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Quantitative Analysis of Cyclin D1 Messenger RNA Expression in Head and Neck Squamous Cell Carcinomas

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Cyclin D1 is thought to play a critical role in the G1/S phase transition of the cell cycle. Amplification of this gene has been reported in several types of human neoplasms including breast, lung, esophageal, and head and neck tumors. In this study, we have analyzed the relative level of expression of cyclin D1 messenger RNA (mRNA) in fresh specimens of head and neck squamous cell carcinoma (HNSCC), and investigated the concordance of the overexpression of cyclin D1 mRNA with gene amplification. Levels of cyclin D1 mRNA were analyzed by a modified method of competitive reverse transcription-polymerase chain reaction and levels of cyclin D1 gene amplification were evaluated by Southern blot hybridization in a series of 23 matched normal mucosas and HNSCC. Overexpression of cyclin D1 mRNA was observed in 10 of 23 cases (43.5%) of HNSCC, ranging from 2 to 50-fold higher than the normal control. Twelve of 23 cases could be evaluated by Southern blot hybridization, and gene amplification was found in only 2 of 12 cases (16.7%). These findings suggest that cyclin D1 plays an important role in tumorigenesis of HNSCC, and gene amplification is not one of the major mechanisms for overexpression of cyclin D1.

Key words: Head and neck squamous cell carcinoma — Cyclin D1 — Quantitative analysis — Competitive RT-PCR — Southern blot hybridization

Cyclins are a group of nuclear proteins that regulate the cell cycle progression by interacting with cyclin-dependent kinases (cdks).[1,2] The gene encoding cyclin D1, first described as the PRAD1 gene, which is rearranged in some parathyroid tumors,[3] is a putative oncogene and plays an important role in regulation of the G1/S phase transition.[1,2,4,5] It has been mapped to human chromosome 11q13, and consists of 5 exons and 4 introns.[6] Amplification of this region has been demonstrated in a variety of malignancies including B-cell leukemia, breast, lung, esophageal, head and neck squamous cell carcinoma (HNSCC).[1,7–9] Overexpression of cyclin D1 has also been demonstrated in HNSCC,[10,11] based on analysis at the protein level by immunohistochemical staining. It would be of interest to examine the situation at the RNA level. RNase-protection assay and northern blot analysis are conventional methods for quantitation of mRNA. However, when the amount of tissue sample is limited or the level of gene expression is low, these methods are not sensitive enough to detect mRNAs of target genes.[12] Reverse transcription-polymerase chain reaction (RT-PCR) has been used for detecting small amounts of mRNA, because of its high sensitivity. A difficulty with quantitative PCR is that the amount of PCR product may not accurately reflect the initial amount of target cDNA because of the variance of amplification efficiency in each reaction and because of the plateau effect in PCR reaction.[13] Therefore, it is necessary to coamplify a target and an internal standard simultaneously in a single tube, with a common primer set (competitive PCR).[14,15]

We aimed to investigate whether there is a relationship between overexpression of cyclin D1 mRNA and gene amplification in HNSCC. For this purpose, we analyzed the level of mRNA expression by a modified method of competitive RT-PCR with double internal standards, and the presence of gene amplification by means of Southern blot hybridization. To our knowledge, this is the first report describing the application of competitive RT-PCR for quantitation of cyclin D1 mRNA in human neoplasms.

MATERIALS AND METHODS

Patients and tissues Both pathologically identified tumor regions and nonneoplastic adjacent mucosas were removed from 23 HNSCC patients who underwent surgery at the Department of Otolaryngology, Okayama University Hospital (Okayama). Specimens were SCC from paranasal sinus (3), tongue (11), oropharynx (4), hypopharynx (1), larynx (3), and salivary gland (1). Samples were snap-frozen in liquid nitrogen and stored at −80°C until studied.

DNA and RNA extraction Genomic DNAs were prepared from frozen tissues by sodium dodecyl sulfate (SDS) and proteinase-K digestion, phenol and chloro-

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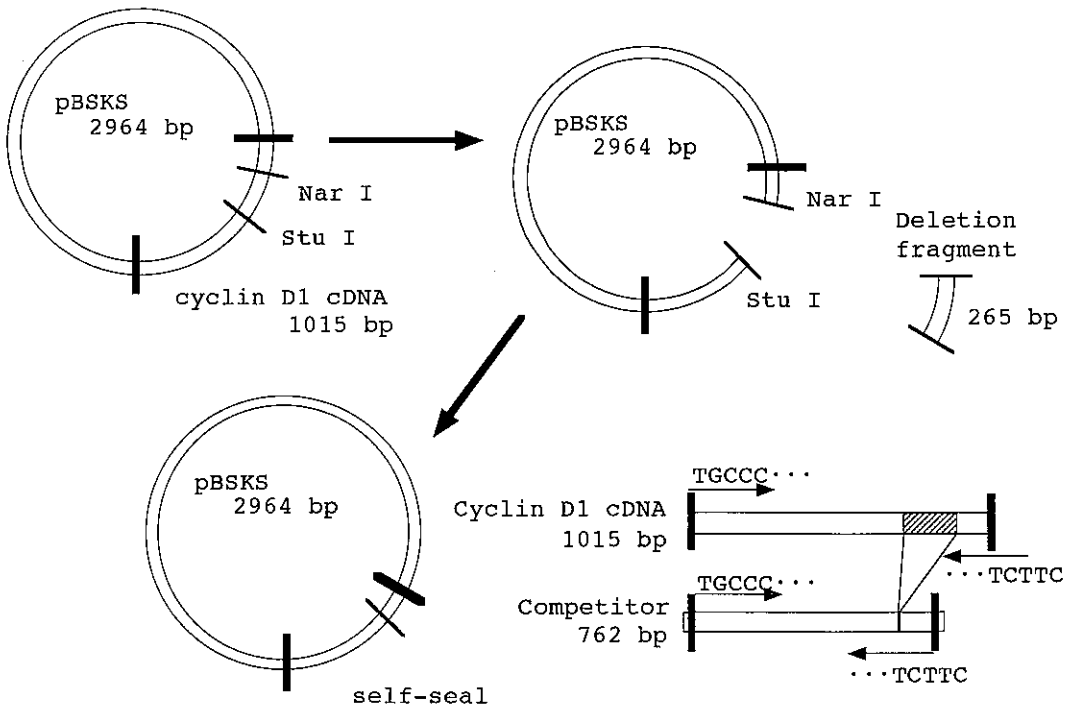

Fig. 1. Construction of the mutant internal control (competitor) used in our quantitative PCR. Details are given in the text.

form extraction and ethanol precipitation. Total RNAs were prepared by means of a modified acid guanidinium phenol chloroform method (ISOGEN kit, Nippon Gene, Tokyo). Nucleic acids were quantitated spectrophotometrically by using an Ultraspec3000 UV/Visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden), and stored at 4°C for DNA or at −80°C for RNA.

Reverse transcription Total RNA was reverse-transcribed with the SuperScript Preamplification System (Gibco BRL, Grand Island, NY). The RNA (5 μg) was dissolved in 11 μl of diethyl pyrocarbonate (DEPC)-treated water, 0.5 μg of oligo (dT) primer was added, and the mixture was incubated at 70°C for 10 min, then chilled on ice for 1 min. Two microliters of 10× PCR buffer (200 m*M* Tris-HCl [pH 8.4], 500 m*M* KCl), 2 μl of 25 m*M* $MgCl_2$, 2 μl of 0.1 *M* DTT, 1 μl of 10 m*M* dNTP mix, and 1 μl of SuperScript II (200 U/μl) were then added, and the whole was incubated at 42°C for 50 min. After heating at 70°C for 15 min, the reaction mixture was treated with 1 μl of RNase H (2 U/μl) at 37°C for 20 min.

Oligonucleotide primers and PCR reaction PCR amplification was carried out with rTth-XL DNA polymerase (GeneAmp XL PCR Kit, Perkin-Elmer-Cetus, Norwalk, CT) in a thermal cycler (PJ-1000, Perkin-Elmer-Cetus). Oligonucleotide primers and PCR conditions were as follows.

Cyclin D1: Primers used for RT-PCR were synthesized by a DNA synthesizer (ABI-380B, Perkin-Elmer-Cetus) based on the published human cyclin D1 cDNA sequences.[3] A full-length cyclin D1 RT-PCR product (1015 bp, covering the entire coding sequence) and its derivative (750 bp) with an internal deletion were amplified from a one-tenth aliquot of the RT-products with S1 primer (5′-TGCCCAGGAAGAGCCCCAGC) and AS1 primer (5′-GGCCTCGTAAAACTATGGTCTTC), under the following standard conditions: 26 cycles of denaturing at 94°C for 30 s, extension at 68°C for 3 min and final elongation at 72°C for 7 min.

Glyceraldehyde phosphate dehydrogenase (GAPDH): To calibrate the exact amount of mRNA, parallel RT-PCR was carried out for each RT-product using a primer set for human GAPDH cDNA: GPS1 (5′-ACGGATTTG-GTCGTATTGGG) and GPAS1 (5′-CGCTCTAGGG-AGGTTTTAGT). The standard PCR conditions were 20 cycles of denaturing at 94°C for 30 s, extension at 64°C for 3 min and final elongation at 72°C for 7 min. As a negative control to eliminate DNA contamination, RT-PCR without the reverse transcriptase was also carried out, because the GAPDH gene does not contain any introns.

Competitor design For quantitative PCR, we raised a mutant cyclin D1 cDNA as a competitor by introducing an internal deletion into the normal cyclin D1 cDNA

(1015 bp) cloned into a plasmid vector, pBluescript KS (−) (pBSKS) (Fig. 1). After digestion of the cyclin D1 cDNA clone with restriction endonucleases, Nar I and Stu I, the main body of the plasmid was flushed with T4 DNA polymerase, and self-sealed by blunt-end ligation. The competitor cDNA (762 bp) was excised from this plasmid by *Eco*R I and *Hin*d III digestion, then purified by phenol and chloroform extraction and ethanol precipitation. After spectrophotometric quantitation, the competitor fragment was serially diluted in sterile water to give concentrations of 100, 50, 20, 10, 5, 2, 1 amol/μl.

Competitive PCR PCR amplification was performed using a GeneAmp XL PCR Kit (Perkin-Elmer-Cetus). Each reaction mixture contained 6 μl of 3.3× XL buffer II (composed of tricine, potassium acetate, glycerol, and dimethyl sulfoxide), 3 μl of 25 m*M* $Mg(OAc)_2$, 0.4 μl of 10 m*M* dNTPs blend, 2.2 μl of sterile water, 0.8 U of rTth DNA Polymerase-XL, 1 μl each of 10 pmol/μl primers (sense and antisense). To the reaction mixture, 5–10% aliquots of the RT-products as the target template and 2 μl of the competitor at various concentrations were added to give the final volume of 20 μl. PCR amplification was carried out for 26 cycles, initiating by the hot start method. The optimum number of cycles for this analysis was determined by control experiments, such that both PCR reactions remained within the exponential phase.

Quantification of the PCR product PCR products were separated through 2% agarose gel and stained with ethidium bromide. As the PCR product derived from the competitor fragment was about 300 bp smaller than that from the target cDNA, these products were readily distinguishable. The intensity of ethidium bromide staining of each band was measured by a CCD image sensor (Gel Print 2000/VGA, Toyobo, Osaka), and analyzed by a computer program for band quantitation (Quantity One, Toyobo).

The degree of tumor-specific cyclin D1 overexpression was determined by calculating the ratio of the expression levels in the tumor and in the normal sample, each of which was normalized for the corresponding GAPDH expression level. Overexpression of cyclin D1 mRNA was considered to be present when this ratio was greater than 2.

Southern blot analysis DNA was extracted from each of 23 HNSCC paired specimens, and 10 μg was digested completely with *Eco*R I. The samples were electrophoresed through 1% agarose gels, transferred to nylon membranes (Hybond-N^+, Amersham Japan, Tokyo), and fixed at 80°C for 2 h. The filters were processed by following the standard Southern blot hybridization procedure.[16] After prehybridization, filters were hybridized for 16–24 h with cyclin D1 cDNA probe which was labeled with [α-^{32}P] dCTP by the random priming labeling method (Megaprime DNA labeling system, Amersham Japan). Subsequently, the filters were washed and exposed to an imaging plate for 12 h, and to a Kodak XAR film in a cassette with an intensifying screen at −80°C for several days. The filters were stripped free of the cyclin D1 probe by washing in 0.5% SDS solution at 100°C, and then rehybridized with a ^{32}P-labeled cDNA probe of human c-K-*ras* gene, which was reported to be rarely amplified in HNSCC,[9] to normalize possible variations in DNA loading or transfer efficiency. For quantitation of signals, densitometric analysis was carried out by a Bioimaging Analyzer System (BAS2000, Fuji, Tokyo). The extent of gene amplification of cyclin D1 was determined by calculating the ratio of intensities of the cyclin D1 band from the tumor and normal samples, after normalization to the corresponding K-*ras* control bands. Gene amplification was considered to exist when this ratio was greater than 2.

RESULTS

Control experiments To determine a suitable number of amplification cycles for analyzing cyclin D1 and GAPDH gene expression, we performed preliminary amplification for each gene. First, we amplified a constant amount of RT-product with GAPDH primers for varying numbers of cycles. We chose 20 cycles for PCR amplification because this was in the middle of the exponential phase (Fig. 2A). Secondly, we coamplified constant amounts of RT-product and the competitor fragment with cyclin D1 primers for varying numbers of cycles. We chose 26 cycles, since the efficiency of PCR amplification was equivalent for both coamplified products and this was within the exponential phase (Fig. 2B). Thirdly, we reverse-transcribed varying amounts of total RNA, and amplified the resulting cDNA under the standard conditions. The concentration of total RNA closely correlated with the amount of PCR products ($r=0.983$, $n=23$, $P<0.0001$), and the efficiency of reverse transcription appeared to be constant (Fig. 2C).

Competitive RT-PCR We quantitated the expression level of cyclin D1 mRNA by coamplifying a constant amount of RT-product and varying amounts of the competitor and by determining the amount of competitor giving an equal molecular yield to that from the target cDNA (Fig. 3). We defined "overexpression" as being the condition when the ratio of the expression level in the tumor to that in the normal sample was greater than 2 (Fig. 4). The cyclin D1 overexpression was found in 10 of 23 cases of HNSCC (43.5%), ranging from 2 to 50-fold higher than the normal control (Table I).

The distribution of patients according to age, sex, tumor site, histological grade, growth pattern, and pathological stage is shown in Table II. No statistically signifi-

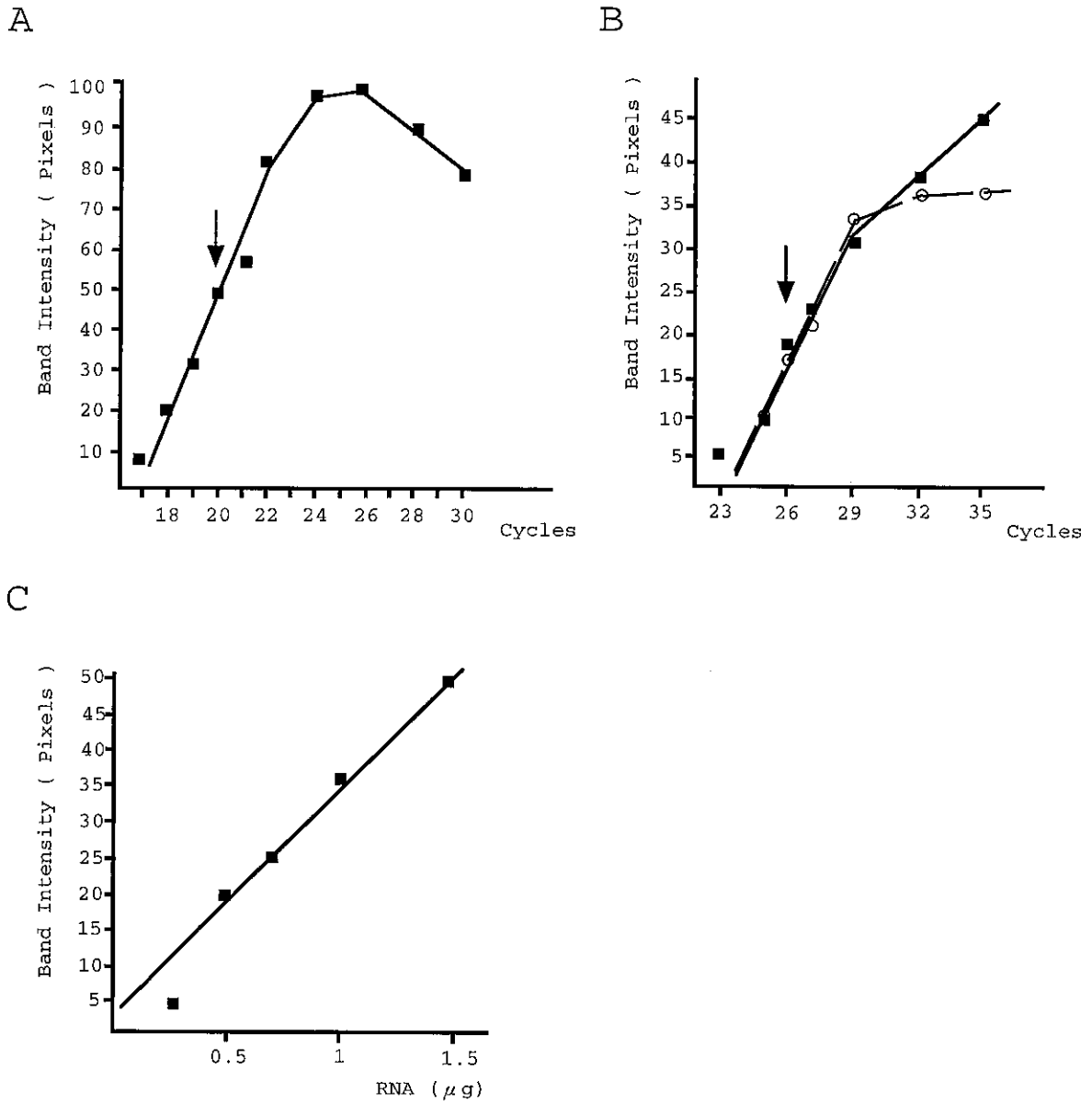

Fig. 2. Control experiments for determination of suitable PCR conditions. A, A constant amount of RT-product (corresponding to 0.5 μg of total RNA) was amplified with GAPDH primers for varying numbers of cycles (14, 16, 17, 18, 19, 20, 21, 22, 24, 26, 28, or 30 cycles). The intensity of ethidium bromide staining of each band was plotted against the number of amplification cycles. Our standard cycle number (20 cycles) is shown by an arrow. B, Constant amounts of RT-product (corresponding to 0.5 μg of total RNA) and the competitor (50 amol) were coamplified with cyclin D1 primers for varying numbers of cycles (20, 23, 25, 26, 27, 29, 32, or 35 cycles). Intensities of ethidium bromide staining of PCR products from the target (■) and from the competitor (○) were plotted against the number of amplification cycles. Our standard cycle number (26 cycles) is shown by an arrow. C, Varying amounts of total RNA were reverse-transcribed and the resulting cDNA was amplified with cyclin D1 primers under our standard conditions.

cant correlation between overexpression and any of these clinicopathological characteristics of the patients was found.

Southern blot analysis To investigate whether overexpression of cyclin D1 mRNA is associated with amplification of the gene, we also quantitated the cyclin D1

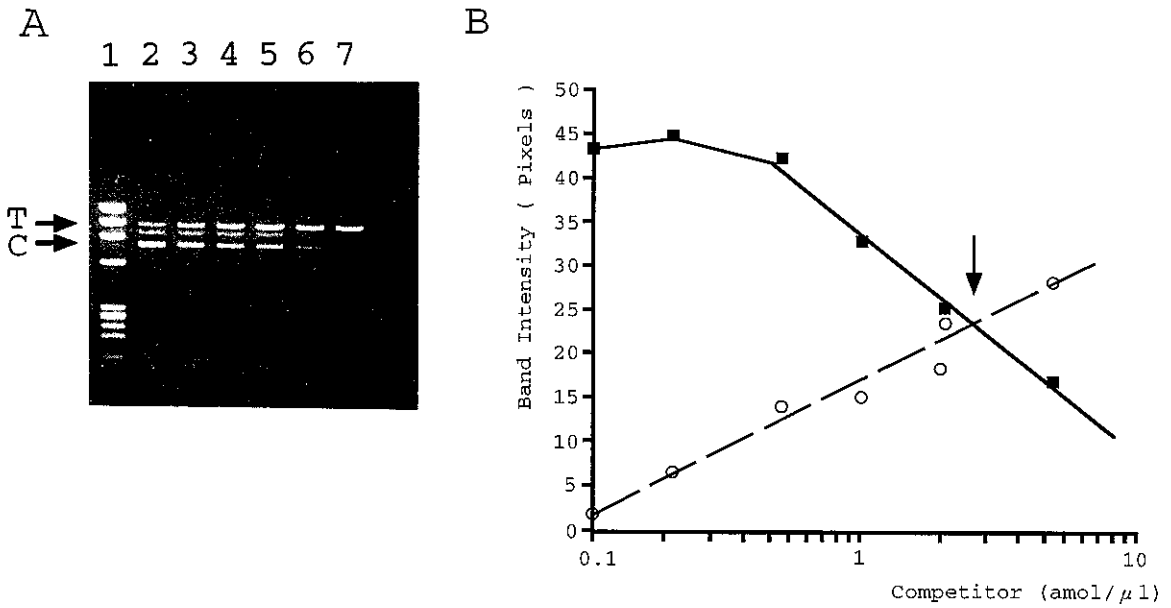

Fig. 3. Quantification of cyclin D1 mRNA by competitive PCR. A, Photograph of ethidium bromide-stained gel. Varying amounts of the competitor (5, 2, 1, 0.5, 0.2, or 0.1 amol/μl) were coamplified with a constant amount of RT-product (corresponding to 0.5 μg of total RNA). Size markers (ϕX174DNA/Hae III) are shown in lane 1. The amount of competitor was 5 amol/μl (lane 2), 2 amol/μl (lane 3), 1 amol/μl (lane 4), 0.5 amol/μl (lane 5), 0.2 amol/μl (lane 6), or 0.1 amol/μl (lane 7). The leftmost arrows denote PCR products derived from the target (upper) and from the competitor (lower), respectively. B, Intensities of ethidium bromide staining of PCR products from the target (■) and from the competitor (○) were plotted against the amount of the competitor. The equivalent point of titration is shown as an arrow.

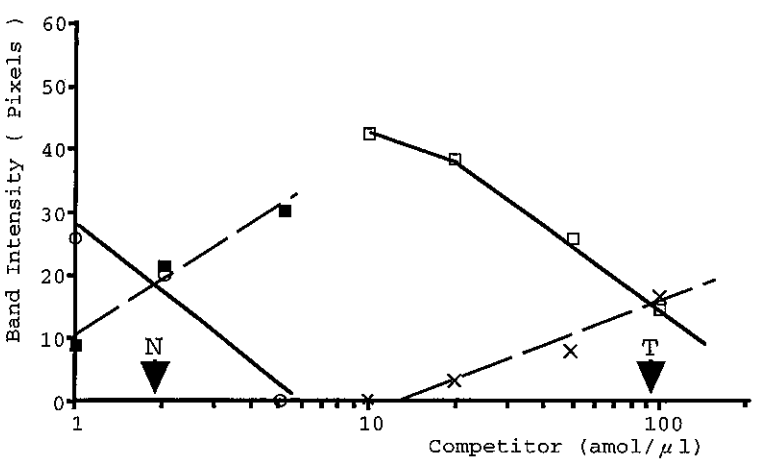

Fig. 4. A representative case (case No. 1) showing overexpression of cyclin D1 mRNA. Intensities of PCR products of the target (■) and of the competitor (○) from normal tissue were plotted against the amount of the competitor. Those of the target (□) and of the competitor (×) from tumor tissue are also plotted here. The equivalent points of titration are indicated by "N" in normal and by "T" in tumor. The ratio of the expression level in the tumor to that in the normal sample was calculated to be about 50.

copy number by Southern blot hybridization analysis. We were only able to evaluate twelve of 23 cases owing to the limitations of sample quantity and the quality of genomic DNA. The cyclin D1 probe detected two *Eco*R I fragments (7.5 kb and 5.5 kb), and the K-*ras* probe detected three fragments (6.7 kb, 3 kb, and 2.4 kb), as shown in Fig. 5. We evaluated the tumor/normal ratio of the intensities of the 7.5 kb fragment of cyclin D1 to obtain the amplification and that of the 3 kb fragment of K-*ras* to obtain the factor for normalization. Two of 12 cases of HNSCC (16.7%) were found to have amplification of cyclin D1 ranging from 2 to 10-fold higher than the normal control. No rearrangement of the gene was detected.

Table I. Overexpression and Amplification of Cyclin D1 Gene

No.	Sex	Age	Tumor site	Histological differentiation	TNM stage	LN meta	Prognosis	Overexp.	Gene amp.
1	M	78	paranasal sinus	moderate	IV	+	dead/T[a]	+ (50×)	+ (9.5×)
2	F	70	tongue	well	I	−	alive/T	−	−
3	M	70	oropharynx	moderate	III	−	alive/T	−	−
4	M	61	tongue	well	II	−	alive	−	−
5	M	55	hypopharynx	well	IV	−	alive	−	−
6	M	66	oropharynx	moderate	III	+	alive	−	−
7	M	51	oropharynx	moderate	II	−	alive	+ (5×)	NI[c]
8	M	58	salivary gland	moderate	IV	+	alive/T[b]	+ (2.4×)	−
9	M	64	larynx	moderate	IV	+	alive	−	NI
10	M	81	larynx	moderate	IV	+	alive	−	NI
11	M	59	paranasal sinus	moderate	IV	−	alive	+ (9×)	−
12	F	53	tongue	well	IV	+	dead/T	−	−
13	M	45	tongue	moderate	IV	+	dead/T	+ (8×)	−
14	F	71	tongue	moderate	IV	−	alive/T	−	NI
15	M	71	larynx	moderate	IV	+	alive	−	NI
16	F	65	tongue	well	IV	+	dead/T	−	NI
17	M	61	tongue	well	II	−	alive	+ (8×)	−
18	F	72	tongue	moderate	IV	+	dead/T	−	NI
19	F	79	tongue	well	III	+	alive	+ (2.6×)	+ (2.2×)
20	F	65	paranasal sinus	well	IV	−	alive/T	−	NI
21	M	62	tongue	well	IV	+	alive/T	+ (3,2×)	NI
22	M	67	oropharynx	moderate	IV	+	alive	+ (4.6×)	NI
23	F	71	tongue	well	III	+	alive	+ (6.6×)	NI

a) Dead due to tumor recurrence.
b) Alive with tumor recurrence.
c) Not informative.

Table II. Overexpression of Cyclin D1 and Clinicopathological Characteristics

Category		Overexpression of cyclin D1		*P*-value[a]
		Positive ($n=10$)	Negative ($n=13$)	
Age		63.1	66.5	NS[b]
Sex	male	8	7	NS
	female	2	6	NS
Tumor site	paranasal sinus	2	1	NS
	tongue	5	6	NS
	oropharynx	2	2	NS
	hypopharynx	0	1	NS
	larynx	0	3	NS
	salivary gland	1	0	NS
Histology	well differentiated	4	7	NS
	moderate or poorly differentiated	6	6	NS
Clinical stage	I–II	2	2	NS
	III	2	2	NS
	IV	6	9	NS
Lymphnode metastasis	positive	7	7	NS
	negative	3	6	NS
Growth pattern	invasive	4	7	NS
	not invasive	6	6	NS

a) Fisher's exact test was used for statistical analysis.
b) Not significant.

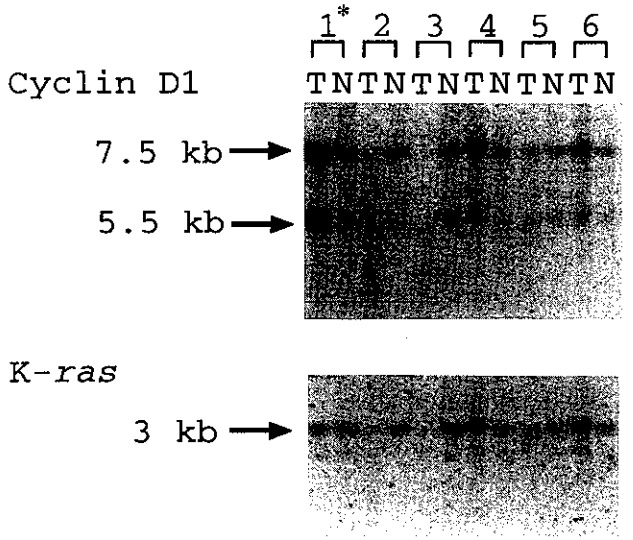

Fig. 5. Southern analysis of the cyclin D1 gene: Southern blot hybridization profiles with cyclin D1 cDNA probe are shown on the top panel. Two *Eco*R I fragments (7.5 kb and 5.5 kb) were detected, and the 7.5 kb fragment was evaluated as representing a cyclin D1 gene copy. Hybridization profiles with K-*ras* probe are shown below as an internal control. * Gene amplification positive.

DISCUSSION

The purpose of this study was to analyze the expression of cyclin D1 mRNA by competitive RT-PCR in HNSCC, and to investigate whether there is a relationship between overexpression and gene amplification. Our results indicated that the levels of cyclin D1 mRNA are frequently increased in HNSCC, but that gene amplification does not seem to be the major mechanism contributing to this overexpression. That is, overexpression of cyclin D1 was observed in 43.5% of 23 cases of HNSCC, while gene amplification was found only in 2 of 12 cases (16.7%). Although both of the cases with the gene amplification exhibited overexpression of cyclin D1, overexpression of the mRNA was not always accompanied with gene amplification.

Overexpression of cyclin D1 has also been demonstrated in 45–65% of HNSCC.[10, 11] These were analyzed only by immunohistochemical staining, but the results are essentially in accordance with ours. Northern blot analysis is the conventional method for quantitation of mRNA, when the quantity of tissues is sufficient, but to our knowledge, only Jares *et al.*[17] have succeeded in measuring the cyclin D1 mRNA in HNSCC by this method, suggesting that cyclin D1 expression is relatively low in general. In fact, cyclin D1 mRNA was scarcely detectable in many specimens of HNSCC by northern blot analysis in our experiments (data not shown).

In this study, therefore, we have aimed to measure the expression level of cyclin D1 gene by means of RT-PCR, which is widely used for amplifying small amounts of mRNA. A previous report described an increased level of cyclin D1 mRNA in HNSCC as determined by RT-PCR with GAPDH as a control.[18] However, in order to quantitate target mRNA more accurately by RT-PCR, several points are important.

Firstly, measurements must be performed within the exponential phase of amplification. The amount of PCR product increases exponentially in early cycles of the reaction, but subsequently reaches a plateau level owing to shortage of primers or dNTPs, inhibition by reactive products and the inactivation of polymerase.[15] We confirmed in control experiments that our PCR amplification was performed within the exponential phase.

Secondly, it is important to coamplify a target template and an internal control (competitor) which is designed so that it may be amplified at the same efficiency as the target. Because of the extraordinarily high sensitivity of PCR, subtle differences in amplification efficiency may dramatically affect the amount of PCR product at the end of the reaction.[14] Coamplification of a target and a competitor is very useful for quantitation of the target.

Thirdly, it is necessary to standardize the variable amounts of RNA applied for analysis. We did this by means of control experiments using RT-PCR of GAPDH mRNA, which is expressed in all cells and does not vary much in expression level. The double internal standard protocol that we have employed here should increase the accuracy of the quantitation of mRNA. A drawback of the technique we have described is that quantitation of GAPDH was done by non-competitive PCR. By adopting standardized competitive PCR for the second internal standard to adjust the total amounts of RNA, much more accurate analysis should be possible.

Another point to consider is the variation of efficiency of reverse transcription. We analyzed the relative quantity of cyclin D1 mRNA in the tumor region and in normal tissue using a cDNA competitor, to confirm that the variables of reverse transcription were within a permissive range. If it is necessary to determine the absolute quantity of the target mRNA, this problem should be resolved by using an RNA competitor.[19] However, the RNA competitor is very unstable and the protocol is rather tedious. In this respect, the cDNA competitor protocol described here is more convenient for quantitative PCR.

Taking into account these points, we were able to quantitate cyclin D1 transcripts by a modified competitive RT-PCR with coamplification of a target template and a competitor cDNA template under the same PCR

conditions, with the same primer sets, in a single reaction tube.

There are several potential mechanisms which may lead to overexpression of cyclin D1 mRNA without gene amplification. One possibility is abnormality of receptor-dependent-transcriptional regulation. Gene amplification and rearrangement of the epidermal growth factor receptor (EGFR) gene have been found in 10–20% of HNSCC.[20-22] Overexpression of H-*ras* mRNA, which encodes a key factor in the tyrosine-kinase cascade, has also been reported in HNSCC.[23] Albanese *et al.*[24] reported that the specific promoter region of the cyclin D1 gene is affected by tyrosine-kinase cascade factors such as EGFR, $p21^{ras}$, $p42^{MAPK}$, c-ETS-2 and c-Jun, and that transcription of cyclin D1 is upregulated by them.

Another possible mechanism is abnormality of cell cycle-dependent transcriptional regulation. In cell cycle regulation, cyclin D1, together with specific kinases (cdk 4 and cdk6), inactivates the product of the retinoblastoma gene (pRb) by phosphorylation.[2] Inactivated pRb releases transcriptional factors, E2Fs, which activate many genes necessary for cell cycle progression, for example, DNA polymerase α, thymidine kinase, and so on.[2, 25, 26] In turn, pRb could bind to cyclin D1 promoter and downregulate its transcription and protein expression, as reported recently.[27] Moreover, E2F binding sites are found in cyclin D1 and cyclin E promoters, and both promoters are activated by E2F.[26, 28] These reports suggest the presence of cross-regulatory mechanisms among cyclin D1, pRb and E2F, and thus loss of heterozygosity and inactivation of the Rb gene or amplification/activation of E2F genes may influence cyclin D1 transcription.

A third possible mechanism is posttranscriptional abnormality. A previous study suggested that the 3′AU-rich region containing sequences involved in mRNA stability and translational control was eliminated by gene rearrangement within the 3′-untranslated region of the cyclin D1 gene in mantle-cell lymphoma and that the half-life of the cyclin D1 mRNA was elongated 6-fold compared with that of the normal control.[29] Any or all of the above possibilities could account for the observed overexpression of cyclin D1 mRNA. It is also likely that the mechanisms involved vary from tumor to tumor.

We could not find any statistically significant correlation between overexpression of cyclin D1 mRNA and clinicopathological characteristics of the patients. In previous reports, it was noted that gene amplification or overexpression of cyclin D1 in HNSCC was significantly associated with high proliferative activity, high cytological grade, infiltrative growth pattern, clinical stage, and primary tumor site (hypopharynx).[7-9] However, the relationship of cyclin D1 overexpression and clinicopathological factors has not been fully established. Jungehulsing *et al.*[9] reported that gene amplification was not associated with histology, disease development, tumor site or other clinical parameters. Gaffey *et al.*[10] reported that the overexpression was not associated with pathological stage, cytological grade, architectural pattern or disease-free or overall survival periods. Therefore, these and our results collectively suggest that cyclin D1 may play an important role in the early stage of tumorigenesis of HNSCC. Very recently, cyclin D1 overexpression has been found at an early stage of tumor development (papilloma region) in a rat esophageal carcinogenesis model, and no difference in cyclin D1 expression levels during tumor progression (papillomas and carcinomas) has been found.[30] A clinical cohort study with a large number of specimens is needed to confirm the involvement of cyclin D1 in the early stage of carcinogenesis.

In conclusion, we have found that the transcription of cyclin D1 is frequently upregulated without gene amplification in HNSCC. This study provides circumstantial support for the hypothesis that the cyclin D1 gene is a putative oncogene in HNSCC. Further exploration is required to clarify the factors influencing the expression of cyclin D1. We wish to emphasize that competitive RT-PCR, particularly our modified simple procedure employing a cDNA competitor, together with another internal control, RT-PCR of GAPDH, provides a useful and convenient method for accurate quantitation of target mRNAs in small amounts of samples.

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