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Main Article

The immunohistochemical expression of $p21^{WAF1/Cip1}$ and Proliferating cell nuclear antigen in laryngeal squamous cell carcinomas

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Abstract

The purpose of this study was to assess the significance of the expression of $p21^{WAF1/Cip1}$ and proliferating cell nuclear antigen (PCNA) in human laryngeal squamous cell carcinomas. Forty-eight patients (25 supraglottic, 23 glottic) who had undergone operations between 1998 and 2003 were included in this study. The Envision immunohistochemistry method was utilized to stain these tissue specimens. The results showed that the immunostaining of $p21^{WAF1/Cip1}$ do not show any correlation with differentiation, N stage, metastasis, survival, recurrence or the laryngeal site of involvement. A significant inverse correlation was detected between $p21^{WAF1/Cip1}$ and the T stage. In contrast, the percentage of PCNA labelled cells showed a significant correlation with the T stage, but not with other clinicopathological parameters. There were differences in the expression of $p21^{WAF1/Cip1}$ and PCNA between the supraglottic and the glottic carcinomas. In conclusion, our findings suggest that $p21^{WAF1/Cip1}$ and PCNA may play an important role in the progression of carcinoma of the supraglottic larynx.

Key words: P21 Cyclin Kinase Inhibitor; Proliferating Cell Nuclear Antigen; Head and Neck Neoplasms; Larynx Neoplasms

Introduction

Laryngeal squamous cell carcinoma is the most common cancer of the head and neck region, which seriously threatens people's lives and has long attracted the attention of head and neck surgeons. For the past 20 years, radiation therapy as well as function-preserving surgery of the larynx has been widely developed and the quality of patients' lives has greatly improved. However, the progress of surgery and other remedies has not resulted in a marked increase in the survival rate of patients with advanced laryngeal cancer. For this reason, we focused our attention on a molecular genetics study involving the pathogenesis of laryngeal squamous cell carcinoma.

The cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ is a member of the kinase interacting protein (KIP) family of cell cycle proteins, which inhibits several cyclin-dependent kinase (CDK) complexes, and plays a central role in inducing cellular growth arrest, terminal differentiation, and apoptosis.[1–4] The transcription of this gene is activated by p53-dependent and independent mechanisms.[5,6] The gene product also binds to proliferating cell nuclear antigen (PCNA) through its C-terminal domain and blocks the ability of PCNA to activate deoxyribonucleic acid (DNA) polymerase δ, the principal replicative DNA polymerase.[7–9] PCNA regulates the DNA synthesis and controls G1 to S phase transition.[10–13] Several recent studies have suggested the loss of $p21^{WAF1/Cip1}$ is strongly associated with the failure of the irradiation response, and it has also been shown to be a prognostic factor in many carcinomas.[14–16] However, cell cycle regulation in laryngeal squamous cell carcinoma is still poorly understood. The aim of our study was to analyse the immunohistochemical expression, and the distribution of $p21^{WAF1/Cip1}$ in laryngeal carcinoma as well as the interaction between the expression of $p21^{WAF1/Cip1}$ and PCNA. As the epidemiological and clinical characteristics are different between the two types (supraglottic and glottic) of laryngeal carcinomas, we also paid attention to the pattern of expression in each sub-type.

Materials and methods

Patients and samples

The study group consisted of 48 patients (46 males, two females), aged from 43 to 84 years (mean: 70 years)

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who were operated on for laryngeal squamous cell carcinomas at Kurume University Hospital between January 1998 and January 2003. Twenty-five cases had supraglottic cancer while 23 cases had glottic cancer. All patients underwent a total laryngectomy either with or without a radical or modified radical neck dissection. The tumour-node-metastasis (TNM) stages of the patients were determined based on the clinical data and histopathological examinations according to the 2002 TNM classification. None of the patients received previous radiation therapy. The characteristics of all patients are presented in Table I.

Immunohistochemical staining

The tumour tissues were initially screened at a low magnification and the representative part of the tumourous lesions with surrounding normal tissues were extracted from the original paraffin blocks for further examination. Four μm-thick sections were cut and then placed on slides, and they were deparaffinated and dehydrated in xylol with graded alcohol. After rinsing with distilled water, they were then placed in hot 10 mmol/l citrate buffer with a pH of 6.0 for antigen retrieval, and then were heated in a microwave oven for five minutes. Using DAKO TechMate^{TM} Horizon Automated Immunostainer, the slides were incubated with primary antibodies for 60 minutes. The primary antibodies against p21$^{WAF1/Cip1}$ (DAKO M 7202, Tokyo, Japan), and PCNA (DAKO M0879) were used. They were all diluted at 1:50, and DAKO Envision^{TM} System Peroxidase was used during the immunohistochemical stain process. Consecutive sections were also stained with hematoxylin-eosin (HE) for routine histopathological examinations.

TABLE I

CHARACTERISTICS OF THE PATIENTS

Variables	Laryngeal cancer (supraglottic) $n = 25$ n (%)	Laryngeal cancer (glottic) $n = 23$ n (%)	Total $n = 48$ n
Age, median	72	68	70
Gender			
Males	23(92)	23(100)	46
Females	2(8)	0(0)	2
Differentiation			
Well	10(40)	17(74)	27
Moderate	15(60)	6(26)	21
Poor	0(0)	0(0)	0
T			
T_{1-2}	3(12)	1(4)	4
T_3	12(48)	12(52)	24
T_4	10(40)	10(44)	20
N			
N_0	13(52)	22(96)	35
N_1	1(4)	0(0)	1
N_2	10(40)	1(4)	11
N_3	1(4)	0(0)	1
M			
M_0	22(88)	22(96)	44
M_1	3(12)	1(4)	4
Stage			
I	1(4)	0(0)	1
II	2(8)	1(4)	3
III	12(48)	12(52)	24
IV	10(40)	10(44)	20
Survival status			
Alive	20(80)	21(91)	41
Died	5(20)	2(9)	7
Recurrence			
No	19(76)	21(91)	40
Yes	6(24)	2(9)	8

The expression of the immunostaining was determined using a light microscope (Nikon, Tokyo, Japan) by the same investigator who did not know the patient's history. The scores, based on the intensity of the staining and the percentage of stained cells, were counted from three high-power fields. At least 200 cancer cells were counted in each microscopic field. The expression of p21$^{WAF1/Cip1}$ was graded as negative (0 per cent) and positive (>0 per cent), and a positive expression was further classified as mild (+), moderate (++) and marked (+++) when the percentage was less than 10 per cent, 10–20 per cent and more than 20 per cent, respectively.[17] The expression of PCNA was graded as negative (0 per cent) and positive (>0 per cent), and a positive expression was further classified as mild (+), moderate (++) and marked (+++) when the percentage was less than 60 per cent, 60–80 per cent and more than 80 per cent, respectively.[17,18]

Statistical analysis

Any correlations between immunohistochemical scoring results and various clinicopathological features were assessed using the chi-square test and Student's test. Any correlation amongst the groups of parameters was evaluated by the Spearman test and a linear regression analysis. A p value of less than 0.05 (two sided) was considered to indicate statistical significance.

Results

Nuclear immunoreactivity to p21$^{WAF1/Cip1}$ was detected in cancer cells from 20 (41.67 per cent) of 48 patients (mean 7.91 per cent; range 0–53.25 per cent) (Figure 1). In some normal mucosal areas as well as in some dysplastic areas of the examined

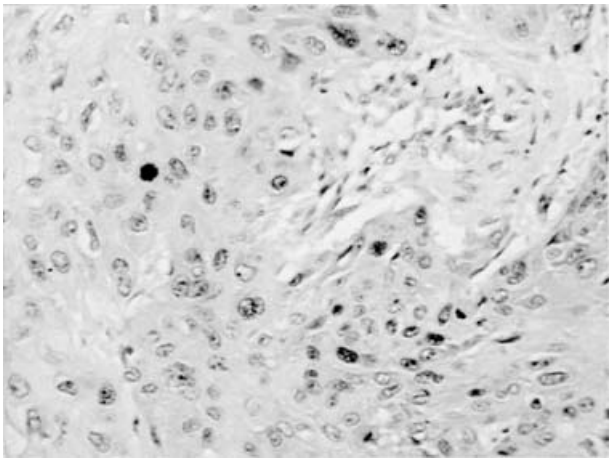

FIG. 1

More than 50 per cent of the cells are stained positively for p21$^{WAF1/Cip1}$ nuclear immunoreactivity in cancer cells (×400).

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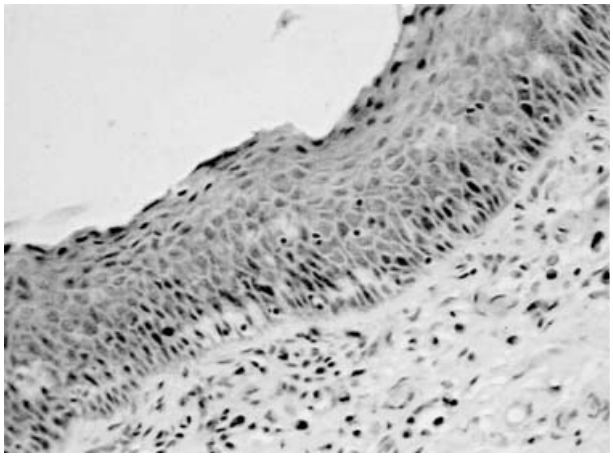

FIG. 2
A normal mucosal area close to the tumour tissue stained negatively for $p21^{WAF1/Cip1}$ (×400).

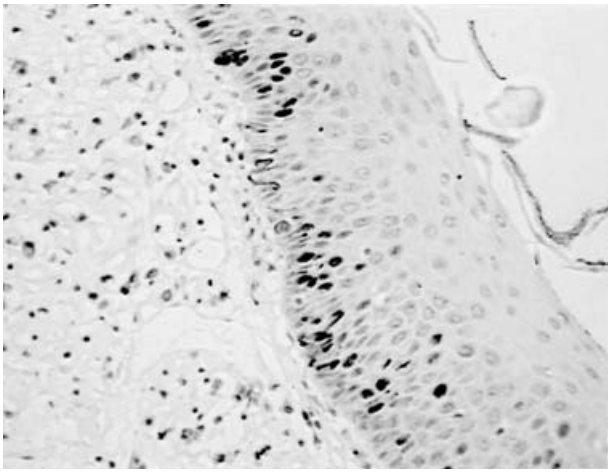

FIG. 3
Cells staining positively for PCNA in the basement of the epithelia showed a weak staining intensity (×400).

patients, no $p21^{WAF1/Cip1}$ positive cells were observed (Figure 2). The correlations between the clinical features and $p21^{WAF1/Cip1}$ are summarized in Table II. A significant inverse correlation was only found between the $p21^{WAF1/Cip1}$ and T stage, but the expression of $p21^{WAF1/Cip1}$ showed no correlation with differentiation, N stage, metastasis, survival, recurrence or laryngeal site of involvement. It was interesting to note, however, that patients whose tumour showed a marked reaction (+++) to $p21^{WAF1/Cip1}$ all survived without any recurrence.

The nuclear expression of proliferating cell nuclear antigen (PCNA) was detected not only in the tumour tissue specimens but also in the normal tissue or dysplastic mucosa specimens. In the normal mucosa with mild dyplasia, the expression of PCNA was observed in some nuclei at the basement of the epithelia (Figure 3). In contrast, a strong staining intensity for PCNA was observed in almost all cancer cells (Figure 4). Indeed, the cancer cells from 47 (97.92 per cent) of 48 patients (mean 55.20 per cent; range 0–90.4) showed a positive reaction to PCNA. The percentage of PCNA labelled cells showed a statistically significant correlation with the T stage, but no significant difference was found amongst the other clinicopathological parameters (Table III). There was also a significant inverse correlation between the expression of $p21^{WAF1/Cip1}$ and the PCNA labelled cells ($r = -0.332$, $p < 0.005$) (Figure 5).

In order to further see the clinical role of $p21^{WAF1/Cip1}$ and PCNA, we investigated the expression according to the subsite (glottic and supraglottic larynx). As a result, a significant inverse correlation

TABLE II
CORRELATION BETWEEN THE EXPRESSION OF $p21^{waf1/cip1}$ AND THE CLINICAL FEATURES OF LSCC

	(−) 0%	(+) <= 10%	(++) 10–20%	(+++) >= 20%	*p* value	
Site of tumour						
Supraglottic	15	2	3	5		
Glottic	13	4	2	4	$\chi^2 = 1.04$	$0.75 < p < 0.90$
Differentiation						
Well	14	6	2	5		
Moderate	14	0	3	4	$\chi^2 = 5.65$	$0.1 < p < 0.25$
T stage						
T_{1-2}	0	0	3	1		
T_3	10	5	2	7		
T_4	18	1	0	1	$\chi^2 = 31.34$	$p < 0.005$
N stage						
N_0	20	5	4	6		
N_x	8	1	1	3	$\chi^2 = 0.67$	$0.75 < p < 0.90$
M stage						
M_0	25	6	4	9		
M_1	3	0	1	0	$\chi^2 = 2.46$	$0.25 < p < 0.5$
Survival status						
Alive	22	5	5	9		
Died	6	1	0	0	$\chi^2 = 3.46$	$0.25 < p < 0.5$
Recurrence						
No	22	5	4	9		
Yes	6	1	1	0	$\chi^2 = 2.30$	$0.5 < p < 0.75$
Total	28	6	5	9		

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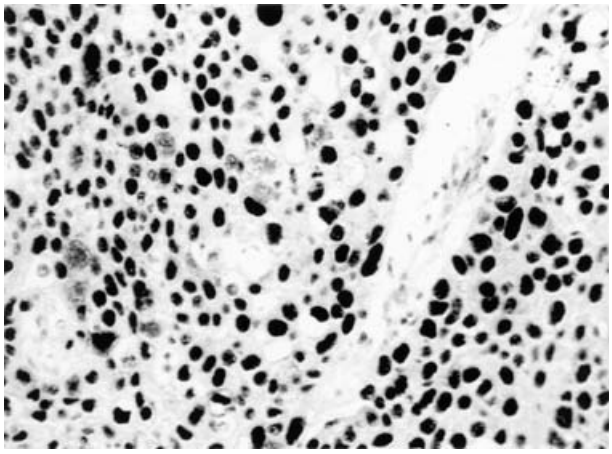

FIG. 4
A strong staining intensity for PCNA was observed in almost all tumour tissue specimens (×400).

between the expression of p21$^{WAF1/Cip1}$ and T was seen only in supraglottic cancer (Table IV). In such analysis, even the N status of the supraglottic carcinoma showed a significant different inverse correlation with the expression of p21$^{WAF1/Cip1}$. Significant correlation between PCNA labelled cells and T was also detected in supraglottic cancer (Table V). These results indicate that p21$^{WAF1/Cip1}$ and PCNA may therefore play an important role in the progression of carcinoma of the supraglottic larynx.

Discussion

In the normal cell cycle, there is a restriction point (R) in the later G1 phase which regulates the cell cycle without interruption until it is completed.

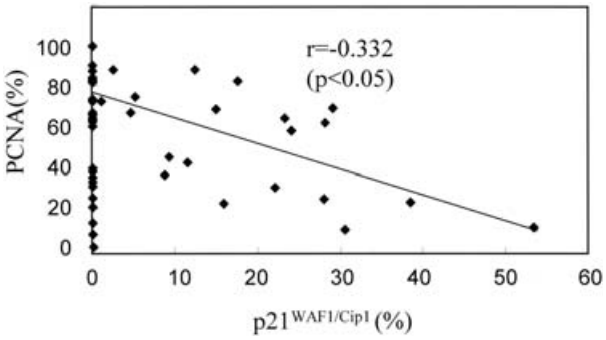

FIG. 5
Correlation between the expression of p21$^{WAF1/Cip1}$ and PCNA. There was a significant inverse correlation between the two factors ($r = -0.332$).

One of the most important properties of cancer cells is the aberrant regulation of the cell cycle. The braking activity of the cell cycle depends on the activity of the tumour suppressor genes.[19–21] Tumour suppressor genes are thought to play a relatively more important role in head and neck tumour development than other oncogenes. Among the several suppressor genes previously reported, p53 has been the subject of extensive studies and it is the most important agent known to suppress transition through the R point. p21$^{WAF1/Cip1}$, which is the main downstream effector gene mediating the p53 induced cell cycle arrest, is transcriptionally regulated by p53.[1–4] p21$^{WAF1/Cip1}$ inhibits the action of cyclin D1-CDK complexes and the p21$^{WAF1/Cip1}$ containing cells are able to repair any damage to DNA, and act as a decisive mediator of p53 response to DNA damage. Several recent studies have suggested that p21$^{WAF1/Cip1}$ plays a role in apoptosis.[14–16] The data in this study

TABLE III
CORRELATION BETWEEN THE EXPRESSION OF PCNA AND THE CLINICAL FEATURES OF LSCC

	(−) 0%	(+) < = 60%	(++) 60–80%	(+++) > = 80%	p value	
Site of tumour						
Supraglottic	1	11	8	5		
Glottic	0	8	9	6	$\chi^2 = 1.54$	$0.5 < p < 0.75$
Differentiation						
Well	1	10	10	6		
Moderate	0	9	7	5	$\chi^2 = 0.94$	$0.75 < p < 0.90$
T stage						
T_{1-2}	1	2	1	0		
T_3	0	15	7	2		
T_4	0	2	9	9	$\chi^2 = 27.02$	$p < 0.005$
N stage						
N_0	1	15	11	8		
N_x	0	4	6	3	$\chi^2 = 1.30$	$0.5 < p < 0.75$
M stage						
M_0	1	17	16	10		
M_1	0	2	1	1	$\chi^2 = 0.35$	$p = 0.95$
Survival status						
Alive	1	17	13	10		
Died	0	2	4	1	$\chi^2 = 1.78$	$0.5 < p < 0.75$
Recurrence						
No	1	17	13	9		
Yes	0	2	4	2	$\chi^2 = 1.31$	$0.5 < p < 0.75$
Total	1	19	17	11		

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suggest the presence of a low expression of $p21^{WAF1/Cip1}$ in laryngeal carcinoma, and negative staining in some types of normal tissue and dysplasia tissue near the cancer tissue. The loss of $p21^{WAF1/Cip1}$ expression may be associated with carcinogenesis and cancer progression.

An aberrant expression of $p21^{WAF1/Cip1}$ has been reported in a number of cancers, including ovarian, uterine, pancreatic cancer and head and neck carcinomas.[22–26] Xie *et al.*[26] reported that the expression of $p21^{WAF1/Cip1}$ correlated inversely with the T classification and clinical stage, but not with N classification or tumour differentiation. Our study also showed the down-regulation of $p21^{WAF1/Cip1}$ in advanced laryngeal carcinoma (Table II). In particular, only two (10 per cent) out of 20 tumours stained positively for $p21^{WAF1/Cip1}$, and these results were statistically lower than those for T_1–T_2 tumours (100 per cent). The prognostic value of $p21^{WAF1/Cip1}$ expression has been reported in tumours of various organs such as the breast[27] and pancreas.[28] Even in squamous cell carcinomas of the head and neck, $p21^{WAF1/Cip1}$, particularly in combination with p53 accumulation, showed a good prognostic value.[26,29–32] In our study, there was no statistical difference between the $p21^{WAF1/Cip1}$ expression and survival status. However, it was interesting to note that no cases of death or recurrence were observed in the group with a marked expression.

The proliferating cell nuclear antibody (PCNA) functions as a co-factor for DNA-polymerase in both the S phase and in DNA synthesis associated with DNA repair. A very low level of PCNA may be found even in non-cycling cells with up-regulation occurring after the start of the cell cycle. An over expression of PCNA has been observed in a number of cancers.[10–13] Our findings suggest the expression of PCNA to be related to the T stage of laryngeal carcinoma. PCNA might thus play an important role in the development of laryngeal carcinoma. In normal cells, $p21^{WAF1/Cip1}$ exists predominantly in quaternary complexes in combination with cyclins and CDKs, while PCNA regulates the DNA synthesis and controls the G1 to S phase transition. $p21^{WAF1/Cip1}$ contains both PCNA binding and inhibitor activities while also inducing growth arrest, terminal differentiation, or apoptosis. In this study, we sought to examine the relationship between the expression of $p21^{WAF1/Cip1}$ and PCNA. As a result, our findings showed an inverse correlation to exist between them.

It is a well known clinical process that supraglottic-type laryngeal carcinoma is more prone to metastasize to lymph nodes and induce a poor prognosis. This is a markedly different clinical characteristic as compared to glottic type. The significant (positive or inverse) correlations between $p21^{WAF1/Cip1}$ or PCNA and T or N that were detected only in supraglottic-type laryngeal carcinoma indicate that the genetic as well as the cytological backgrounds are different in cancer cells of the two subsites of the larynx. In addition, as the role of $p21^{WAF1/Cip1}$ in tumour cell proliferation is complex, further studies are called for.

- **This study assesses the significance of the expression of p21 and proliferating cell nuclear antigen (PCNA) in human laryngeal squamous carcinoma**
- **A significant correlation between p21 or PCNA was detected only in supraglottic laryngeal carcinoma**
- **p21 and PCNA may play a part in the progression of supraglottic laryngeal carcinoma**

TABLE IV

CORRELATION BETWEEN THE EXPRESSION OF $P21^{WAF1/cip1}$ AND THE CLINICAL FEATURES OF LSCC ACCORDING TO SUBSITE

	Supraglottic cancer ($n = 25$)					Glottic cancer ($n = 23$)		
	(−) 0%	(+) $< = 10\%$	(++) 10–20%	(+++) $> = 20\%$	p	(−) 0%	(+) $< = 10\%$	(++) 10–20%
Differentiation								
Well	6	2	1	1	$\chi^2 = 3.75$	8	4	1
Moderate	9	0	2	4	$0.25 < p < 0.5$	5	0	1
T stage								
T_{1-2}	0	0	3	0	$\chi^2 = 25$	0	0	0
T_{3-4}	15	2	0	5	$p < 0.005$	13	4	2
N stage								
N_0	7	2	2	2	$\chi^2 = 9.5$	13	3	2
N_x	8	0	1	3	$0.01 < p < 0.025$	0	1	0
M stage								
M_0	13	2	2	5	$\chi^2 = 2.28$	12	4	2
M_1	2	0	1	0	$0.5 < p < 0.75$	1	0	0
Survival status								
Alive	11	1	3	5	$\chi^2 = 3.38$	11	4	2
Died	4	1	0	0	$0.25 < p < 0.5$	2	0	0
Recurrence								
No	11	1	2	5	$\chi^2 = 2.58$	11	4	2
Yes	4	1	1	0	$0.25 < p < 0.5$	2	0	0
Total	15	2	3	5		13	4	2

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TABLE V

CORRELATION BETWEEN THE EXPRESSION OF PCNA AND THE CLINICAL FEATURES OF LSCC ACCORDING TO SUBSITE

	Supraglottic cancer ($n = 25$)					Glottic cancer ($n = 23$)				
	(−) 0%	(+) < = 60%	(++) 60–80%	(+++) > = 80%	p	(−) 0%	(+) < = 60%	(++) 60–80%	(+++) > = 80%	p
Differentiation										
Well	1	4	2	3	$\chi^2 = 3.25$	0	6	8	3	$\chi^2 = 2.97$
Moderate	0	7	6	2	$0.25 < p < 0.5$	0	2	1	3	$0.25 < p < 0.5$
T stage										
T_{1-2}	1	1	1	0	$\chi^2 = 17.5$	0	1	0	0	$\chi^2 = 1.909$
T_{3-4}	0	10	7	5	$p < 0.005$	0	7	9	6	$0.5 < p < 0.75$
N stage										
N_0	1	7	3	2	$\chi^2 = 2.5$	0	8	8	6	$\chi^2 = 1.38$
N_x	0	4	5	3	$0.25 < p < 0.5$	0	0	1	0	$0.5 < p < 0.75$
M stage										
M_0	1	9	8	4	$\chi^2 = 2.07$	0	8	8	6	$\chi^2 = 1.38$
M_1	0	2	0	1	$0.5 < p < 0.75$	0	0	1	0	$0.5 < p < 0.75$
Survival status										
Alive	1	10	5	4	$\chi^2 = 2.75$	0	7	8	6	$\chi^2 = 0.92$
Died	0	1	3	1	$0.25 < p < 0.5$	0	1	1	0	$0.75 < p < 0.9$
Recurrence										
No	1	9	5	4	$\chi^2 = 1.25$	0	8	8	5	$\chi^2 = 1.38$
Yes	0	2	3	1	$0.5< p < 0.75$	0	0	1	1	$0.5 < p < 0.75$
Total	1	11	8	5		0	8	9	6	

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