cases, 68%). Staging of tumors was established according to the American Joint Committee on Cancer (1992) (27). Thus, 2 patients had stage I carcinoma (4%), 3 had stage II (6%), 20 had stage III (44%), and 21 had stage IV (46%).

DNA Extraction and Southern Blot Analysis. High-molecular-weight DNA was isolated from frozen tissue of 46 carcinomas and matched normal mucosas. Cryostat sections of these cases were previously examined in order to determine the proportion of normal and neoplastic tissue present in each sample. Tumor samples were selected only if more than 75% of the section had carcinoma. DNA was extracted by conventional methods (28), and 10 µg were
digested with EcoRI. DNA from selected cases was also digested with BgIII and HindIII. The samples were separated on 0.8% agarose gels and transferred to Hybond-N membranes (Amersham Int., Amersham, United Kingdom). The membranes were prehybridized with 50% formamide, 5X SSC, 5X Denhardt’s, 500 μg/ml denatured salmon sperm DNA, at 42°C for 6 h, and hybridized with 50% formamide, 5X SSC, 1X Denhardt’s, 100 μg/ml salmon sperm DNA, 10% dextran sulfate, and 106 cpm/ml of 32P-labeled PRAD-1 probe for 24 h. After hybridization, membranes were washed with 2X SSC, 0.1% SDS at room temperature for 30 min followed by 2X SSC, 0.1% SDS at 60°C for 30 min, and 0.1X SSC, 0.1% SDS at 60°C for 1 h. The filters were then autoradiographed by using intensifying screens at −70°C for 24–72 h. After being stripped free of the PRAD-1 probe, the same blots were hybridized with a 32P-labeled β-actin probe to normalize against possible variations in loading or transfer of DNA.

The autoradiograms were analyzed by using a UVP-5000 video densitometer (UVP, San Gabriel, CA). Intensities of PRAD-1/cyclin D1 bands were calculated from these normalized values. Amplification was considered when the signal of the tumor band was ≥2-fold the value of its matched normal mucosa.

**RNA Extraction and Northern Blot Analysis.** Total RNA was isolated from frozen tissue of 43 carcinomas and matched normal mucosa by guanidine isothiocyanate extraction and cesium chloride gradient centrifugation (28, 29). Genomic DNA had been previously obtained in all cases. Eight μg of RNA from each sample were electrophoresed on a 1.2% agarose formaldehyde gel and transferred to Hybond-N membranes (Amersham). The PRAD1 complementary DNA probe was 32P-labeled by random priming and hybridized to the blots at 45°C overnight. Washes after hybridization included a final step under stringent conditions in 0.1x SSC, 0.1% SDS at 65°C for 30 min. After being stripped free of the PRAD-1 probe, the same blots were hybridized with a 32P-labeled 28S rRNA probe to normalize against possible variations in loading or transfer of RNA.

Hybridization signals of different radioautographic exposures within the linear response range were quantified as previously described, and the values for each case were normalized to the respective 28S rRNA signal. Overexpression was considered when the signal of the tumor band was 2-fold the value of the matched normal mucosa.

**Probes.** Probes were radiolabeled with the use of a random primer DNA-labeling kit (Promega Corp., Madison, WI) with [32P]dCTP. The PRAD-1 probe used was a 1.4-kilobase EcoRI fragment (AP1-4) of the pPL-8 partial complementary DNA clone of PRAD-1 gene (kindly provided by Dr. A. Arnold, Massachusetts General Hospital, Boston, MA) (18).

**Statistical Analysis.** χ² test and Fisher’s exact test were used for comparison between qualitative or categorical data. RR was computed according to previously described methods (30). Linear correlation of DNA amplification against RNA overexpression was carried out by means of the Pearson product moment correlation. Data were analyzed with the BMDP statistical software package (BMDP Statistical Software, Inc.).

**RESULTS**

**PRAD-1/Cyclin D1 Gene Amplification in Laryngeal SCC.** The PRAD-1 probe detected 3 EcoRI fragments of 4.0, 2.2, and 2.0, and 1 BgIII fragment of 15 kilobases as previously described (25). **PRAD-1/Cyclin D1 gene amplification was detected in 17 of the 46 cases analyzed (37%).** The degree of amplification was heterogeneous from tumor to tumor with a 2- to 12-fold variation when compared with the signal of the respective normal mucosa. Amplifications were confirmed with other restriction enzymes (Fig. 1 and 2). No rearrangements were seen in any tumor sample.

The correlations between gene amplification and the clinical and pathological characteristics of the patients are summarized in Tables 1–4. **PRAD-1/cyclin D1 gene amplification was significantly associated with an advanced stage of the tumors (Table 2). Amplification was found in 13 of the 21 (62%) stage IV carcinomas while only in 4 of the 25 (16%) stage I–III carcinomas (P = 0.0021; RR = 2.4). Similarly, DNA amplification was detected in 61% (11 of 18) of the carcinomas with extensive local invasion (T4 carcinomas) but only in 22% (6 of the 28) of the carcinomas with limited invasion (T1–T3 tumors) (P = 0.0015; RR = 3.4) (Table 3). There was also a significant correlation between the PRAD-1/cyclin D1 gene amplification and the presence of lymph node metastases (P = 0.0078; RR = 5.9) (Table 4). Nonkeratinizing carcinomas were more frequently amplified (50%) than keratinizing tumors (29%) (Table 1). This difference, however, was not statistically significant. No association was observed between amplification and the age of the patient, histological grade, or localization of the tumor (Table 1).

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**Fig. 1. A.** Southern blot analysis of seven representative squamous cell carcinomas of the larynx (T) and normal mucosa (N) specimens. Numbers correspond to specific patients. Genomic DNA was digested with BgIII and fractionated by electrophoresis in a 0.8% agarose gel. After transfer, the membranes were hybridized with PRAD-1 probe and subsequently with a β-actin probe for loading control. Tumors 10, 44, 3, 46, 73, and 98 show different degrees of PRAD-1/cyclin D1 amplification, whereas tumor 35 was not amplified. **B.** Northern blot analysis of the same matched carcinomas (T) and normal mucosas (N) shown in A. Eight μg of total RNA were electrophoresed on a 1.2% agarose/formaldehyde gel and transferred to nylon membranes. The filters were hybridized to the PRAD-1 probe and subsequently to a 28S rRNA probe for loading control. Two transcripts of 4.5 and 1.5 kilobases were observed in all the samples. Tumors 10, 44, 3, 46, and 73 with DNA amplification also showed mRNA overexpression when compared to their respective normal mucosas. Tumor 98 was amplified but not overexpressed, and in case 35 neither amplification nor overexpression was detected.
**PRAD-1/Cyclin D1 mRNA Overexpression in Laryngeal SCC.** In order to determine whether the **PRAD-1/cyclin D1** gene was transcribed in laryngeal carcinomas, total RNA was obtained from additional tissue available in 43 carcinomas and the respective nonneoplastic mucosa. On Northern blot analysis, the **PRAD-1** signal showed two transcripts of 4.5 and 1.5 kilobases. Low levels of these two transcripts were detectable in all normal samples. Fifteen carcinomas (35%) showed from 2- to 18-fold overexpression when compared to the signal of the matched mucosa. No anomalous transcripts were detected in any of the cases (Fig. 1).

The finding of mRNA overexpression in carcinomas correlated significantly with the presence of DNA amplification (Table 5) \( (P < 0.0001) \). Discordance between amplification and overexpression was observed in only three cases: two amplified tumors did not show mRNA overexpression whereas high levels of transcription were observed in one carcinoma in which Southern blot analysis did not detect gene amplification. The relationship between the degree of gene amplification and the level of mRNA expression was analyzed in the 14 cases with amplification and overexpression (Fig. 3). Linear analysis showed a significant correlation between these two parameters with an r value of 0.6 \( (P = 0.024) \).

As in gene amplification, a significant correlation was found between **PRAD-1/cyclin D1** overexpression and clinical progression of the disease (Tables 2, 3, and 4). mRNA overexpression was observed in 12 of the 20 (60%) stage IV carcinomas, but in none of the 5 stage I/II tumors, and in only 3 of the 18 (17%) stage III cases \( (P = 0.0032, \text{RR} = 2.8) \). The local invasion of carcinomas with high mRNA levels was also significantly more advanced than in tumors with normal expression of this gene \( (P = 0.0025, \text{RR} = 3.3) \) (Table 3). Overexpressed carcinomas were more frequently associated with lymph node metastases (62%) than tumors which were not overexpressed (28%) (Table 4), although this difference did not reach statistical significance. Nonkeratinizing carcinomas were more frequently overexpressed (53%) than keratinizing tumors (23%) \( (P = 0.047; \text{Table 1}) \). No correlation was observed between overexpression and the age of the patients, histological grade, or localization of the tumors (Table 1).

**DISCUSSION**

Amplification of 11q13 genes has been observed in 10–50% of squamous cell carcinomas of the head and neck, lung, and esophagus.
indicates that genes located on this amplicon may play a role in the development and/or progression of these tumors (5–8, 10, 11). Occasionally laryngeal carcinomas have been included in some of the head and neck series. However, no specific analysis has been performed in this group of carcinomas. In this study we have demonstrated that PRAD-1/cyclin D1 is amplified in a relatively high number of SCC of this group of carcinomas. In this study we have demonstrated that PRAD-1/cyclin D1 is amplified in a relatively high number of SCC of the larynx and this amplification is associated with overexpression of the gene. Interestingly, amplification and overexpression were significantly more frequent in carcinomas with advanced local invasion, lymph node metastases, and, consequently, with an advanced tumor stage.

Amplification of the 11q13 region has been correlated with more advanced primary tumors and shortened survival in breast and esophageal carcinomas (11, 14, 31). There are some indications that amplification of 11q13 may also be associated with poorly differentiated and advanced head and neck carcinomas (6, 7). In these studies, however, the series examined was too small to show statistical significance. Two genes localized on this amplicon, int-2 and hst-1, had been considered as candidates to be activated by this amplification. However, in contrast to the overexpression of PRAD-1, their levels of mRNA were consistently undetectable (6, 17). Our study confirms the relationship between 11q13 amplifications and poor prognosis parameters in SCC of the larynx. In addition, the consistent mRNA up-regulation of the PRAD-1/cyclin D1 gene in our cases supports the idea that this gene plays an important role in the aggressive behavior of these lesions.

How overexpression of cyclin D1 may participate in the progression of these tumors is unknown. Recently, several studies have found that cyclin D1 overexpressing cells have abnormal proliferative characteristics with a shortened G1 phase and less dependence on growth factors (20, 32). Cyclin D1 may also function as an oncogene cooperating with other oncogenes in cellular transformation (22). However, the tumorigenic and transforming properties of cyclin D1 seem to be less effective than the conventional oncogenes (20, 22). The overexpression of cyclin D1 in the late steps of the progression of human laryngeal carcinomas observed in our study suggests that this gene does not play a role in the initial transformation of squamous cells. Its up-regulation, however, may confer some growth advantage to cells already transformed by other mechanisms contributing to an increase in the aggressiveness of the tumor.

Association between PRAD-1/cyclin D1 gene amplification and overexpression has been observed in some cell lines (17, 23, 24). However, this correlation is more difficult to demonstrate in solid tumors because of tumor heterogeneity and the possible dilutional effect of nonneoplastic tissue present in the sample (23, 24). To minimize this problem only samples with more than 75% of tumor cells have been included in this study. The high correlation between overexpression and amplification observed in our study indicates that amplification is the main mechanism leading to PRAD-1/cyclin D1 gene activation in SCC of the larynx. However, overexpression was observed without gene amplification in one case. Similarly, mRNA up-regulation with no gross genetic abnormalities has also been observed in some human breast carcinomas and solid tumor cell lines (17, 24). These findings suggest that alternative mechanisms of activating PRAD-1/cyclin D1 gene such as translocations, mutations in gene regulatory regions, or stabilization of mRNA may also occur. PRAD-1/cyclin D1 expression is activated by chromosome translocations and inversions in lymphoid neoplasms and parathyroid adenomas (18, 19). 11q13 translocations have been also described in some squamous cell carcinomas, suggesting that this mechanism may also activate this gene in this type of tumors (3, 20). Although no rearrangements were detected in the PRAD-1/cyclin D1 gene in our case with the probe used in this study, we cannot rule out the presence of rearrangements involving more distant breakpoints as they occur in lymphoid neoplasms.

In our study two carcinomas showed PRAD-1/cyclin D1 gene amplification without mRNA overexpression, suggesting that other genes in this amplicon may also be involved in the progression of some laryngeal carcinomas. EMS1 and EXP1 are recently identified genes in the 11q13 region which are frequently coamplified with PRAD-1/cyclin D1 in breast and squamous cell carcinomas (24, 33, 34). In some cases, however, amplification of EMS1 may occur with lower number of copies of the PRAD-1/cyclin D1 gene (24, 26). Amplification of these genes is associated with their overexpression in some tumor cell lines and human carcinomas, suggesting that they may also be targets of this amplicon. Further studies are required to know whether these genes are also amplified and overexpressed in laryngeal carcinomas and to determine their possible cooperation in the progression of these tumors.

In conclusion, PRAD-1/cyclin D1 gene is amplified in a high number of laryngeal carcinomas and this amplification is significantly associated with mRNA overexpression. Furthermore, PRAD-1 amplification and overexpression correlate with advanced tumor invasion, lymph node metastasis, and advanced tumor stage. Our findings suggest that this gene is a relevant target of the 11q13 amplification in SCC of the larynx and may be involved in the progression of these tumors. The detection of cyclin D1 in SCC of the larynx may be a useful marker of prognostic significance.

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REFERENCES


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