Title: Prognostic value of cyclin D1 expression in tumor-free surgical margins in head and neck squamous cell carcinomas.

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Running title: Prognostic value of cyclin D1 in tumor-free surgical margins.

Abstract

Conclusion. It was proved that cyclin D1-positive status in surgical margins was an independent prognostic indicator of local recurrence. The expression of cyclin D1 in tumor-free surgical margins may better predict local recurrence in head and neck squamous cell carcinoma (HNSCC) patients after surgical treatment with curative intent.

Objectives. This retrospective study was aimed at determining the prognostic indicator for local recurrence in HNSCC.

Method. A total of 116 HNSCC patients who underwent surgical treatment with curative intent and had histopathologically tumor-free margins were eligible for this study. The expression of p53 and cyclin D1 was assessed by the immunohistochemical staining in surgical margins as well as in tumor specimens.

Results. Sixty-three patients (54.3%) had p53-positive tumor specimens, and 34 patients (29.3%) had p53-positive margins. Seventy-six patients (65.6%) had cyclin D1-positive tumor specimens, and 54 patients (46.6%) had cyclin D1-positive margins. A significant difference in local control rates was observed between patients with cyclin D1-positive and -negative margins (77.2% vs. 91.5%, log rank test, p=0.0139). Multivariate Cox proportional hazards testing indicated that the hazard ratio of cyclin D1-positive margins for local recurrence was 4.58 (95% confidence interval 1.14- 21.69, p=0.0304).

Introduction

Surgical treatment for head and neck squamous cell carcinoma (HNSCC) is undertaken with curative intent. The primary goal of surgery is to obtain tumor-free margins while sparing as much normal tissue as possible. The histopathological status of the resected margins is a potential indicator of tumor recurrence. However, even when surgical margins are revealed to be histopathologically clear, the local recurrence rate has reported to be 10 to 30% [1,2].

Local recurrences and/or multiple primary tumors develop despite an apparently complete excision and histopathologically tumor-free surgical margins. It was well known that the development of HNSCC is the result of a multistep process characterized by the accumulation of genetic and epigenetic alterations [3]. These genetic and epigenetic alterations in the tumor-free surgical margins may not be detectable by conventional microscopic histopathological analysis, but may be detected using immunohistochemical (IHC) staining.

The oncogene cyclin D1 is involved in the regulation of the G1 checkpoint and may play an important role in the tumorigenesis of head and neck cancers. The tumor suppressor gene p53 encodes a nuclear transcription factor that is a critical regulator of cell growth and proliferation through its role in cell-cycle checkpoint control. Mutations in or overexpression of p53 have been reported in approximately 40-60% of head and neck cancers [4,5].

This retrospective study was aimed at exploring prognostic indicators for local recurrence in HNSCC patients treated by surgical resection. We examined the expression of p53 and cyclin D1 in surgical margins as well as in tumor specimens.

Material and methods

Patients. A total of 162 retrospectively identified HNSCC patients were surgically treated with histologically tumor-free margin specimens at the Hokkaido University Hospital from 1995 to 2007. Sixteen of the 162 patients who underwent salvage surgery for recurrent tumors after definitive radiation therapy and 30 patients who underwent salvage surgery after concomitant chemoradiotherapy were excluded. The remaining 116 patients were eligible for this study.

Table 1 shows patient characteristics. The median age of patients was 61 years old (range 29-86 years old). The primary site was the oral cavity in 43 patients, oropharynx in 11 patients, hypopharynx in 39 patients, and larynx in 23 patients. Patients were also evaluated by a multidisciplinary team consisting of head and neck surgeons, radiation oncologists, and medical oncologists, and tumors were classified according to the American Joint Committee on Cancer (AJCC) staging system (6^{th} edition, 2002). Histologic tumor grade was well differentiated in 56 patients, moderately in 50 patients, and poorly in 10 patients.

Adjunctive treatment was performed for 57 patients. Of 57 patients, 39 patients underwent pre-operative radiotherapy and/or chemotherapy. Eighteen patients underwent post-operative radiotherapy. Total irradiation dose ranged from 20 to 50 Gy (median 40 Gy, mean 44 Gy).

Approval for this study was obtained from the institutional review board at Hokkaido University. Completion of the survey was considered as implied consent for participation.

Immunohistochemistry. Primary tumor resection was performed at the time of surgical treatment. Following primary tumor resection, surgical margin specimens (two or three

superficial mucosal margin samples and one deep connective and/or muscle tissue margin sample) were harvested from the surgical defect. Tumor specimens and surgical margins were assessed for IHC staining. The specimens were embedded in paraffin and cut into 4 μm-thick sections. After deparaffinization with xylene and rehydration through an ethanol series, the specimens were placed in 0.1% hydrogen peroxide to quench any endogenous peroxide activity. The slides were then incubated with an anti-human cyclin D1 monoclonal antibody (NCL-L-CYCLIN D1-GM; Novocastra Laboratories Ltd., Newcastle, UK), and a p53 monoclonal antibody (NCL-p53-DO7; Novocastra Laboratories Ltd., Newcastle, UK) in a humid chamber at 4℃ overnight. The sections were then incubated with a biotin-labeled rabbit anti-mouse secondary antibody (Histofine SAB-PO (M) kit; Nichirei, Tokyo, Japan) for 30 minutes at 37℃ followed by incubation with a streptavidin-biotin horseradish peroxidase complex. We determined the percentage of p53- and cyclin D1-positive basal or suprabasal cells in the mucosal epithelium by calculating the ratio of positively stained cells to the total number of cells in the most highly stained area of each slide.

Following previously published criteria, we considered a specimen with more than 10% of cells demonstrating p53 IHC staining to be p53-positive [6,7], and a specimen was considered to be cyclin D1-positive if more than 10% of the cells demonstrated positive nuclear staining for cyclin D1, based on a previous study [8]. Figure 1 demonstrated representative IHC staining samples of surgical margins. These evaluations were performed by three investigators (T.S., N.O, and S.S.), who were blinded to clinical outcome.

Statistics. The Kaplan-Meier method was applied for overall survival and local control curves, and the survival and control rates were compared using the log rank test. The time of interest

was the duration from the start of treatment to death or failure. A Cox proportional hazard regression model was used to assess the effects of each factor on overall survival or local control rate. Cyclin D1- and p53-positive rates were compared using the Pearson's chi-squared test. A p-value of less than 0.05 was considered statistically significant. JMP 9.0.2 statistical software (SAS Institute, Cary, NC) was used for the statistical analysis.

Results

The median follow-up period for surviving patients was 69.6 months (range 12-164.4, mean 77.9 months). All of the surgical margin specimens were histopathologically shown to be tumor free, there was neither cancer cells nor a dysplasia.

Sixty-three patients (54.3%) had p53-positive tumor specimens, and 34 patients (29.3%) had p53-positive margins. Seventy-six patients (65.6%) had cyclin D1-positive tumor specimens, and 54 patients (46.6%) had cyclin D1-positive margins. Thirty-four patients died of HNSCC, 10 patients died of the other diseases, and 71 patients were alive without disease at the end of the follow-up. One patient was alive with distant metastasis.

Figure 2 shows local control curves. A significant difference in local control rates was observed between patients with cyclin D1-positive margins and those with cyclinD1-negative margins (77.2% vs. 91.5%, p=0.0139).

The overall survival and local control rates associated with each categorical variable were summarized in Table 2. There was a significant difference in overall survival with regard to the primary site of the tumor (p= 0.0373). In addition, patients with advanced T stage, with nodal metastases, with poorly or moderately differentiated histologic tumor grade, and with adjunctive treatment had worse overall survival (p=0.0191, p=0.0138, p=0.0135, and

p=0.0062, respectively). Moreover, patients with cyclin D1-positive margins had significantly worse local control (p=0.0139). There was no significant difference in local control between patients with and without adjunctive treatment (81.7% vs. 88%, p=0.2172). Table 3 shows an analysis of prognostic indicators using a multivariate Cox proportional hazards test. There was a significant difference in overall survival between patients with well differentiated histologic tumor grade and with poorly or moderately differentiated grade (p=0.0350). Besides, there was a significant difference in local control between patients with cyclin D1-positive margins and -negative margins (p=0.0306). The hazard ratio for cyclin D1-positive margins for local recurrence was 4.58 (95% confidence interval 1.14- 21.69).

Figure 3 shows local control rates according to the IHC status in tumor specimens and surgical margins. A significant difference was observed in local control rates among groups classified according to cyclin D1 expression in tumor specimens and surgical margins (p=0.0170, log rank test for trend). Cyclin D1 expression in surgical margins remained positive in 39 (51.3%) of 76 patients with cyclin D1-positive tumor specimens. Five-year overall survival and local control rates of them were 52.5% and 72.1%, respectively. Cyclin D1 expression in surgical margins changed into negative status in 37 (48.7%) of 76 patients with cyclin D1-positive tumor specimens. Five-year overall survival and local control rates of them were 78.8% and 92.8%, respectively. There were significant differences in both overall survival rate and local control rate between patients with both tumors and margins positive for cyclin D1 and those with cyclin D1-positive tumors but cyclin D1-negative margins (p=0.0484 and p=0.0281, respectively).

The rates of p53-positive margins in patients with and without adjunctive treatment were 36.8% (21/57) and 22.0% (13/59), respectively (p=0.0798), and the rates of cyclin

D1-positive margins in patients with and without adjunctive treatment were 63% (36/57) and 30.5% (18/59), respectively (p=0.0004).

Discussion

Even in surgical margins classified as histopathologically negative, the local recurrence rate is reported to be 10 to 30% [1,2]. Some groups, therefore, investigated alternative approaches to the detection of occult tumor cells in the surgical margins of patients with HNSCC [9,10]. These occult tumor cells may express factors associated with local recurrence and some of these factors, which are the result of genetic or epigenetic alterations, can be detected by IHC staining. It is widely accepted that IHC analysis along with pathological assessment is a feasible alternative to other molecular genetic techniques, such as quantitative real-time PCR, immunoblotting and DNA sequencing. Therefore, we applied an IHC approach to the assessment of the expression of a cell-cycle regulator, cyclin D1 and a tumor suppressor gene, p53, in tumor specimens and surgical margins, and found that margin specimens without tumor cells expressed these factors in varying degrees.

It is well known that p53 is a tumor suppressor gene and that it is phosphoprotein phosphorylated at multiple sites by a variety of kinases. Alterations in p53 have commonly been found in HNSCCs, but its influence on prognosis remains controversial. In recent reports, p53 mutations, detected by molecular assays such as DNA sequencing, LigAmp assay, and real-time PCR, were considered a useful prognostic factor [9-12]. However, we previously reported that the IHC assay of p53 was not useful in predicting the prognoses of patients undergoing chemoradiation therapy [13]. In the current study, we also failed to find any evidence supporting the idea that the IHC analysis of p53 in surgical margins could be useful

for predicting the prognoses for patients undergoing surgical treatment.

The most critical point in cell-cycle regulation is the G1 checkpoint. It is here that complex interactions take place to determine whether the cell will exit the cell cycle and enter a quiescent state (G0) or enter into the S phase and proceed to cell division [14]. These complex interactions involve a large number of regulatory proteins such as cyclins, cyclin dependent kinases (CDKs), and CDK-inhibitors. Among the cyclins involved in the G1 phase, cyclin D1 appears to be the most strongly implicated in HNSCC carcinogenesis. Cyclin D1, through its interaction with cyclin dependent kinase-4 or 6 (CDK-4/6), forms a complex that inactivates the tumor suppressor protein retinoblastoma (Rb) through phosphorylation [15]. An increased level of cyclin D1 expression has been reported in a number of malignancies [15]. The overall survival rate of patients with cyclin D1-positive tumor specimens was reported to be decreased [8,16]. However, few reports evaluating cyclin D1 expression in surgical margins have been published to date. Our data proved that the identification of cyclin D1-positive tumor specimens did not indicate a worse prognosis, but cyclin D1-positive margins could be a worse prognostic factor for local recurrence. It was proved that the cyclin D1-positive status in surgical margins was found to be an independent prognostic indicator for local recurrence with the use of a multivariate Cox proportional hazards test from our results. In 37 (48.7%) of 76 patients with cyclin D1-positive tumor specimens, cyclin D1-negative margins were observed. It is considered that surgical margin analysis has an advantage over tumor analysis due to this discrepancy.

On the other hand, cyclin D1-positive margins were observed in 37.5% of patients with cyclin D1-negative tumor specimens. In this situation, cyclin D1 overexpression in surgical margins might not be originated by present cancer. Cyclin D1 overexpression in

surgical margins may cause other future carcinogenesis. However, the survival rate of patients with cyclin D1-negative tumor specimens and -positive margins was not downgraded from our results. The rate of cycin D1-positive margin in patients with adjunctive treatment was higher than that in patients without adjunctive treatment. Adjunctive treatment was indicated for patients with multiple nodal metastasis, extracapsular spread, or massive primary cancer. These oncological characteristics may relate to cyclin D1 abnormalities in histopathologically normal mucosa. Additionally, it was speculated that patients with adjunctive treatment might have worse overall survival because of this bias.

In the univariate analysis of various factors, the effect of cyclin D1-positivity in the surgical margins was significant for the rate of local control, whereas no other factors showed any significance. The presence of cyclin D1-positive surgical margins did not have a significant influence on overall survival ($p=0.0596$), but might have a tendency to worsen overall survival. A type II error could probably derive this discrepancy due to insufficient number of samples. Based on our results, we considered that overall survival was influenced by various factors, such as T and N stage, tumor differentiation, primary site, and the presence of adjunctive treatment. In our long-term observation study, 10 patients died of other diseases, and one patient died during the perioperative period. Regional recurrence was observed in 17 patients, and distant metastasis was observed in 9 patients. Overall survival could be influenced by various factors apart from local recurrence. On the other hand, local recurrence is observed less frequently in patients with histopathologically tumor-free surgical margins [1,2]. Therefore, it could be that the effect of cyclin D1-positivity in the surgical margins was masked by these various factors during the analysis of overall survival.

It was reported that patients with human papilloma virus (HPV) positive

oropharyngeal cancer showed an improved prognosis when compared to patients with HPV negative tumors [17-19]. We examined IHC p16 expression of tumor specimens in patients with oropharyngeal cancer as a surrogate marker of HPV DNA. In only one of 11 patients, p16 overexpression was observed. It was speculated that many of patients with HPV negative oropharyngeal cancer might undergo surgical treatment because we tended to perform chemoradiation therapy for patients with HPV positive and multiple nodal metastases. Because of a small number of p16 overexpression, we considered the influence of HPV infection could be small in the current study.

Cyclin D1 overexpression exists in multiple types of cancer and is a potential chemopreventive or therapeutic target. Molecular targeting interventions, using antisense oligonucleotides were reported for non-small cell lung cancer in the previous literature [20]. Cyclin D1 targeting therapy is also a potential future strategy.

Conclusions

The cyclin D1-positive status in tumor-free surgical margins was found to be an independent prognostic indicator for local recurrence. It is possible that the assessment of cyclin D1 expression in surgical margins is more valuable than that in tumor specimens for patients undergoing surgical treatment. The IHC analysis of surgical margins can augment standard histopathological assessment and may improve the prediction of local recurrence. These data may have a major impact for future diagnostic workups for patients with HNSCC after surgical treatment.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure legends

Figure 1. Immunohistochemical staining. A representative example of a p53-positive specimen (A), a cyclin D1-positive specimen (B). Original magnification 200×

Figure 2. Kaplan-Meier curves for local control were drawn for the expression of p53 in tumor specimens (A), cyclin D1 in tumor specimens (B), p53 in surgical margins (C), and cyclin D1 in surgical margins (D).

Figure 3. Kaplan-Meier curves for local control were drawn comparing patients according to the immunohistochemical status.

Abbreviations: t+, positive in tumor specimens; t–, negative in tumor specimens; m+, positive in surgical margins; m–, negative in surgical margins

Characteristic	n
Total	116
Gender	
Male	101 (87.1%)
Female	15 (12.9%)
Age, years	
Median	61
Range	29-86 (Ave. 61.7)
Follow up period, months	
Median	69.6
Range	12-164.4 (Ave. 77.9)
Primary site	
Oral cavity	43 (37.1%)
Oropharynx	11 (9.5%)
Hypopharynx	39 (33.6%)
Larynx	23 (19.8%)
Smoking behavior	
absent	15 (12.9%)
present	101 (87.1%)
Daily alcohol consumption	
absent	21 (18.1%)
present	95 (81.9%)
T stage	
T1	20 (17.2%)
T2	45 (38.8%)
T3	34 (29.3%)
T4a	17 (14.7%)
N stage	
N0	64 (55.2%)
N1	19 (16.4%)
N2a	5 (4.3%)
N2b	24 (20.7%)
N2c	4 (3.4%)
Tumor differentiation	

well differentiated	56 (48.3%)
moderately differentiated	50 (43.1%)
poorly differentiated	10 (8.6%)
Adjunctive treatment	
absent	59 (50.9%)
present	57 (49.1%)

Table 1. Patient Demographics

		Overall survival		Local control	
Characteristics	n	5-year OS (%)	p	5-year LC (%)	p
Age					
<60	54	66.5		85.2	
>60	62	64.4	0.3125	84.7	0.8996
Sex					
Male	101	63.3		82.7	
Female	15	79.0	0.3572	100.0	0.1260
Primary site					
Oral	43	73.6		88.5	
Others	73	59.8	**0.0373**	82.9	0.2599
Smoking behavior					
absent	15	53.0		71.3	
present	101	66.8	0.7481	86.3	0.3989
Daily alcohol consumption					
absent	21	80.7		94.7	
present	95	62.1	0.1947	82.6	0.2030
T stage					
T1-2	65	74.5		87.3	
T3-4	51	53.3	**0.0191**	82.0	0.2748
N stage					
N0	64	74.3		87.0	
N1-2	52	53.8	**0.0138**	82.5	0.3158
Tumor differentiation					
well	56	77.0		89.8	
poor+moderate	60	54.4	**0.0135**	80.1	0.1006
Adjunctive treatment					
absent	59	78.7		88.0	
present	57	51.1	**0.0062**	81.7	0.2172
p53-					
negative tumors	53	70.1		88.1	
positive tumors	63	61.2	0.2447	82.1	0.6303
cyclin D1-					
negative tumors	40	63.5		90.6	
positive tumors	76	65.6	0.5492	82.1	0.2273
p53-					

negative margins	82	63.8		83.0	
positive margins	34	67.8	0.7694	89.2	0.4103
cyclin D1-					
negative margins	62	72.0		91.5	
positive margins	54	56.6	0.0596	77.2	**0.0139**

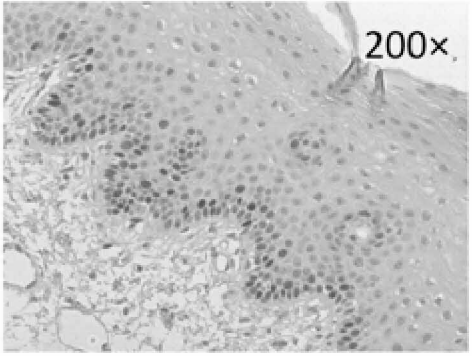
Table 2. Clinicopathologic characteristics and overall survival (OS) and local control (LC) rates

		Overall survival		Local control	
Variable (score)	n	HR (95% CI)	p	HR (95% CI)	p
Primary site					
Oral (0)	43				
Others (1)	73	0.81 (0.29-2.25)	0.6978	0.36 (0.06-2.36)	0.2921
T stage					
T1-2 (0)	65				
T3-4 (1)	51	1.64 (0.81-3.44)	0.1721	1.65 (0.49-6.31)	0.4219
N stage					
N0 (0)	64				
N1-2 (1)	52	1.49 (0.75-3.02)	0.2516	0.99 (0.31-3.30)	0.9865
Tumor differentiation					
well (0)	56				
poor+moderate (1)	60	**2.00 (1.05-3.94)**	**0.0350**	2.39 (0.77-8.37)	0.1335
Adjunctive treatment					
absent (0)	59				
present (1)	57	1.51 (0.72-3.36)	0.2817	1.34 (0.39-5.04)	0.6491
cyclin D1-					
negative margins (0)	62				
positive margins (1)	54	1.15 (0.55-2.48)	0.7124	**4.58 (1.14-21.69)**	**0.0304**

Table 3. Multivariate Cox proportional hazard regression model analysis for overall survival and local control

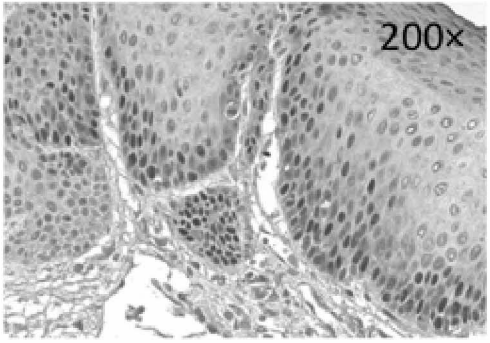
Figure 1.

A)



p53-positive

B)



cyclin D1-positive

Figure 2.

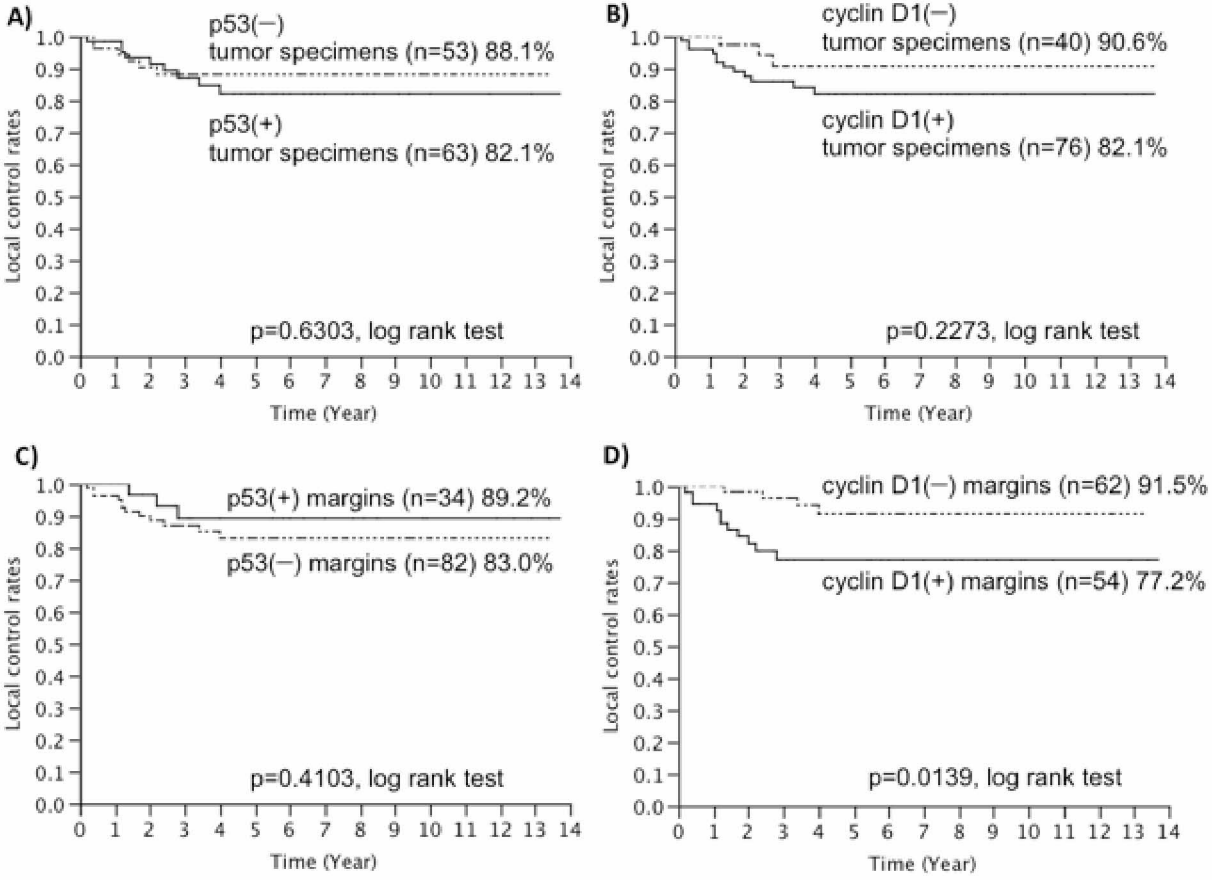
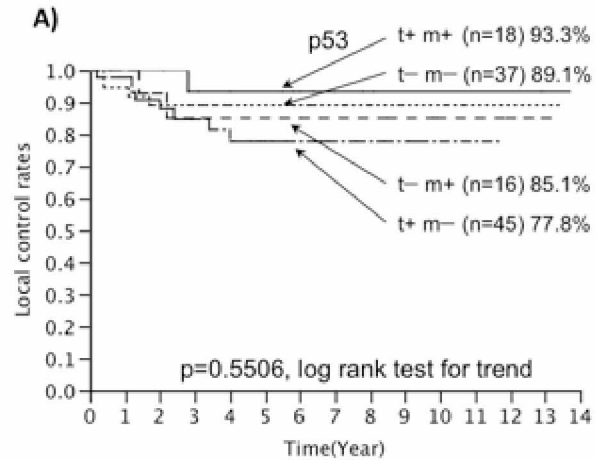
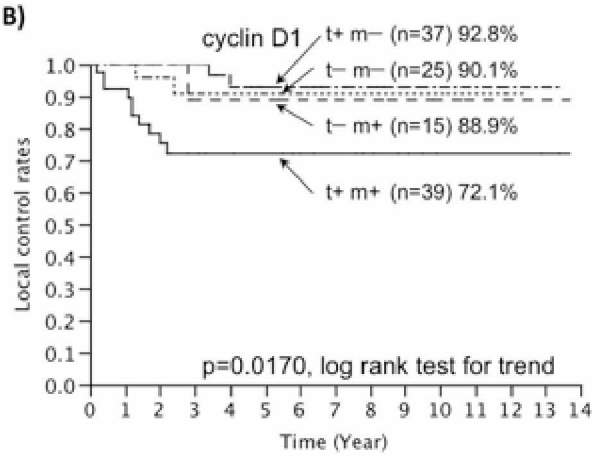

A)
p53(−)
tumor specimens (n=53) 88.1%
p53(+)
tumor specimens (n=63) 82.1%
p=0.6303, log rank test
Local control rates
Time (Year)
B)
cyclin D1(−)
tumor specimens (n=40) 90.6%
cyclin D1(+)
tumor specimens (n=76) 82.1%
p=0.2273, log rank test
Local control rates
Time (Year)
C)
p53(+) margins (n=34) 89.2%
p53(−) margins (n=82) 83.0%
p=0.4103, log rank test
Local control rates
Time (Year)
D)
cyclin D1(−) margins (n=62) 91.5%
cyclin D1(+) margins (n=54) 77.2%
p=0.0139, log rank test
Local control rates
Time (Year)

Figure 3.


A)
p53
t+ m+ (n=18) 93.3%
t− m− (n=37) 89.1%
t− m+ (n=16) 85.1%
t+ m− (n=45) 77.8%
p=0.5506, log rank test for trend
Local control rates
Time(Year)


B)
cyclin D1
t+ m− (n=37) 92.8%
t− m− (n=25) 90.1%
t− m+ (n=15) 88.9%
t+ m+ (n=39) 72.1%
p=0.0170, log rank test for trend
Local control rates
Time (Year)