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Amplification and overexpression of the cyclin D1 gene in head and neck squamous cell carcinoma

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Abstract

***Aims*—To determine cyclin D1 gene amplification and expression levels in head and neck squamous cell carcinoma (HNSCC) patients.**

***Methods*—Total RNA and genomic DNA were isolated from 40 samples of HNSCC tissue and matched normal tissue and were hybridised with a cyclin D1 cDNA probe. Northern and Southern analyses were used to detect mRNA overexpression and cyclin D1 gene amplification, respectively.**

***Results*—15 of the 40 HNSCC samples examined (38%) showed cyclin D1 gene amplification. Of these 15 samples, all 13 from which RNA was available showed increased cyclin D1 expression.**

***Conclusions*—HNSCC patients with both amplification and overexpression of the cyclin D1 gene are at greater risk than patients who showed no cyclin D1 gene amplification; amplification and overexpression of the cyclin D1 gene may play an important role in the progression of HNSCC and in clinical outcome.**

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Keywords: cyclin D1, head and neck squamous cell carcinoma, gene amplification, mRNA overexpression, tumour progression.

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Cyclins are central to the control of the eukaryotic cell cycle. When their expression is unregulated, they are considered likely participants in the oncogenic process.[1-3] Within the cyclin gene family, the involvement of cyclin D1 in various human tumours has often been observed.[4-20] Recently, the capacity of altered cyclin D1 gene expression to transform cells in vitro and in vivo has been demonstrated,[21 22] indicating that cyclin D1 indeed plays an important role in tumorigenesis.

The deregulation of cyclin D1 in tumours occurs as a consequence of either tumour specific gene translocation or amplification of chromosome locus 11q13, where the cyclin D1 gene resides. In the case of parathyroid adenoma and some B cell lymphomas, the cyclin D1 gene undergoes rearrangement.[4 6] In contrast, amplification of this gene has been implicated in other types of human tumours including those of breast,[7-10] oesophagus,[11] lung,[13] liver[14 15] and urinary bladder,[16] and in head and neck squamous cell carcinomas (HNSCC).[7 17-20]

Head and neck carcinomas account for 5-7% of new cases of cancer diagnosed annually and for about 3% of all cancer deaths.[23] Over 90% of these tumours are squamous cell carcinomas. Epidemiological studies have indicated that tobacco is a major factor in the tumorigenesis of HNSCC,[24] while alcohol, environmental exposure, and some viral infections may play a synergistic role.[25] The molecular mechanisms operating in the malignant transformation of these carcinomas are still largely unknown. Although amplification of chromosome band 11q13, including int-2 and hst-1 genes, has been found in HNSCC,[17 26-28] inconsistent and often absent expression of these genes[17 29] suggests that they do not play a key role in oncogenesis. Another possible target gene in the HNSCC is the cyclin D1 gene, which is overexpressed in HNSCC cell lines.[7 18] However, cyclin D1 gene expression in HNSCC patients has not been evaluated systematically in relation to clinical outcome.

In this study we have examined the relation between gene amplification and mRNA overexpression of cyclin D1 in HNSCC patients. During the course of this work, a paper indicating that cyclin D1 gene amplification correlates with higher levels of mRNA in human laryngeal carcinoma was published.[20] Our investigation confirms these results and further indicates a poor prognosis for patients with cyclin D1 gene amplification.

Methods

PATIENTS AND TISSUES

Forty human HNSCC tissue samples were collected from patients who underwent surgery between March 1992 and May 1994. Adjacent normal mucosa was also obtained from the same patient. Of the 40 HNSCC samples used in the study, 33 were primary and seven were recurrent carcinomas. The 33 primary HNSCCs included two stage II, nine stage III, and 22 stage IV carcinomas.

DNA AND RNA ISOLATION

Tissue was pulverised and ground to a fine powder with a pestle in a mortar partially filled with liquid nitrogen. Total RNA and high molecular weight DNA were isolated by guanidine isothiocyanate extraction and caesium chloride gradient centrifugation.[30]

Cyclin D1 gene amplification/overexpression and clinicopathological characteristics of patients with primary head and neck squamous cell carcinoma (a total of 19 patients) who underwent surgery from March 1992 to July 1993

No	*Age/sex*	*Site*	*TNM*	*Clinical stage*	*Gene amplification*	*mRNA overexpression*	**Alive/dead*
2	69/M	Hypopharynx	T4N2M0	IV	+	+	Dead
4	65/F	Subglottic	T3N0M0	III	−	−	Alive
5	54/F	Oral cavity	T4N2M0	IV	+	unavailable	Dead
6	60/F	Oral cavity	T2N2M0	IV	−	−	Alive
7	76/M	Oropharynx	T2N1M0	III	−	−	Dead
8	57/M	Hypopharynx	T3N2M0	IV	−	−	Dead
9	74/M	Oral cavity	T3N0M0	III	+	+	Dead
11	49/M	Oral cavity	T4N0M0	IV	−	−	Dead
12	53/F	Subglottic	T3N0M0	III	−	−	Alive
13	50/M	Parapharynx	T4N1M0	IV	+	+	Alive
16	56/M	Oropharynx	T4N2M0	IV	+	+	Dead
17	40/M	Supraglottic	T2N1M0	III	+	+	Alive
18	72/M	Supraglottic	T3N2M0	IV	+	+	Dead
19	54/M	Oropharynx	T3N2M0	IV	−	−	Alive
21	71/M	Supraglottic	T2N0M0	II	−	−	Alive
23	68/M	Hypopharynx	T4N1M0	IV	−	−	Alive
24	70/M	Oropharynx	T2N0M0	II	−	−	Alive
26	47/M	Hypopharynx	T4N0M0	IV	+	+	Dead
27	60/M	Oropharynx	T3N1M0	IV	−	−	Alive

TNM: staging system for classification of head and neck cancers. Extent of primary cancer (T), status of regional lymph nodes (N), absence or presence of metastatic spread (M).
"+": with cyclin D1 gene amplification or mRNA overexpression; "−": without gene amplification or overexpression.
*Alive: of surviving patients, follow up was 10 to 23 months (average 18 months).

SOUTHERN ANALYSIS

A 10 μg sample of genomic DNA was digested to completion with EcoRI (Life Technology). The digested DNA was then separated on a 0·8% agarose gel and transferred to a Zetabind membrane (CUNO). The membrane was prehybridised at 65°C in 6 × SSC (NaCl/sodium citrate), 5 × Denhard's solution, 0·5% sodium dodecyl sulphate (SDS) (wt/vol), and 100 mg/ml denatured salmon sperm DNA. The human cyclin D1 cDNA probe (provided by Dr D Beach) was labelled with ^{32}P using a Prime-IT II random primer labelling kit (Strategene) and was added to the prehybridisation solution. After hybridisation, the membrane was washed twice with 2 × SSC, 0·1% SDS at room temperature for 30 min, and twice with 0·1 × SSC, 0·1% SDS at 60°C for 20–30 min. The membrane was then autoradiographed. After removing the cyclin D1 cDNA probe, the membrane was reprobed with a p53 cDNA probe (provided by Dr D Givol) as a single copy internal control to normalise possible variation in loading or transfer of DNA.

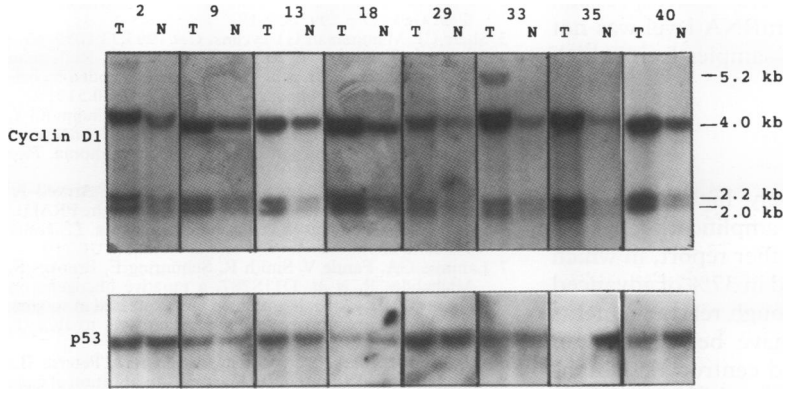

Figure 1 Amplification of cyclin D1 gene from eight representative head and neck carcinomas (HNSCC). T = DNA from HNSCC; N = DNA from normal mucosa. Genomic DNA (10 μg) from each of the numbered specimens was digested with EcoRI and analysed by Southern blotting with a cyclin D1 cDNA probe and a p53 cDNA probe as described in Methods. The size of each band is indicated on the right. Tumours 2 and 33 showed an extra 5·2 kb band.

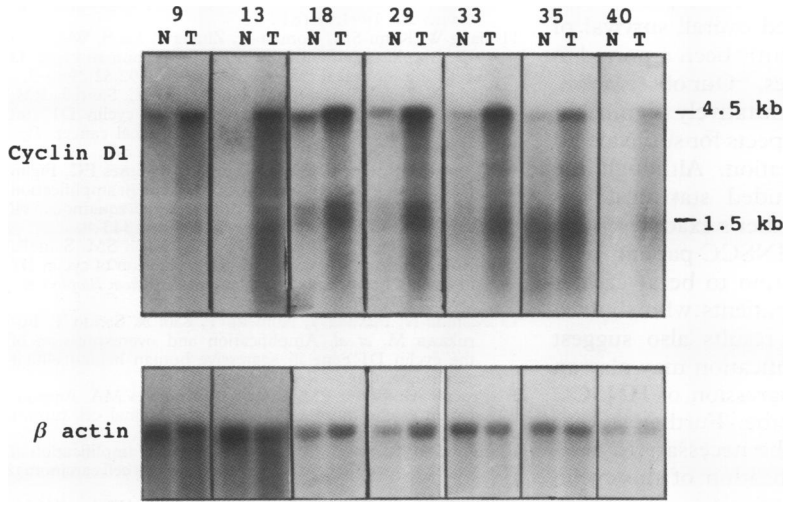

Figure 2 Overexpression of cyclin D1 gene from seven representative head and neck carcinomas (HNSCC). T = RNA from HNSCC; N = RNA from normal mucosa. Total RNA (5 μg) from each of the numbered specimens was analysed by northern blotting with a cyclin D1 cDNA probe and a β actin cDNA probe as described in Methods. The size of each band is indicated on the right.

NORTHERN ANALYSIS

A 5 μg aliquot of total RNA from each sample was fractionated on a 1·2% agarose gel after denaturing with glyoxal/dimethylsulphoxide.[31] The RNA was transferred and hybridised as described above. Washes of membranes were carried out at room temperature in 1 × SSC, 0·1% SDS for 15 min twice, and at 60°C in 0·2 × SSC, 0·1% SDS for 20 min twice. The human β actin cDNA probe was used as an internal control for northern analysis.

Results

The cyclin D1 cDNA probe detected three EcoRI fragments of 4·0, 2·2, and 2·0 kb on Southern blots. In our study cyclin D1 gene amplification was found in 15 (38%) of the 40 HNSCC samples collected. The degree of amplification varied from two- to 18-fold. We also observed two interesting cases of HNSCC which showed not only gene amplification but also an extra 5·2 kb band on the Southern blot, suggestive of cyclin D1 gene rearrangement (fig 1).

For those patients (total of 19) who underwent surgery from March 1992 to July 1993 and had primary tumors with amplified cyclin D1, only 25% (two out of eight) are alive (table). In contrast, seven of 11 patients (64%) whose tumors did not manifest cyclin D1 gene amplification have survived an average of one and a half years (table).

In order to determine the frequency with which the cyclin D1 gene is overexpressed, we examined the level of cyclin D1 transcription by northern analysis. A major transcript of 4·5 kb as well as a minor one of 1·5 kb were detected with the cyclin D1 probe. Of the 15 HNSCC samples with cyclin D1 amplification, all 13 with available RNA showed increased cyclin D1 expression when compared to their normal mucosa counterparts (fig 2). The overexpression ranged from two- to 16-fold. The two samples with an extra 5·2 kb EcoRI band did not show a dramatic increase in expression of cyclin D1. Despite the fact that cyclin D1 overexpression was found in every case of HNSCC with gene amplification, the degree of amplification and overexpression did not seem to correlate fully. The cyclin D1 mRNA level of the 25 HNSCC samples without gene amplification was also examined. Overexpression of cyclin D1 gene at the mRNA level was not detected in any of the 22 samples from which RNA was available.

Discussion

Our finding of a relatively high percentage (38%) of cyclin D1 gene amplification is consistent with that from another report, in which amplification was recorded in 37% of advanced laryngeal tumours.[20] Although rearrangements in the cyclin D1 gene have been shown in parathyroid adenoma[4] and centrocytic lymphoma,[5,6] none has been reported in HNSCC. It would therefore be informative to investigate further the two cases which showed an extra 5·2 kb band on Southern blotting, to establish whether or not the rearrangement was indeed involved in some of the HNSCC cases.

The association between overexpression of cyclin D1 and a shortened overall survival of HNSCC patients has recently been reported in immunohistological studies.[32] Our observation, however, suggests a quantitatively significant relationship between prospects for survival and cyclin D1 gene amplification. Although the small sample size precluded statistical significance ($p = 0{\cdot}170$ by Fisher's exact test), the data suggest a trend for HNSCC patients with cyclin D1 gene amplification to be at greater risk of early death than patients who showed no amplification. These results also suggest that cyclin D1 gene amplification may play an important role in the progression of HNSCC and in the clinical outcome. Further studies with larger numbers will be necessary to evaluate the extent of amplification of the cyclin D1 gene in relation to prognosis.

The mechanism by which overexpression of the cyclin D1 gene affects the progression of HNSCC is not well understood. Overexpression may, by itself or in combination with other oncogene products, contribute to increased cellular proliferation.[21,33–37] It has been suggested that increased expression of cyclin D1 may provide a growth advantage for tumour cells because cells overexpressing this protein have a shortened G1 phase and less dependence on growth factors in vitro.[33–36] It has also been reported that overexpression of the cyclin D1 gene may induce cellular transformation in cooperation with other oncogenes.[21,37] Further, this effect seems to be mediated through the capacity of cyclin D1 to overcome the inhibitory effect of retinoblastoma (RB) protein on cell cycle progression.[21] Indeed, it has been found that in oesophageal cancer overexpression of cyclin D1 is associated with persistent expression of RB protein, whereas normal cyclin D1 expression appears to be associated with loss of RB expression.[12] In view of these findings, we are currently investigating whether or not expression of RB is altered in HNSCC.[38]

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