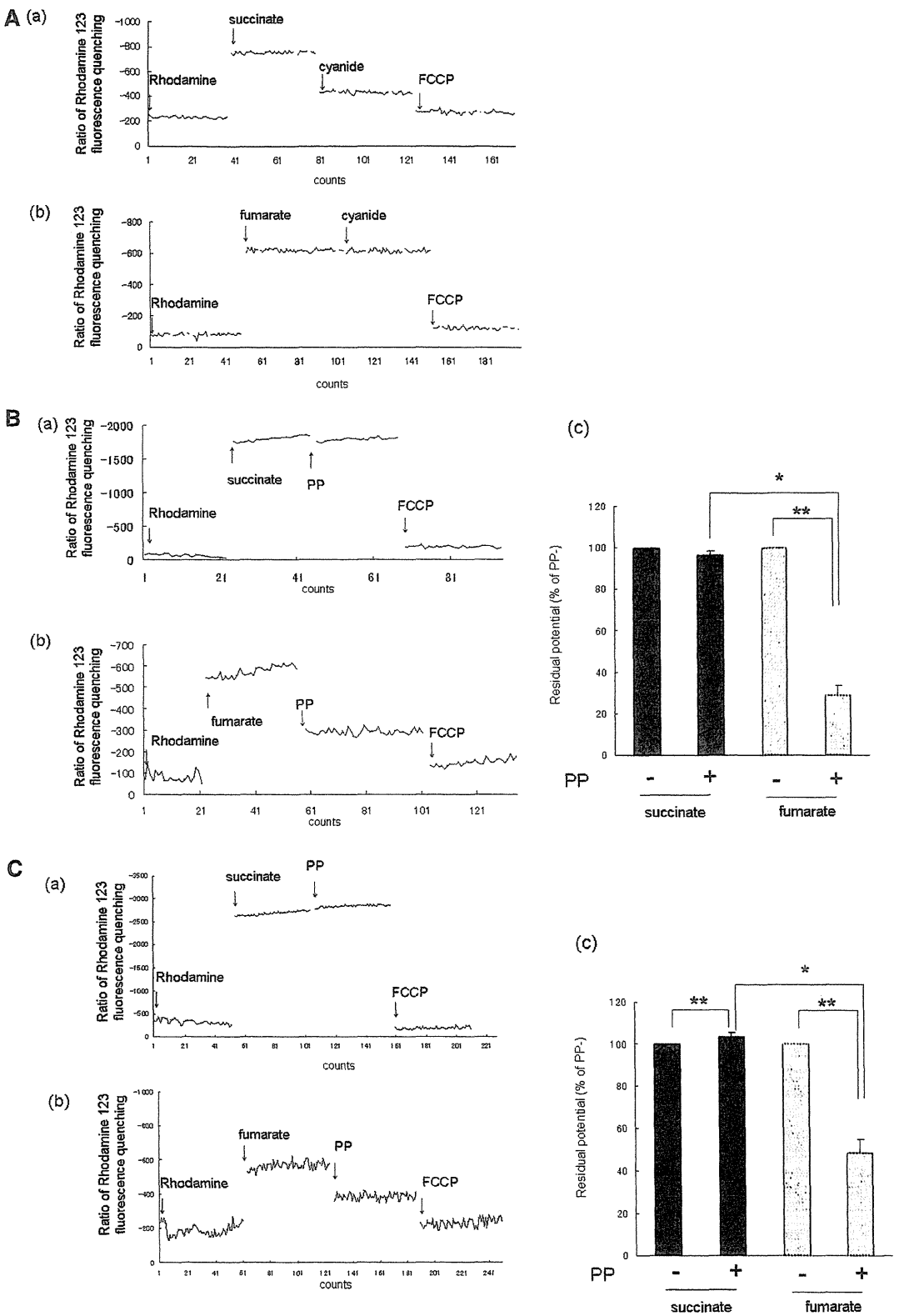

Fig. 7 Effect of pyrvinium pamoate on mitochondrial membrane potentials. (**A**) Effect of 0.1 mM cyanide on the $\Delta\Psi m$ in Panc-1 mitochondria. The values of Rhodamine 123 fluorescence quenching after the addition of succinate in mitochondria from Panc-1 cultured under normoxia–normoglycemic conditions [N+ glc(+)] (**a**) or after the addition of fumarate in mitochondria from Panc-1 cultured with glucose-free medium under 1% O_2 [H+ glc(−)] for 5 days (**b**) are shown. (**B**) Effect of 0.5 μM PP on the $\Delta\Psi m$ in Panc-1 mitochondria. The values of Rhodamine 123 fluorescence quenching starting after the addition of succinate in mitochondria from Panc-1 cultured under N+ glc(+) (**a**) or after the addition of fumarate in mitochondria from Panc-1 cultured under H+ glc(−) for 5 days (**b**) are shown. The percentages of the residual $\Delta\Psi m$ in mitochondria from Panc-1 cultured under N+ glc(+) (succinate as substrate) and cultured under H+ glc(−) for 5 days (fumarate as substrate) are also shown (**c**). (**C**) Effect of 0.5 μM PP on the $\Delta\Psi m$ in DLD-1 mitochondria. The values of Rhodamine 123 fluorescence quenching after the addition of succinate in mitochondria from DLD-1 cultured under N+ glc(+) (**a**) or after the addition of fumarate in mitochondria from DLD-1 cultured under H+ glc(−) for 7 days (**b**) are shown. The percentages of the residual $\Delta\Psi m$ in mitochondria from DLD-1 cultured under the N+ glc(+) (succinate as substrate) and cultured under H+ glc(−) 7 days (fumarate as substrate) are shown (**c**). The values reported represent the mean ± SEM of three independent measurements. Statistically significant differences are shown by the asterisks (*$P<0.05$ succinate versus fumarate, **$P<0.05$ versus PP −, Student's t-test).

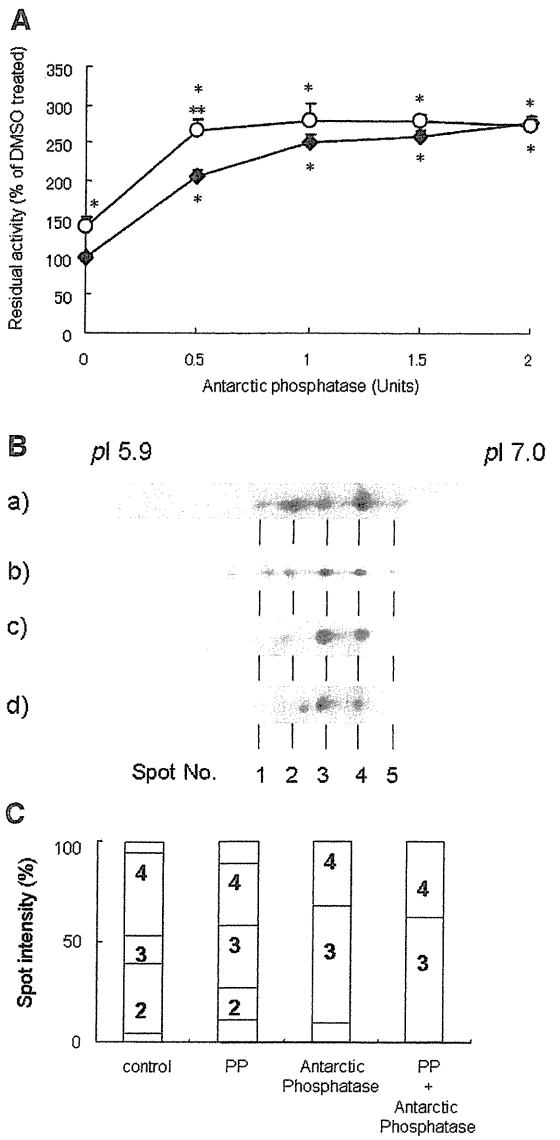

Fig. 8 Effects of pyrvinium pamoate and phosphatase on complex II. (**A**) Effect of PP and Antarctic Phosphatase on SQR activity. The percentages of the residual SQR activities after the addition of DMSO in solubilized mitochondrial proteins from DLD-1 cultured under normoxia–normoglycemic conditions [N+ glc(+)] and treated with various units of Antarctic Phosphatase and/or treated with 25 μM PP are shown. The closed diamonds indicate treatment with Antarctic phosphatase, whereas the open circles indicate treatment with Antarctic phosphatase plus PP. The values reported represent the mean ± SEM of three independent measurements. Statistically significant differences are shown by the asterisks (*$P<0.05$ versus phosphatase −/PP −, **$P<0.05$ versus phosphatase +/PP −, Student's *t*-test). (**B**) Separation and detection of the Fp subunit in solubilized mitochondrial proteins from DLD-1 cultured under N+ glc(+) using 2-D gel electrophoresis. Untreated (**a**), treated with 25 μM PP for 10 min at 30°C (**b**) or treated with 0.5 U of Antarctic phosphatase for 2 h at 37°C (**c**). Treated with 25 μM of PP for 10 min at 30°C and 0.5 U of Antarctic phosphatase for 2 h at 37°C (d). (**C**) Spot intensities of (B). The numbers show the spot No. of (B).

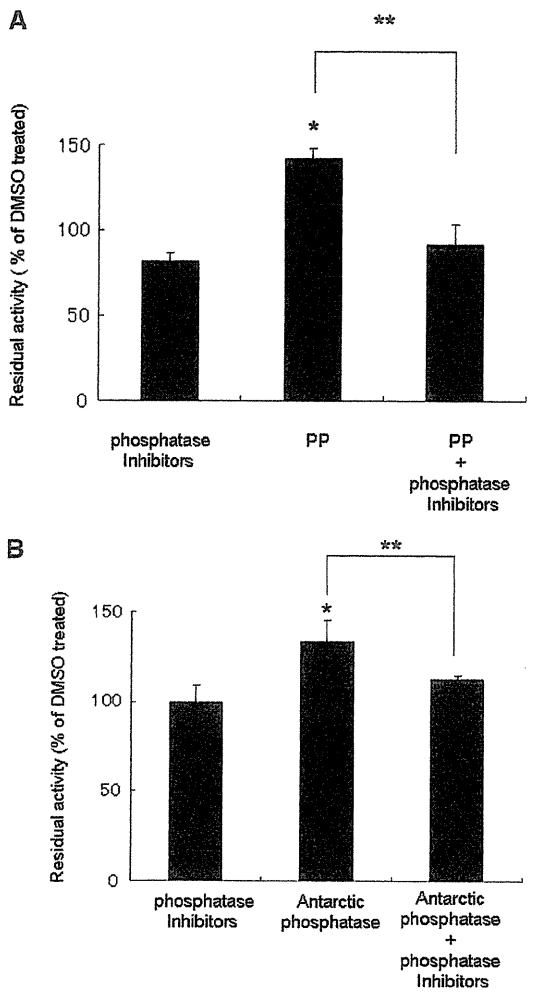

Fig. 9 Effect of pyrvinium pamoate, phosphatase and phosphatase inhibitors on SQR activity. (**A**) Percentages of residual SQR activities after the addition of DMSO in solubilized mitochondrial proteins from DLD-1 cultured under normoxia–normoglycemic conditions [N+ glc(+)] and treatment with a phosphatase inhibitor cocktail and/or 25 μM PP for 10 min at 30°C. Statistically significant differences are shown by the asterisks (*$P<0.05$ versus DMSO treated, **$P<0.05$ phosphatase inhibitors −/PP + versus phosphatase inhibitors +/PP +, Student's *t*-test). (**B**) Percentages of residual SQR activities after the addition of DMSO in solubilized mitochondrial proteins from DLD-1 cultured under N+ glc(+) and treatment with a phosphatase inhibitor cocktail, 0.5 U of Antarctic phosphatase or 0.5 U of Antarctic phosphatase plus a phosphatase inhibitor cocktail for 2 h at 37°C. The values reported represent the mean ± SEM of three independent measurements. Statistically significant differences are shown by the asterisks (*$P<0.05$ versus DMSO treated, **$P<0.05$ phosphatase inhibitors −/Antarctic phosphatase + versus phosphatase inhibitors +/Antarctic phosphatase +, Student's *t*-test).

for anti-parasitic drugs. How PP exerts species-specific effects between parasites and hosts needs to be understood. In our study, PP exerted different effects on complex II in parasites and mammals, with PP inhibiting complex II activities in both the normoxic ETC and the NADH–FR system in parasites and increasing SQR activity in mammals. Complex II in *A. suum* has been well characterized and complex II (SQR) and complex II (QFR) contain different Fp and the small cytochrome *b* (CybS) subunits (*33*, *34*). On the other hand, modification of the Fp subunit in mammalian complex II switches its function from SQR to FRD (*20*). Therefore, one of the species-specific effects of PP is caused by its different effects on complex II in parasitic and mammalian mitochondria, which have different complex II characteristics.

To consider the side effects of PP, the effects of PP on mammalian ETCs should be compared in both

cancer cells and normal cells and under an ordinary environment and tumour microenvironments. In mammalian mitochondria, we showed that PP inhibited NADH–FR activity, but the activity was low in bovine mitochondria and cancer cell lines, such as DLD-1 (*35*), compared with *A. suum* mitochondria. Moreover, SQR activity was increased by PP in bovine mitochondria and human cancers and in the mitochondria of normal cells under normoxia–normoglycemic conditions. In addition, PP did not inhibit the $\Delta\Psi m$ generated by normoxic ETC starting with complex II. These data suggest that PP does not interfere with ATP synthesis via normoxic mitochondrial ETC through complex II, even when complex I is partially inhibited and is unlikely to produce severe side effects in normal human tissues. Moreover, PP exerted an anticancer effect, inhibiting tumour formation in xenografts (*10*) and PP inhibited hypoxic ETC under tumour microenvironmental conditions in the present study. Therefore, PP has a minimal cytotoxicity in normal cells but is effective against cancer cells under tumour microenvironmental conditions and PP may serve as a good anticancer compound with minimal side effects.

To understand why PP is cytotoxic only in tumour microenvironments, we examined the NADH–FR system, a unique energy metabolic pathway, under hypoglycemic and hypoxic conditions. When glucose is limited, amino acids can be used for energy production instead of glucose, *e.g.* aspartate → oxaloacetate → malate → fumarate (*36*) and fumarate is the substrate of the NADH–FR system. Despite the limited glucose concentrations in tumour tissues in tumour microenvironments, the accumulation of amino acids has been observed in tumour tissues (*3*, *37*). One of the reasons for the accumulation of amino acids is thought to be the activation of the autophagic degradation of proteins under nutrient-starved conditions (*38*). The NADH–FR system is only composed of complex I and the reverse reaction of complex II; this system results in succinate formation via the fumarate reductase activity in complex II (*35*). This system does not need oxygen, allowing it to function under hypoxic conditions. The final product in this system, succinate, is known to have an important role in the hypoxic response. Succinate inhibits prolyl hydroxylase (PHD), leading to the stabilization of hypoxia inducible factor (HIF) -1α degradation (*39*). As HIF is a major regulator of the hypoxic response, the NADH–FR system may have an important role in the activation of a pseudo-hypoxic pathway.

We showed that PP inhibited the $\Delta\Psi m$ generated by fumarate, which is a substrate of the NADH–FR system. In contrast, PP did not inhibit the $\Delta\Psi m$ generated by succinate; moreover, a slight activation effect was observed in DLD-1 and HepG2. The $\Delta\Psi m$ generated by fumarate was not inhibited by cyanide, consistent with the action of the NADH–FR system. In this system, complex I functions as a proton pump to form a transmembrane electrochemical proton gradient, the driving force in ATP synthesis. Therefore, the NADH–FR system may be involved in ATP generation under tumour microenvironmental conditions and PP may interfere with ATP synthesis by inhibiting the NADH–FR system in cancer cells in tumour microenvironments. Moreover, the $\Delta\Psi m$ is an important factor in the maintenance of mitochondria. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-induced putative kinase 1 (PINK1) and Parkin reportedly play important roles in the quality control of mitochondria through the clearance of damaged mitochondria via autophagy; after the loss of the $\Delta\Psi m$, PINK1 recruits Parkin into mitochondria and promotes mitophagy in *Drosophila* and both neurogenic and non-neurogenic human cells (*40–42*). Therefore, the generation of $\Delta\Psi m$ by the NADH–FR system in tumour microenvironments likely has important roles in ATP generation and the maintenance of mitochondrial quality.

PP increased the SQR activity in the mitochondria of cancer cells cultured under normoxia–normoglycemic conditions; however, SQR was not activated in cancer cells cultured under hypoxia–hypoglycemic conditions. These observations raise two questions: how does PP affect complex II and how does complex II differ under normal and tumour microenvironmental conditions? Post-translational modifications in mitochondrial proteins, such as phosphorylation and acetylation, have been identified and some of these modifications regulate mitochondrial functions (*43*, *44*). In complex II, the acetylation of the Fp subunit has been reported and the deacetylase Sirtuin 3 deacetylates the Fp subunit (*45*, *46*). The phosphorylation of the Fp subunit has also been shown and we previously reported that the phosphorylation of the Fp changed its activity, with the activity of SQR increasing when Fp was dephosphorylated and the activity of FRD increasing when Fp was phosphorylated. Under tumour microenvironmental conditions, the phosphorylated form of Fp and FRD activity concomitantly increased, whereas the dephosphorylated form of Fp and SQR activity decreased (*20*). Treatment of the cells with PP resulted in the dephosphorylation of the Fp subunit, which might have been mediated by the activation of mitochondrial phosphatase(s). However, several protein kinases and phosphatases have been detected in mitochondria, although the details of their physiological roles are poorly understood (*47*). Regarding complex II, Salvi *et al.* (*48*) reported that Fgr tyrosine kinase, which is a member of the Src kinase family, phosphorylated the Fp subunit, but little information on Fgr tyrosine kinase is available. Therefore, a direct target of PP, which activates SQR, could not be found at present. PP has also been shown to inhibit the phosphorylation of PKB/Akt under hypoglycemic condition (*10*). The inhibition of the PKB/Akt pathway may be involved in cytotoxicity, specifically under hypoglycemic condition, but the inhibition of the PKB/Akt pathway by wortmannin and LY294002 did not cause selective cytotoxicity under hypoglycemic condition (*49*). These data suggest the importance of the PKB/Akt pathway in selective cytotoxicity under hypoglycemic condition even though this pathway is not a direct target of PP. Although the direct target of PP remains

unknown, the key effects of PP such as its anticancer and selective cytotoxic effects, may arise through the inhibition of the NADH–FR system. Therefore, the NADH–FR system is a good target for anticancer therapy.

In this report, we showed that PP affects mitochondrial energy metabolism through the inhibition/activation of ETC enzymes. Therefore, the NADH–FR system is a novel mitochondrial pathway for energy metabolism in tumour microenvironments and PP is a promising leading compound for the development of tumour-microenvironment-specific anticancer agents.

Supplementary Data

Supplementary data are available at *JB* Online.

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Conflict of interest

None declared.

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KRAS mutations in primary tumours and post-FOLFOX metastatic lesions in cases of colorectal cancer

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BACKGROUND: *KRAS* mutations are predictive markers for the efficacy of anti-EGFR antibody therapies in patients with metastatic colorectal cancer. Although the mutational status of *KRAS* is reportedly highly concordant between primary and metastatic lesions, it is not yet clear whether genotoxic chemotherapies might induce additional mutations.

METHODS: A total of 63 lesions (23 baseline primary, 18 metastatic and 24 post-treatment metastatic) from 21 patients who were treated with FOLFOX as adjuvant therapy for stage III/IV colorectal cancer following curative resection were examined. The DNA samples were obtained from formalin-fixed paraffin-embedded specimens, and *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutations were evaluated.

RESULTS: The numbers of primary lesions with wild-type and mutant *KRAS* codons 12 and 13 were 8 and 13, respectively. The mutational status of *KRAS* remained concordant between the primary tumours and the post-FOLFOX metastatic lesions, irrespective of patient background, treatment duration and disease-free survival. Furthermore, the mutational statuses of the other genes evaluated were also concordant between the primary and metastatic lesions.

CONCLUSION: Because the mutational statuses of predictive biomarker genes were not altered by FOLFOX therapy, specimens from both primary tumours and post-FOLFOX tumour metastases might serve as valid sources of DNA for known genomic biomarker testing.

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Keywords: colorectal cancer; genomic biomarker; *KRAS*; anti-EGFR antibody; oxaliplatin

KRAS mutations are predictive markers for the poor efficacy of anti-EGFR antibody therapies in patients with metastatic colorectal cancer (Lievre *et al*, 2006; Benvenuti *et al*, 2007; Di Fiore *et al*, 2007; Frattini *et al*, 2007; Khambata-Ford *et al*, 2007; Amado *et al*, 2008; De Roock *et al*, 2008; Freeman *et al*, 2008; Karapetis *et al*, 2008; Lievre *et al*, 2008). Point mutations in the *KRAS* gene occur early in the progression from colorectal adenoma to carcinoma and are detected in 35–40% of patients, regardless of their Dukes stage (Andreyev *et al*, 1998). More than 90% of the *KRAS* mutations in these patients have been detected in codons 12 (GGT) and 13 (GGC) (Oliveira *et al*, 2004). Activating mutations at codons 61 and 146 have also been reported in a small number of these tumours. In addition, mutations in the molecules involved in signalling pathways downstream of EGFR, such as *NRAS*, *BRAF* and *PIK3CA*, have also been reported in colorectal cancers. These mutations have been suggested to modify the efficacy of anti-EGFR antibody therapies, although their predictive value has not yet been established (De Roock *et al*, 2010).

Oxaliplatin [*trans*-R,R-1,2-diaminocyclohexaneoxalatoplatinum (II), L-OHP] is a third-generation platinum (Pt)-containing anti-tumour compound. It is frequently administered as a component of FOLFOX therapy in combination with 5-FU for patients with metastatic colorectal cancer. Oxaliplatin induces DNA damage associated with intra- and inter-strand cross-links (Pt-GG adducts) and can induce gene mutations (Woynarowski *et al*, 2000; Hah *et al*, 2007; Sharma *et al*, 2007). The mutagenic activity of oxaliplatin has been demonstrated in cultured cells (Silva *et al*, 2005).

The *KRAS* mutation status of primary and metastatic lesions is reportedly highly concordant (Oudejans *et al*, 1991; Losi *et al*, 1992; Suchy *et al*, 1992; Zauber *et al*, 2003; Weber *et al*, 2007; Etienne-Grimaldi *et al*, 2008; Santini *et al*, 2008; Garm Spindler *et al*, 2009; Loupakis *et al*, 2009; Perrone *et al*, 2009; Baldus *et al*, 2010; Italiano *et al*, 2010; Knijn *et al*, 2011). However, whether long-term treatment with genotoxic chemotherapies, such as oxaliplatin, can induce additional mutations in metachronous metastatic lesions has not yet been well examined.

Assuming that FOLFOX therapy has the potential to alter the biomarker mutation profile, it is important to determine whether

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the primary or relapsed tumour represents the more appropriate source of DNA for testing. We examined the mutation status of *KRAS* and other biomarker genes in primary and synchronous/metachronous metastatic lesions in patients with stage III/IV colorectal cancer treated with adjuvant FOLFOX therapy following curative resection.

PATIENTS AND METHODS

Patient selection

A total of 63 lesions from 21 patients who had received adjuvant FOLFOX therapy for stage III/IV colorectal cancer following curative resection at the National Cancer Center Hospital East, Japan, between January 2006 and December 2009 were examined.

All patients were treated with a modified FOLFOX6 regimen, with a reduced oxaliplatin dose of 85 mg m^{-2} administered every 14 days, and 12 cycles were planned as the full therapy course (Andre *et al*, 2004; Allegra *et al*, 2009). FOLFOX therapy was discontinued when tumour relapse was demonstrated by imaging or when intolerable adverse events occurred.

DNA samples and mutational analyses

The DNA samples were obtained from macroscopically dissected formalin-fixed paraffin-embedded specimens cut into 10-μm-thick sections. Genomic DNA was extracted using the EZ1 Advanced XL and EZ1 DNA Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Bando *et al*, 2011). Mutations in *KRAS* codons 12 and 13 were detected using the ARMS/Scorpions technology-based *KRAS* PCR Kit (Qiagen) according to the manufacturer's instructions. Mutations in *KRAS* codons 61 and 146, *NRAS* codons 12, 13 and 61, *BRAF* codon 600 and *PIK3CA* codons 542, 545, 546 and 1047 were detected using the multiplex PCR-Luminex method-based MEBGEN Mutation Kit (Medical & Biological Laboratories, Nagoya, Japan). Mutations detected with the MEBGEN Mutation Kit were confirmed by direct sequencing. Mutations in *PIK3CA* codons 542, 545 and 546 were further confirmed using the ARMS/Scorpions technology-based PI3K Mutation Test Kit (Qiagen). The study was approved by the Institutional Review Board of the National Cancer Center.

RESULTS

Patient and tumour site characteristics

We reviewed 151 consecutive cases of stage III/IV colorectal cancer treated with an adjuvant FOLFOX therapy after curative resection. Among these cases, 21 patients developed metastatic tumours that were diagnosed during or after the FOLFOX therapy and surgically resected. The patient and tumour site characteristics are shown in Table 1. The primary tumour sites were the colon and rectum in 8 and 13 patients, respectively. The most abundant primary tumour histopathological type was differentiated adenocarcinoma. Well- and moderately differentiated adenocarcinomas and mucinous adenocarcinomas were observed in 5, 14 and 2 patients, respectively. All metastatic tumours exhibited histology concordant with that of the associated primary colorectal adenocarcinoma.

In all, 12 patients had stage III disease, whereas the remaining 9 patients had synchronous metastatic lesions and were diagnosed as stage IV at the initial operation. There were 12 synchronous metastatic lesions in the patients with stage IV disease. In addition, six metastatic lesions were detected in five patients with stage III disease at operation that were resected prior to the start of FOLFOX therapy. These 18 lesions were regarded as 'pre-FOLFOX' metastatic lesions. The pre-FOLFOX metastases were found in the liver (11 lesions), lung (5 lesions), as a local recurrence (1 lesion) and as a subcutaneous recurrence (1 lesion). Meanwhile, 24 metastatic lesions in the 21 patients were detected during or after FOLFOX therapy. These lesions were regarded as 'post-FOLFOX' metastatic lesions. The post-FOLFOX metastases were found in the liver, lung, as a local recurrence and lymph node in 6, 14, 3 and 1 patients, respectively.

Table 1 Characteristics

Patient characteristics	Number
Sex (female/male)	8/13
Median age (range)	64 (36–75) years
Primary tumour site	
Colon	8
Rectum	13
Histopathological type of primary site	
Well-differentiated adenocarcinoma	5
Moderately differentiated adenocarcinoma	14
Mucinous adenocarcinoma	2
Stage before initial operation	
III	12
IV (synchronous metastases)	9
Tumour site characteristics	
Metastases	
Pre-FOLFOX	18
Synchronous	12
Metachronous	6
Post-FOLFOX	24
Sites of metastases	
Pre-FOLFOX	
Liver	11
Lung	5
Local recurrence	1
Subcutaneous	1
Post-FOLFOX	
Liver	6
Lung	14
Local recurrence	3
Lymph node	1

The median number of FOLFOX therapy cycles administered was 9 (3–12 cycles). Five patients experienced relapse during FOLFOX therapy (case 1, 2, 3, 7 and 12), whereas the remaining 16 patients experienced relapse after the end of FOLFOX therapy. The median disease-free survival, calculated from the time of the last operation until post-FOLFOX recurrence, was 409 days (97–1077). The median period from the start of FOLFOX therapy until recurrence was 373 days (35–1029). Relapses developed within 180 days after the end of FOLFOX therapy in 10 of the 21 patients (Table 2).

Mutational status of *KRAS* and other genes

The mutational statuses of *KRAS* and other genes in primary and metastatic lesions are shown in Table 3. Mutations in *KRAS* codons 12 and 13 were detected in 13 of the 21 primary colorectal tumours. Among the remaining eight tumours with wild-type *KRAS* codons 12 and 13, two tumours exhibited *KRAS* codon 146 mutations (A146V and A146T) and one tumour exhibited *NRAS* codon 61 mutation (Q61H). Two tumours exhibited mutations in *PIK3CA* codon 542 (E542K), one tumour exhibited a *KRAS* G12S mutation and one tumour had no mutations in any of the genes examined. No apparent mutations of *KRAS* codon 61, *NRAS* codon

Table 2 FOLFOX treatment, metastasis status and tumour recurrence sites

Case	Primary site	Histopathological type	Pre-FOLFOX metastatic site	Synchronous/ metachronous	FOLFOX cycles	DFS (days)	Days from end of FOFLOX until recurrence	Post-FOLFOX recurrence site
1	Rectum	Mode		—	3	124	6	Liver
2	Colon	Mode	Liver	Synchronous	4	97	−16[a]	Liver
3	Colon	Mode	Liver	Synchronous	4	116	26	Liver
4	Rectum	Well	Local recurrence	Metachronous	4	469	363	Local recurrence
5	Rectum	Mode	—	—	5	827	603	Lung
6	Colon	Mode	—	—	5	350	244	Lymph node
7	Rectum	Mode	Liver	Synchronous	8	214	1	Lung
			Lung	Synchronous				
8	Rectum	Muc	—	—	8	538	318	Lung
9	Colon	Well	—	—	8	1077	903	Liver
10	Colon	Mode	Liver	Synchronous	8	344	120	Lung
			Liver	Synchronous				Lung
			Lung	Synchronous				
11	Colon	Muc	Lung	Synchronous	9	721	401	Lung
12	Rectum	Well	Liver	Synchronous	9	109	−88[a]	Liver
13	Rectum	Mode	Liver	Metachronous	11	328	120	Liver
			Lung	Metachronous				
14	Rectum	Mode	Subcutaneous	Metachronous	12	519	156	Lung
15	Colon	Mode	—		12	388	176	Local recurrence
16	Rectum	Mode	Liver	Synchronous	12	466	210	Lung
17	Rectum	Well	Lung	Synchronous	12	556	264	Lung
18	Colon	Mode	Liver	Metachronous	12	531	231	Lung
								Lung
19	Rectum	Mode	Liver	Synchronous	12	409	217	Lung
20	Rectum	Mode	—	—	12	455	243	Local recurrence
21	Rectum	Well	Liver	Metachronous	12	346	71	Lung
								Lung

Abbreviations: DFS = disease-free survival; mode = moderately differentiated adenocarcinoma; muc = mucinous adenocarcinoma; well = well-differentiated adenocarcinoma.
[a]The cases that FOLFOX therapies were administered after recurrence.

Table 3 Mutational status of *KRAS* and other genes

Case	Primary site	Mutation status	Pre-FOLFOX metastatic site	Mutation status	Post-FOLFOX recurrence site	Mutation status
1	Rectum	KRAS G12D	—	—	Liver	KRAS G12D
2	Colon	KRAS G12D	Liver	KRAS G12D	Liver	KRAS G12D
3	Colon	KRAS G12D	Liver	KRAS G12D	Liver	KRAS G12D
4	Rectum	KRAS G12R	Local recurrence	KRAS G12R	Local recurrence	KRAS G12R
5	Rectum	KRAS G12D	—		Lung	KRAS G12D
6	Colon	WT			LN	WT
7	Rectum	KRAS G12S	Liver	KRAS G12S	Lung	KRAS G12S
			Lung	KRAS G12S		
8	Rectum	WT	---	—	Lung	WT
9	Colon	WT	--		Liver	WT
10	Colon	KRAS G12A	Liver	KRAS G12A	Lung	KRAS G12A
			Liver	KRAS G12A	Lung	KRAS G12A
			Lung	WT		
11	Colon	KRAS G13D	Lung	KRAS G13D	Lung	KRAS G13D
12	Rectum	KRAS A146V	Liver	KRAS A146V	Liver	KRAS A146V
13	Rectum	KRAS G12V	Liver	KRAS G12V	Liver	KRAS G12V
			Lung	KRAS G12V		
14	Rectum	KRAS G12D	Subcutaneous	KRAS G12D	Lung	KRAS G12D
15	Colon	WT	—	—	Local recurrence	WT
16	Rectum	KRAS G12S, PIK3CA E542K	Liver	KRAS G12S, PIK3CA E542K	Lung	KRAS G12S, PIK3CA E542K
17	Rectum	KRAS G12D	Lung	KRAS G12D	Lung	KRAS G12D
18	Colon	KRAS G12D	Liver	KRAS G12D	Lung	KRAS G12D
					Lung	KRAS G12D
19	Rectum	NRAS Q61H	Liver	NRAS Q61H	Lung	NRAS Q61H
20	Rectum	PIK3CA E542K	—		Local recurrence	PIK3CA E542K
21	Rectum	KRAS A146V	Liver	KRAS A146V	Lung	KRAS A146V
					Lung	KRAS A146V

Abbreviations: LN = lymph node; WT = wild-type.

12 or 13, *BRAF* codon 600, or *PIK3CA* codon 1047 were detected in any sample in this study.

The degree of concordance of the gene mutations in primary and pre-FOLFOX metastatic lesions was examined. In case 10, a *KRAS* G12A mutation was detected in the primary lesion, whereas the metastatic lesion in the lung had wild-type *KRAS*. Although the histological features of the lung lesion were consistent with metastatic adenocarcinoma of the colon, no mutations in the metastatic lesion were detected, even after repeated high-sensitivity examinations. The remaining 17 metastatic lesions in 14 patients, including 2 liver metastatic lesions in case 10, showed the same mutational statuses as the primary tumours for all of the genes examined.

Then, the mutational statuses of the post-FOLFOX metastatic lesions were examined. The mutational statuses of all genes examined were identical in the 21 primary tumours and the corresponding 24 post-FOLFOX metastatic lesions, regardless of the sites involved, duration of FOLFOX treatment or disease-free survival period.

DISCUSSION

Previous studies have reported a high concordance rate of the *KRAS* mutations in primary and metastatic tumours (Oudejans *et al*, 1991; Losi *et al*, 1992; Suchy *et al*, 1992; Zauber *et al*, 2003; Weber *et al*, 2007; Etienne-Grimaldi *et al*, 2008; Santini *et al*, 2008; Garm Spindler *et al*, 2009; Loupakis *et al*, 2009; Perrone *et al*, 2009; Baldus *et al*, 2010; Italiano *et al*, 2010; Knijn *et al*, 2011). However, in patients receiving long-term chemotherapy, the effects of genotoxic chemotherapies, such as oxaliplatin, have not been well investigated.

In this study, we examined 21 patients with metastatic colorectal cancer who received adjuvant FOLFOX therapy. The recurrent tumours in three patients who showed relapse within 4 months after the primary surgery or during the first 3 or 4 cycles of adjuvant FOLFOX therapy (cases 1–3) were regarded as synchronous metastases arising from micrometastases that likely existed prior to the start of the adjuvant chemotherapy. The remaining 18 patients who developed relapses more than 8 months from the end of adjuvant FOLFOX therapy or after more than 6 cycles of adjuvant FOLFOX therapy were regarded as having metachronous metastatic tumours that had developed after exposure to oxaliplatin. Among these cases, tumour relapse occurred within 180 days after FOLFOX therapy in 7 patients and more than 180 days after FOLFOX therapy in the remaining 11 patients. Regardless of the treatment duration, 8 of the primary tumours with wild-type *KRAS* codons 12 and 13 did not acquire *KRAS* mutations. The remaining tumours with *KRAS* mutations also did not show additional mutations after FOLFOX therapy. Furthermore, none of the other genes that might potentially affect the efficacy of anti-EGFR antibody therapy were altered.

KRAS, *NRAS* and *BRAF* mutations are all regarded as strong driver mutations that induce cell proliferation. These mutations might be acquired in the early stages of carcinogenesis and have generally been reported as mutually exclusive (Andreyev *et al*, 1998). Consistent with this observation, the *KRAS* and *NRAS* mutations in this study were found to be mutually exclusive. In the rest of the tumours, other unidentified driver mutations or amplifications may have activated the signalling pathways promoting cell proliferation. Considering the exclusive nature of the tested mutations, the acquisition of additional driver mutations may not be advantageous to these tumour cells for clonal selection. This could be one explanation for why the mutational statuses of *KRAS* and other genes were not altered during the development of metastatic tumours.

Our findings suggest that both the primary tumours and metastatic tumours arising during or after FOLFOX therapy could be valid sources of DNA for *KRAS* testing prior to treatment with anti-EGFR antibodies, although the number of cases in this study was limited. This finding should be further confirmed in a larger number of cases. Though collecting surgically resected metastatic tumour tissues is often difficult, circulating tumour cells may be a useful alternative DNA source for highly reliable and sensitive mutation detection systems such as the ARMS/Scorpion method for further analyses.

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ORIGINAL ARTICLE

Prospective trial of chemotherapy-enhanced accelerated radiotherapy for larynx preservation in patients with intermediate-volume hypopharyngeal cancer

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ABSTRACT: *Background.* Altered fractionation radiotherapy (RT) improves locoregional control in head and neck cancer without aggravation of late adverse events. To improve successful larynx-preservation rates in patients with resectable, intermediate-volume hypopharyngeal cancer, a prospective trial of chemotherapy-enhanced accelerated RT was conducted.

Methods. Patients with T2 to T4 hypopharyngeal cancer received 40 Gray (Gy)/4 weeks to the entire neck followed by boost RT administering 30 Gy/2 weeks (1.5 Gy twice-daily fractionation). Cisplatin and 5-fluorouracil were administered concomitantly only during boost RT.

Results. Thirty-five patients were enrolled in this study. All patients completed this protocol as planned. After a median follow-up period for surviving patients of 59 months (24–90 months), overall survival and local control rates at 3 years were 91% (95% confidence interval, 81% to 100%), and 88% (79% to 99%), respectively. All surviving patients maintained normalcy of diets.

Conclusion. This regimen was feasible with encouraging oncological and functional outcomes. © 2011 Wiley Periodicals, Inc. *Head Neck* 34: 1363–1368, 2012

KEY WORDS: hypopharyngeal cancer, accelerated fractionation radiotherapy, chemotherapy, larynx preservation, long-term swallowing function

Approximately one fourth of oral cavity or pharyngeal cancers in Japan originate from the hypopharynx, and the estimated incidence of patients with hypopharyngeal cancer is about 2500 per year.[1,2] Larynx-preserving approaches for hypopharyngeal cancer showed no obvious difference in overall survival compared to other surgical approaches in a randomized study[3] as well as in a large population-based study.[4] Conventional fractionation radiotherapy (RT) alone for patients with early-stage hypopharyngeal cancer with T2 disease achieved a local control rate of approximately 60%.[5,6] RT alone using altered fractionation significantly improved local control rates without deterioration of serious late adverse events.[7,8] This approach could achieve favorable larynx-preservation rates in selected patients with hypopharyngeal cancer with low-volume, T1 or T2 primary tumors which had tumor volumes of 7 mL or smaller.[9,10] However, less favorable results were expected for other patients with larger hypopharyngeal cancers that required total laryngectomy.[10,11] Therefore, the combination of chemotherapy with RT is required to improve larynx-preservation rates in these patients.[12]

Because hypopharyngeal cancer originates from the narrowest part of the upper digestive tract, late dysphagia and aspiration due to consequential late effects are not uncommon even after successful eradication of the disease after intensive chemoradiotherapy (CRT).[13–16] Therefore, special attention should be paid to minimize severity and duration of serious mucosal toxicity by a deliberate combination of altered fractionation RT and/or chemotherapy with meticulous patient selection according to the morphology and volume of the primary tumor.[10,17]

High incidence of distant metastasis in patients with hypopharyngeal cancer was mainly because of the high frequency of advanced nodal disease at the time of initial presentation.[18] If a patient has an intermediate-volume tumor without advanced nodal metastasis, tumor control above the clavicle instead of prevention of systemic tumor dissemination should be prioritized. We previously reported a favorable local cure rate after conventional fractionation RT in patients with intermediate-volume disease at the pharyngolarynx; however, further improvement of local control with good function is needed.[19] This study was based on the principle that use of chemotherapy as a radiation sensitizer should not compromise the benefit of altered fractionation RT alone in terms of long-term swallowing function in these patients.[7,8]

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Accelerated fractionation that delivers dose-dense RT during the latter part of the entire treatment is a reasonable strategy to overcome accelerated repopulation of the tumor, which is supposed to begin at approximately 4 weeks after commencement of RT.[8,20,21] The aim was to enhance the effect of treatment using both twice-daily RT and chemotherapy only during the period when acceleration of tumor repopulation is to be expected. Although the incidence of these patients was expected to be limited, and the true efficacy should be tested in multiinstitutional collaborative studies, preliminary results of this "chemotherapy-enhanced" accelerated fractionation RT were promising.[22] Therefore, matured results of a prospective, single institutional trial were reported here to demonstrate the safety and validity of conducting a larger trial of this regimen.

MATERIALS AND METHODS

Patient population

Patients were required to have previously untreated, histologically proven squamous cell carcinoma of the hypopharynx that was judged amenable to margin-free resection with total laryngopharyngectomy and neck dissection by expert head and neck surgeons in our institution. Those who were considered as candidates for partial laryngectomy or who had T1 disease were ineligible for this study. In addition, the following eligibility criterion were required: age ranging from 20 to 75 years; no bilateral lymph node metastasis on CT and/or MRI scans; no evidence of distant organ metastasis (clinically M0); Zubrod Performance Status (PS) of 0 to 2; no history of RT for the head and neck area; adequate bone marrow and organ function; no history of other malignancies within 5 years before enrollment; and no history of ischemic heart disease and/or symptomatic cerebrovascular accident within 3 months before enrollment. Patients who had simultaneous superficial esophageal and/or gastric cancers that were judged amenable to margin-free resection using endoscopic mucosal resections were eligible for the study. All patients provided written informed consent. We received approval for this study from our institutional ethics committee.

Pretreatment evaluation

Disease was staged according to the American Joint Committee on Cancer Staging Manual (6th edition). Staging procedures consisted of physical examination, head and neck fiberscope, CT and/or MRI of the head and neck region, chest X-ray, upper abdominal ultrasound, and gastroesophageal endoscopy. CT of the chest and bone scans were performed as indicated. Laboratory studies included a complete blood cell count, routine liver and kidney function tests, and electrocardiography. All patients underwent pretreatment dental examinations, and dental therapy was done as indicated before the start of RT. Nutritional support by using a nasogastric tube or percutaneous gastrostomy was not done in this protocol.

Radiotherapy

A total dose of 40 Gray (Gy)/4 weeks using 2 Gy once-daily fractionation was administered to the primary tumor, bilateral level II to IV lymph node stations, and retropharyngeal lymph nodes according to the American Joint Committee on Cancer Staging Manual (6th edition). This was followed by boost RT administering 30 Gy/2 weeks (1.5 Gy twice-daily fractionation) to the primary tumor with 2-cm margins. Interfraction interval was set as $\geq$6 hours. Maximum efforts were taken, if appropriate, to exclude the base of tongue and cervical esophagus at >2 cm below the caudal edge of the cricoid cartilage from irradiated volume of the boost RT. If a patient had gross nodal disease extending above the posterior belly of the subdigastric muscle and/or to level IV, which necessitated a larger irradiated volume than that described above during boost RT, neck dissection was performed before the start of RT. This was followed by a total of 55 Gy of RT that was administered to the surgical bed of this upfront nodal dissection, followed by additional 15 Gy to the primary tumor. Maximum dose to the spinal cord was restricted to 46 Gy/24 fractions. RT was delivered using 6 MV X-rays in all patients with 3-dimensional RT planning. Intensity-modulated radiotherapy was not used in this group of patients.

Chemotherapy

A single course of chemotherapy was concomitantly administered during the boost RT in expectation of a radiosensitizing effect.[23,24] Cisplatin 80 mg/m^2 was administered with intravenous hydration on the first day of chemotherapy, and 4-day continuous infusion of 5-fluorouracil 400 mg/m^2/day was started on the same day. Patients were hospitalized during the course of chemotherapy and received hydration and antiemetic therapy as indicated.

Dose modifications

Grade 4 hematological toxicity or grade $\geq$3 dysphagia and/or swallowing pain required treatment break until these toxicities became grade $\leq$2. Chemotherapy was started only when the following criterion were fulfilled: white blood cell count $\geq$2000/mm^3, hemoglobin level $\geq$8.0 g/dL, platelet count $\geq$100,000/mm^3, any gastrointestinal toxicities of less than grade 3, serum bilirubin level $\leq$1.5 mg/dL, serum creatinine level $\leq$1.5 mg/dL, and dermal toxicity of less than grade 2. If patients did not meet these criterion, chemotherapy was postponed without RT break and administered only when patients satisfied the criterion within 7 days.

Outcome measures and statistical considerations

Low accrual rate was expected in a single institutional setting, and experience of administering altered fractionation RT for hypopharyngeal cancer was limited in Japan at the time of protocol development.[6] Therefore, this trial was conducted as a feasibility study to plan a multiinstitutional trial of this regimen to evaluate the true efficacy with a sufficient number of patients. The primary endpoint was completeness of the protocol treatment without unplanned treatment break or dose modification. This trial used a 2-stage design wherein the expected rate of completeness was defined as 80%. This was tested against the threshold rate of 60% or lower with an alpha level of 5% and a power of 80%, which required an initial enrollment of 13 patients. If <8 of these 13 patients

completed the protocol without unplanned break and dose modifications, the trial would be stopped. Otherwise, enrollment would be extended to 35 patients and the rate of completeness determined. Secondary endpoints were local control rate, progression-free survival, overall survival, and adverse events. Follow-up visits were requested monthly within 2 years after completion of RT, at least once per 3 months during the third year, and once per 6 months thereafter. Radiological examinations including CT and/or MRI of the head and neck were done at least twice within 6 months immediately after treatment, and at regular intervals of 6 to 12 months thereafter. Positron-emission tomography was not routinely done in this protocol. Time-to-event analyses from the start of RT were done using Kaplan–Meier estimates. Biopsy-proven recurrence of the primary tumor was considered as an event for calculating the local control rate, and patients who died without this event were censored at the time of last follow-up examinations. Death of any cause was defined as events in calculating overall survival. Also, recurrence at any site or death of any cause was used in estimating progression-free survival. Adverse events were estimated according to the National Cancer Institute Common Toxicity Criteria, version 2.0, and Radiotherapy Oncology Group/European Organization for Research and Treatment of Cancer Late Radiation Morbidity Scoring Scheme.

Although not required in the protocol, volumetry of the primary tumor was estimated from CT scans during RT planning in all patients retrospectively using RT planning software (Xio, version 4.4, Elekta CMS Software, St. Louis, MO) by the principal investigator (M.K.).

RESULTS

Patients

Between October 2002 and March 2008, 35 patients were enrolled. Patient characteristics are listed in Table 1. Thirteen of 15 patients with T3/4 disease had fixation of the vocal cord at presentation, and disease in 2 patients was defined as T3 because of estimated tumor diameter on CT/MRI scan that exceeded 4 cm. Among 20 patients with node-positive disease, 3 had lymph node metastasis at level IV, and the others were confined to level II and/or level III. Two patients had histories of esophagectomy due to esophageal cancer at 7 and 10 years before enrollment, and 2 other patients had simultaneous superficial esophageal cancers that were successfully treated with endoscopic mucosal resections thereafter. Three patients were classified as Zubrod PS 2, otherwise all patients were PS 1. At the time of this analysis, 1 patient was lost to follow-up at 24 months without evidence of disease recurrence. Otherwise, all patients were followed for more than 2 years or until death, and the median follow-up period for surviving patients was 59 months (24–90 months).

Completeness of the protocol

Eight patients received up-front nodal dissection at 8 to 25 days (median, 17 days) before start of RT without serious postoperative complications. All of the 35 patients completed RT and chemotherapy as planned with a median overall treatment time of 44 days (range, 40–48 days). All prolongations of overall treatment time for more than 6 weeks were due to public holidays and/or maintenance of the RT machine. Adverse events that were observed within 90 days after start of the treatments are listed in Table 2. It should be noted that all of the grade 3 adverse events were observed after the end of the treatment and no patients required interruption of RT and/or chemotherapy. Five patients required transient parenteral hyperalimentation to supplement decreased oral intake. However, 24 (69%), 33 (94%), and 35 (100%) patients recovered their normalcy of diet within 2, 4, and 7 weeks, respectively, after completion of the treatments. Although 3 patients required tracheostomy before start of the treatments because of tumor-related airway stenoses, all of them were able to achieve complete resolution of the tumor and were decannulated within 2 months after completion of the treatments. One patient experienced transient grade 3 thrombocytopenia immediately after completion of RT but recovered spontaneously within a week without suffering a symptomatic hemorrhagic accident.

TABLE 1. Patient characteristics.

Characteristics	No. of patients	%
Sex		
Male	32	91
Female	3	9
Age		
Median (range), y	61 (46–73)	
Subsite		
Pyriform sinus	30	86
Post cricoid	4	11
Posterior wall	1	3
Differentiation		
Moderately	17	49
Poorly	6	17
Not specified	12	34
T/N classification		
T2	20	57
N0	7	20
N1	3	9
N2a	1	3
N2b	8	23
N3	1	3
T3	12	34
N0	6	17
N1	1	3
N2a	1	3
N2b	4	11
T4	3	9
N0	2	6
N1	1	3
Stage		
II	7	20
III	10	29
IV	18	51
Volume of the primary tumor (mL)		
Median (range)	15 (3–49)	
<10	5	14
≥10, <20	17	49
≥20, <30	9	26
≥30	4	11

TABLE 2. Acute events.

Grade	0	1	2	3	4	5
White blood cell	18	12	5	0	0	0
Anemia	25	6	4	0	0	0
Thrombocytopenia	33	0	1	1	0	0
Mucositis due to radiation	0	6	17	12	0	0
Dysphagia-pharyngeal due to radiation	1	8	18	8	0	0
Creatinine	24	10	1	0	0	0
Nausea/vomiting	31	2	2	0	0	0
Worst overall	0	4	18	13	0	0

Patterns of failure

Four patients experienced local persistence or recurrence with ($n = 3$) or without ($n = 1$) nodal metastases. Otherwise, 3 patients, all of whom had node-negative disease at presentation, experienced nodal recurrences as first sites of relapses within irradiated volume of the boost RT in 2 patients, and at the periphery in 1 patient. All but 1 patient with unresectable, isolated nodal failures underwent successful salvage without serious postoperative complications. However, 1 patient died of subsequent nodal failure. Two other patients experienced distant metastases in the lungs as first site of relapse without evidence of locoregional recurrence. Both of these 2 patients originally had node-negative disease (T2 in 1 patient and T4 in 1 patient). One patient died of ischemic heart disease without evidence of disease recurrence at 41 months. Overall survival rate at 3 years was 91% (95% confidence interval, 81% to 100%). All of the disease recurrences at the primary sites were observed within 2 years, and local control rate at 2 years was 88% (79% to 99%), as shown in Figure 1. Nodal and distant metastasis rates at 2 years were 14% (3% to 26%) and 6% (0% to 15%), respectively. Progression-free survival at 2 years was 77% (63% to 91%). All of the 20 patients who had T2 disease did not experience local recurrence. However, 1 patient died of nodal recurrence at 13 months. On the other hand, 4 of the 15 patients who had T3/4 diseases experienced local recurrences. Local control rate at 2 years for patients with T3/4 disease was 73% (51% to 96%).

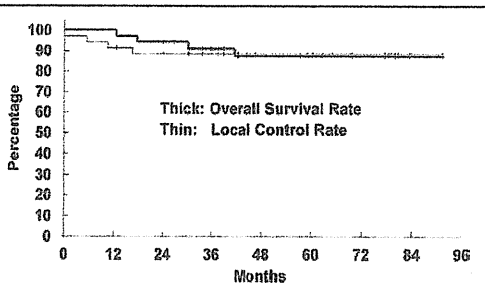

FIGURE 1. Kaplan-Meier estimates of overall survival and local control rates in patients with T2 to 4 primary tumor that was amenable to margin-free resection without bilateral or unresectable nodal metastasis who underwent chemotherapy-enhanced accelerated radiotherapy. Thick and thin lines represent overall survival and local control rates, respectively.

Primary tumors that showed superficial spread with an exophytic growth pattern had tumor volumes of less than 7 mL ($n = 5$), whereas patients that presented with endophytic tumors ($n = 30$) had tumor volumes of at least 10 mL on CT volumetry. Local control rate at 2 years for the former patients was 100%, contrasted to 87% (95% confidence interval, 74% to 99%) for the latter.

Late adverse events

One patient required 3 months of gastrostomy tube feeding and antibiotics for exposure of the thyroid cartilage to the pharyngeal cavity at 10 months, and another patient underwent repetitive balloon dilatation for pharyngeal stenosis without the need of taking a soft diet at 9 months. Both patients recovered their normalcy of diet thereafter and were alive and recurrence-free at 83 and 58 months, respectively. Otherwise, late radiation morbidities of grade 2 or greater were not observed. As a whole, all of the patients who were alive with their larynx retained their normal understandable speech without the need for a tracheostomy. Furthermore, all patients who were alive, including those who underwent salvage total laryngopharyngectomy, maintained their normal diets.

DISCUSSION

For laryngeal cancer, a clinical practice guideline for larynx-preserving approach was presented[17] based on data accumulated from landmark studies.[25,26] The same principles are thought to be applicable to hypopharyngeal cancer. In this study, patients who had primary tumors that mostly localized within the hypopharynx and larynx without penetration of the thyroid cartilage and pharyngeal constrictor muscle were enrolled. These hypopharyngeal cancers had tumor volumes of approximately $\leq$30 mL, and better local control rate after RT than in patients with larger primary tumors as was suggested from retrospective studies.[19,27,28] Measurement of tumor volume is significantly influenced by interobserver variation and imaging modality used in volumetry.[29] However, the required precision for tumor volumetry to adequately predict radiocurability was considered as $\pm$50% in a review of the literature.[30] Therefore, it is conceivable that most patients enrolled in this study had "intermediate-volume" tumors requiring total laryngopharyngectomy as a curative surgical approach and amenable to margin-free resections, but which were not categorized as having "low-volume" tumors.

When this study was being developed, however, a high percentage of patients with intermediate-volume hypopharyngeal cancers without advanced nodal diseases did not receive definitive CRT in many academic centers in Japan.[1,31] This was because the safety and efficacy of possible salvage surgery after CRT was empirically expected to be poor.[32,33] In addition, a recent multiinstitutional larynx-preserving trial using intensive CRT showed that, at 1 year, 23% of the patients were able to swallow only soft foods or liquids, and 3% could not swallow at all.[26] Other detrimental effects of concomitant high-dose chemotherapy with altered fractionation RT on long-term

swallowing functions were also documented.[34,35] For patients with hypopharyngeal cancer with intermediate-volume primary tumors, clinical clarification of the following points were sought in this study: (1) altered fractionation RT alone is insufficient to satisfy the result; (2) however, 2 or more courses of concomitant chemotherapy not only could result in deterioration of function, but is unnecessary to achieve the outcome comparable to altered fractionation RT alone for early-stage, low-volume tumors[10,11]; (3) efforts to minimize the irradiated volume receiving CRT may be needed to prevent excessive vascular and/or connective tissue damage at the expected anastomosis site in possible salvage surgery; (4) for this purpose, up-front nodal dissection should be positively considered in patients with nodal disease spreading outside of the target volume for boost RT encompassing only the primary tumor with margins. Concomitant chemotherapy during the former part of RT was not done to eliminate unexpected local and/or systemic toxicity of chemotherapy that possibly interrupt timely administration of accelerated RT.[21] As a result, all patients completed the protocol treatment without an unplanned break, and all of the 4 patients who experienced local recurrences safely underwent salvage total laryngopharyngectomy.

More than 5 years were required to accumulate the 35 patients as expected at the time of protocol development. The principal conclusion of this study was the feasibility of this protocol. However, it should be emphasized that none of the 20 patients with T2 disease experienced local recurrence. Although 73% of local control rate at 2 years for T3/4 disease was observed in only 15 patients, the lower limit of the 95% confidence interval was 51%, which exceeded the results of a previous randomized study for larynx-preserving treatment in patients with resectable hypopharyngeal cancer.[3] Overall survival rate at 3 years was 91% with acceptable distant failure rate. These results showed that this regimen can become an alternative to more intensive CRT in patients who were eligible for this study.

This regimen is in contrast to the widely accepted benefit of concomitant chemotherapy delivered throughout RT. However, for certain patients with stage III/IV disease, low-volume disease could achieve satisfactory results after treatment without using intensive chemotherapy.[14,36] Incidence of grade $\geq$3 mucositis was 34% (12 of 35), which was comparable to results in previous studies regarding CRT with higher dose of chemotherapy.[26] However, it should be noted that all of the grade 3 mucositis occurred after completion of the protocol and most of the patients recovered their normalcy of the diet within 4 weeks, probably because of no additional injury to the mucous membrane after occurrence of serious mucositis in this regimen. In addition, grade $\geq$3 hematologic toxicity was observed in only 1 patient (3%). Given that bacterial colonization in patients with compromised immune reaction aggravates and prolongs severe acute mucositis,[37] lower bone marrow toxicity of this regimen is preferable to ameliorate chronic dysphagia as a consequential late effect.[16] As a result, no surviving patient experienced feeding tube dependency at $\geq$2 years in this study. Upfront nodal dissection followed by definitive RT with or without substandard chemotherapy for appropriately selected patients with small pharyngolaryngeal cancer with bulky N2/3 disease could achieve locoregional control rates equal to those who had N0/1 disease without compromise of survival.[38–41] Six percent of distant failure rate (none in patients who underwent up-front nodal dissection) in this study was in good agreement with these previous reports.[38,40,41] The survival benefit of adding intensive chemotherapy for the purpose of preventing distant failure had never been observed in patients with resectable disease and, at present, the value of intensive chemotherapy for these patients is recognized as improvement of locoregional control.[3,12,26] In this context, because of 88% preservation rate of functioning larynx with a low distant failure rate, this study including 20 patients with node-positive disease who were amenable to margin-free resections (8 required up-front nodal dissection) should not be criticized based solely on substandard use of chemotherapy. Involvement of nodal metastasis outside of the sentinel area (ie, ipsilateral levels II and III) was reported as a significant factor of developing distant failure.[42] Because only 3 of 35 patients had gross nodal disease at the level IV, the results of this study might be relevant to a subsection of hypopharyngeal cancer patients having T2 or small T3/4 primary tumor with N0 to resectable N2 disease localized to the sentinel area (incipient N2), which should be considered in subsequent studies. The necessity of up-front nodal dissection only for prevention of excessive tissue damage may be negated in the intensity-modulated radiotherapy era.

Whether the results of this study were merely due to our patient selection in a single institutional setting, must be elucidated in larger, multi-institutional trials. However, the survival benefit of altered fractionation RT was already demonstrated in a meta-analysis.[43] Patients with hypopharyngeal cancer have relatively poor health status and high propensity of developing acute and/or late toxicities such as pneumonia and dysphagia. In this context, the benefit of intensive chemotherapy added to RT may be diminished and negated by its toxic effect when patients with hypopharyngeal cancer having relatively small tumor burdens are included in larynx preserving trials.[30] Therefore, testing separate strategies for patients with intermediate-volume primary tumor with N0 to incipient N2 disease was considered justifiable. Appropriateness of chemotherapy-enhanced accelerated RT was thought to be applicable even when intermediate-volume tumors were categorized as T3/4 disease in the current staging system with sophisticated imaging modalities. However, dosing of chemotherapy, role of induction chemotherapy, and molecular targeted therapy should be studied further with careful patient selection in these patients. In patients who have larger tumor burdens, reduced dose chemotherapy no longer achieved satisfactory tumor cure.[44,45]

In conclusion, accelerated fractionation RT with delayed concomitant chemotherapy as a radiation sensitizer was feasible and showed encouraging oncological and functional outcomes in patients with intermediate volume hypopharyngeal cancer who would otherwise have required total laryngopharyngectomy. Further study is warranted to test the appropriateness of this regimen for patients with hypopharyngeal cancer who have intermediate-volume, especially T2, primary tumor with N0 to incipient N2 disease in multi-institutional collaborations.

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ORIGINAL ARTICLE

Salvage surgery for recurrent oropharyngeal cancer after chemoradiotherapy

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Abstract

Background The current study aimed to assess the role of salvage surgery for failure cases of oropharyngeal cancer (OPC) undergoing initial chemoradiotherapy (CRT).

Methods The data for 523 patients with previously untreated OPC were gathered from 12 institutions belonging to the Head and Neck Cancer Study Group in Japan Clinical Oncology Group (JCOG).

Results Of the 170 patients who received CRT, 35 patients (21 %) had local recurrence or residual disease. Only 11 patients underwent further salvage surgery, and 24 patients received nonsurgical treatment. There were statistically significant differences between the two groups in terms of patient age and the presence of a simultaneous regional recurrence. The 5-year overall survival rates for the patients who underwent salvage surgery were 49.1 %, whereas those for the patients who received nonsurgical treatment were 16.3 %.

Conclusion The initial treatment method for OPC should be decided carefully and the limitations of salvage surgery should be fully considered.

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Introduction

In recent years, the initial treatment strategy for advanced head and neck cancer has shifted from surgery toward chemoradiotherapy (CRT) [1, 2]. This paradigm shift is particularly marked for oropharyngeal cancer (OPC), because OPC has high sensitivity to radiation and chemotherapy, and extended resection of the oropharynx leads directly to swallowing and speech disorders. The meta-analysis reported by Parsons et al. [3] revealed that organ preservation protocols have comparable survival rates, improved functional outcomes, and decreased severe complications compared to open surgery. Additionally, the relationship between human papilloma virus (HPV) and carcinogenesis of the oropharynx has been confirmed, and its treatment sensitivity has expedited a further paradigm shift [4, 5]. Although CRT is reported to show good results, patients with OPC are at risk of recurrence after initial therapy. Bachar et al. [6] reported that 239 of 640 patients (37 %) with tonsillar cancer recurred post radiotherapy.

Salvage surgery is the only curative treatment for patients with recurrence. However, the rate of successful surgical salvage has remained modest. Previously, we analyzed the effectiveness of salvage surgery for local recurrence after CRT or radiotherapy (RT) in hypopharyngeal cancer and reported that the successful salvage rate was only 17.1 % [7]. Goodwin [8] conducted a meta-analysis of 532 patients with recurrent pharyngeal cancer undergoing salvage surgery after definitive radiotherapy and reported a recurrence-free survival rate of only 25 % at 2 years and a 5-year overall survival rate of 26 %. Furthermore, although reconstructive surgery for oral and pharyngeal surgical defects, such as microvascular reconstructive techniques, has developed over the past several decades, Agra et al. [9] reported that postoperative complications after en bloc salvage surgery for head and neck cancer occurred in 53.2 % of patients, including 42.7 % of patients with minor complications, 18.5 % of patients with major complications, and 3.2 % of patients who died within the postoperative period.

Recently, a large-scale multi-institutional joint research for OPC was performed in Japan for the first time. Twelve institutions, mainly treating patients with cancer, participated in this research, and the data for 523 patients were obtained. In this study, we focused on the patients initially treated with CRT, and retrospectively analyzed the treatment failures, salvage surgeries, and survival rates of these patients.

Patients and methods

Patients

The data for 523 patients with previously untreated OPC from April 2005 to March 2007 were gathered from 12 institutions belonging to the Head and Neck Cancer Study Group in Japan Clinical Oncology Group (JCOG). Therapeutic strategy varied widely among the institutions, with the proportion of surgical interventions varying between 6 % and 59 % and that of RT with or without chemotherapy being 41–94 %. This study was a retrospective analysis, so the criteria of selection of therapeutic modality was decided by the institutional policies or patients' preference. In all, 37 patients who received palliative therapy were excluded from further analysis, and the data for the remaining 486 patients were analyzed retrospectively. Of the 486 patients with OPC treated with curative intent, 199 patients (41 %) were treated with surgery, 117 (24 %) with RT alone, and 170 (35 %) with CRT (Table 1). Between each therapeutic modality, there was no statistical difference in age, gender, subsite, or histology. However, the patients with advanced disease tended to undergo CRT compared to surgery or RT alone. The rate of T3 or T4 disease was 48 % in the group of CRT (37 % in surgery, 29 % in RT alone), that of neck lymph node metastasis was 77 % in the group of CRT (60 % in surgery, 64 % in RT alone), and that of clinical stage III or IV was 88 % in the

Table 1 Characteristics of patients treated initially with surgery, radiotherapy (RT), or chemoradiotherapy (CRT)

Variable	No. of patients (%)		
	Surgery ($n = 199$)	RT ($n = 117$)	CRT ($n = 170$)
Age (years)			
Median (range)	64 (36–84)	66 (38–96)	60 (37–80)
Gender			
Male/female	167/32 (84/16)	100/17 (85/15)	147/23 (86/14)
Subsite			
Lateral/anterior/ superior/posterior	106/52/34/7 (53/26/17/4)	78/21/11/7 (67/18/9/6)	105/45/8/12 (62/26/5/7)
T classification			
1, 2/3, 4	126/73 (63/37)	83/34 (71/29)	87/82 (51/48)
N classification			
0/1–3	80/119 (40/60)	42/75 (36/64)	39/131 (23/77)
Stage			
I, II/III, IV	64/135 (32/68)	35/82 (30/70)	21/149 (12/88)
Histology			
SCC/others	189/10 (95/5)	115/2 (98/2)	166/4 (98/2)

SCC squamous cell carcinoma

Table 2 T and N classification for patients treated with CRT

T classification	No. of patients by N classification						Total (%)
	0	1	2a	2b	2c	3	
1	4	0	2	5	2	5	18 (11)
2	17	9	9	22	8	4	69 (41)
3	9	7	1	10	8	3	38 (22)
4a	7	3	0	6	15	4	35 (21)
4b	2	0	0	1	2	4	9 (5)
X	0	0	0	0	1	0	1 (1)
Total (%)	39 (23)	19 (11)	12 (7)	44 (26)	36 (21)	20 (12)	170

group of CRT (68 % in surgery, 70 % in RT alone). The T and N classification for patients treated with CRT is shown in Table 2.

Time of assessment and evaluation method for tumors after CRT depend on the institution policies. It is difficult to differentiate between radiographic changes related to the treatment and scar tissue from persisting tumors. Over time, scar tissue remains stable, but persistent tumor tissue will progress, so a patient with radiologic changes that remained stable with no signs or symptoms of disease was considered to be progression free. Recurrence or persistent tumor was judged by apparent radiologic findings or proved by biopsy.

This multi-institutional joint research has been representatively approved by the appropriate ethical committees of National Hospital Organization Tokyo Medical Center, Tokyo, Japan, and written informed consent was obtained from all patients before entry into the study.

Statistical analysis

Associations between patient characteristics were tested using the unpaired Student's t test or the chi-square test, as appropriate. Overall survival curves were constructed using the Kaplan–Meier method and were analyzed using the log-rank test. A two-tailed P value < 0.05 was considered statistically significant. Statistical analyses were performed using XLSTAT 2011 (Addinsoft, NY, USA).

Results

Details of initial treatment

Table 3 shows details of initial treatment in the CRT group. The median irradiation dose was 70 Gy (range, 55–72 Gy). Most patients received conventional radiotherapy and 2 patients were treated with brachytherapy. Although the concomitant chemotherapy consisted of various regimens, about 76 % of patients treated with those regimens received cisplatin and 92 % received platinum-containing anti-cancer drugs. Intraarterial (IA) cisplatin infusion was performed for 16 patients with OPC, including 13 with anterior wall cancer. Forty-one patients (24 %) received induction chemotherapy and 5 patients (3 %) underwent neck dissection (ND) followed by CRT.

Table 3 Details of initial treatment

Irradiation dose	55–72 Gy (median 70 Gy) No. of patients (%)
Concomitant chemotherapy regimen	
Cisplatin, 5-FU	64 (38)
Cisplatin	39 (23)
Nedaplatin	14 (8)
Docetaxel	11 (7)
Cisplatin, 5-FU, docetaxel	9 (5)
Carboplatin, 5-FU	5 (3)
Nedaplatin, 5-FU	4 (2)
Carboplatin	3 (2)
S1	3 (2)
Cisplatin, etoposide	2 (1)
Cisplatin (IA)	16 (9)
Induction chemotherapy	41 (24)
ND followed by CRT	5 (3)

IA intraarterial, *ND* neck dissection

Survival by initial treatment

The median follow-up period was 4.4 years (range, 0.3–5.9 years). The 3-year overall survival rate for patients treated initially with surgery, RT, and CRT was 81.8, 75.4, and 75.8 %, respectively. The 5-year overall survival rate for patients treated initially with surgery, RT, and CRT was 74.8, 66.0, and 67.1 %, respectively (Fig. 1).

Local recurrence and salvage surgery

Of the 170 patients who received CRT, 35 patients (21 %) had local recurrence or residual disease regardless of neck

lymph node and distant metastasis. The median interval of local failure after CRT was 126 days (range, 0–715 days). Of the patients with local failure, 11 patients underwent salvage surgery. The most common surgical approach was open surgery, requiring microvascular free flap reconstruction (10 patients), whereas only 1 patient, who developed recurrence at the lateral wall, underwent transoral surgery. Following salvage surgery, 1 patient received postoperative reirradiation and 4 patients received adjuvant chemotherapy. Twenty-four patients received nonsurgical treatment, including reirradiation in 1, chemotherapy in 9, and best supportive care in 14 patients. Of 134 patients without local failure, 24 patients developed regional recurrences, 12 patients developed distant metastasis, and 7 patients developed both. Seventeen of the 134 patients were successfully salvaged. Additionally, there was 1 treatment-related death. The final outcome for each group is shown in Fig. 2.

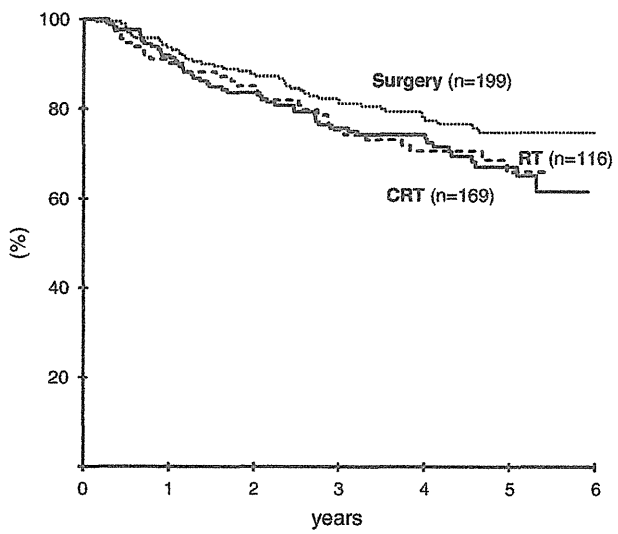

Fig. 1 Overall survival in 169 patients with oropharyngeal cancer treated with surgery, radiotherapy (*RT*), and chemoradiotherapy (*CRT*)

Characteristics of the patients undergoing salvage surgery or nonsurgical treatment for local recurrence or residual disease are summarized in Table 4. Statistically significant differences in patient age and the presence of a simultaneous regional recurrence were observed between the two groups. In addition, the patients who had more aggressive initial disease and developed distant metastasis tended to belong to the nonsurgical treatment group, although the difference was not significant.

Of the 35 patients with local failure, only 11 patients (31 %) underwent further salvage surgery, of whom only 8 (23 %) were successfully salvaged for local failure. Tables 5 and 6 show the successful salvage rates by T classification and subsite, respectively.

There was no perioperative death among the patients who underwent salvage surgery. As to swallowing function, two patients depended on a feeding tube just after CRT, whereas five patients required tube-feeding support after salvage surgery. Furthermore, three patients required the removal of their larynxes (Table 7).

For the patients treated with CRT, the 3- and 5-year overall survival rates for those without local failure were 83.8 % and 75.5 %, respectively (Fig. 3). For the patients with local failure, the 3- and 5-year overall survival rates for those who underwent salvage surgery were 61.8 and 49.1 %, respectively; those for the patients who received nonsurgical treatment were 24.4 and 16.3 %, respectively. The overall survival rate for patients treated with salvage surgery was significantly higher than that for patients

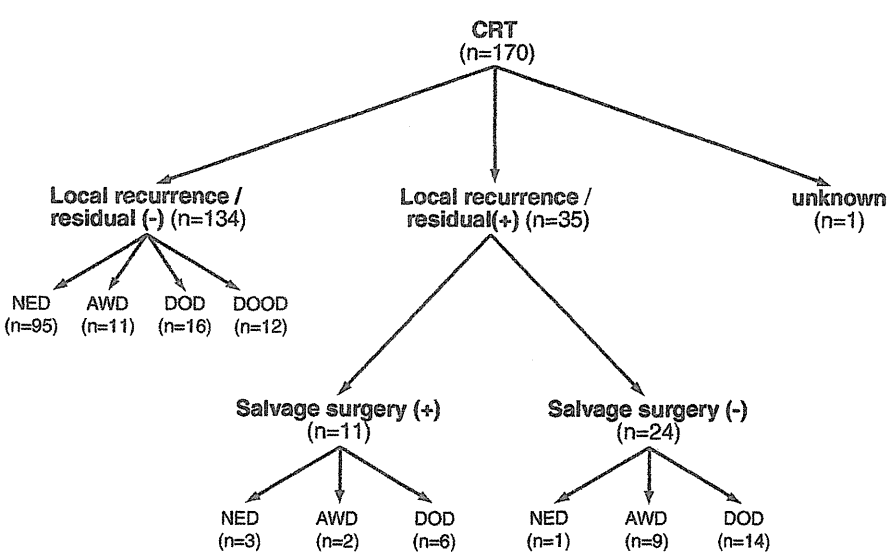

Fig. 2 Flowchart of 170 patients who received chemoradiotherapy for oropharyngeal cancer. *NED* no evidence of disease, *AWD* alive with disease, *DOD* dead of disease, *DOOD* dead of other disease