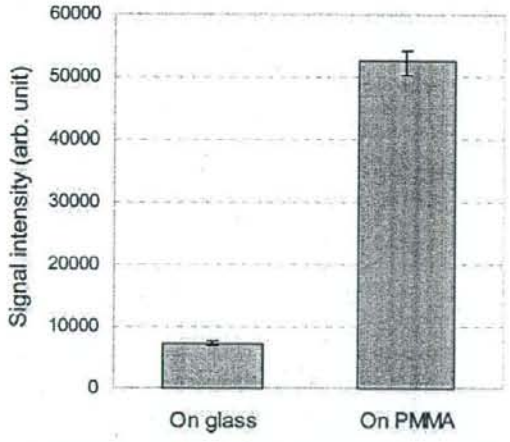

Fig. 8. **Comparison of the hybridization signal intensities of oligonucleotides immobilized on glass and PMMA.** The target DNA was synthesized from the pKF3 template by the random priming method and the oligonucleotide comprised 60 bases.

two possibilities for the signal increase. One possibility is that the PMMA surface might be much rougher than that of glass. Hence, the surface area of a plastic chip might be larger than that of a glass chip. The other possibility is that the influence of steric hindrance might be smaller when a large target DNA hybridizes with an immobilized oligonucleotide on PMMA. When a 20 bases oligonucleotide was immobilized on glass and PMMA plates, and a fluorescent-dye–labeled complementary oligonucleotide (20 bases) was used as the target DNA, interestingly, the signal intensity of PMMA was 0.7–1.2 times as high as that of glass (data not shown). This result implies that a large target DNA can efficiently hybridize with an oligonucleotide on PMMA in comparison with glass. The reason PMMA shows higher hybridization efficiency might be the uneven height of the exposed side chains (carboxyl groups) on PMMA surfaces. This might lead to a lower three-dimensional density of immobilized oligonucleotides on PMMA, resulting in higher hybridization efficiency of a large target DNA.

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REFERENCES

1. Flaim, C.J., Chien, S., and Bhatia, S.N. (2005) An extracellular matrix microarray for probing cellular differentiation. *Nature Methods* **2**, 119–125
2. Maekawa, M., Yamamoto, T., Tanoue, T., Yuasa, Y., Chisaka, O., and Nishida, E. (2005) Requirement of the MAP kinase signaling pathways for mouse preimplantation development. *Development* **132**, 1773–1783
3. Soronen, P., Laiti, M., Torn, S., Harkonen, P., Patrikainen, L., Li, Y., Pulkka, A., Kurkela, R., Herrala, A., Kaija, H., Isomaa, V., and Vihko, P.J. (2004) Sex steroid hormone metabolism and prostate cancer. *Biochem. Mol. Biol.* **92**, 281–286
4. Jamshisidi-Parsian, A., Dong, Y., Zheng, X., Zhou H.S., Zacharias, W., and McMasters, K.M. (2005) Gene expression profiling of E2F-1-induced apoptosis. *Gene* **344**, 67–77
5. Zhou, X.J., Kao, M.C., Huang, H., Wong, A., Nunez-Iglesias, J., Primig, M., Aparicio, O.M., Finch. C.E., Morgen, T.E., and Wong, W.H. (2005) Functional annotation and network reconstruction through cross-platform integration of microarray data. *Nature Biotechnol.* **23**, 238–243
6. Katsuma, S., Nishi, K., Tanigawara, K., Ikawa, H., Shiojima, S., Takagaki, K., Kaminishi, Y., Suzuki, Y., Hirasawa, A., Ohgi, T., Yano, H., Murakami, Y., and Tsujimoto, G. (2001) Molecular monitoring of bleomycin-induced pulmonary fibrosis by cDNA microarray-bassed gene expression profiling. *Biochem. Biophys. Res. Commun.* **288**, 747–751
7. Yamada, M., Katsuma, S., Adachi,T., Hirasawa, A., Shiojima, S., Kadowaki, T., Okuno, Y., Koshimizu, T., Fujii, S., Sekiya, Y., Miyamoto, Y., Tamura, M., Yumura, W., Nihei, H., Kobayashi, M., and Tsujimoto, G. (2005) Inhibition of protein kinase CK2 prevents the progression of glomerulonephritis. *Proc. Natl. Acad. Sci. USA* **102**, 7736–7741
8. Van't Veer, L.J., Dai, H., Van de Vijver, M.J., He, Y.D., Hart, A.A.M., Mao, M., Peterse, H.L., Van Der Kooy, K., Marton, M.J., Witteveen, A.T., Schreiber, G.J., Kerkhoven, R.M., Roberts, C., Linsley, P.S., Berarnrds, R., and Friend, S.H. (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**, 530–536
9. Gerhold, D.L., Jensen, R.V., and Gullans, S.R. (2002) Better therapeutics through microarrays. *Nature Genetics* **32**, 547–551
10. Margalit, O., Somech, R., Amargio, N., and Rechavi, G. (2005) Microarray-based gene expression profiling of hematologic malignancies: basic concepts and clinical applications. *Blood Rev.* **19**, 223–234
11. Assersohn, L., Gangi, L., Zhao, Y., Dowsett, M., Simon, R., Powles, T.J., and Liu, E.T. (2002) The feasibility of using fine needle aspiration from primary breast cancers for cDNA microarray analyses. *Clin. Cancer Res.* **8**, 794–801
12. Kern, W., Kohlmann, A., Schnittger, S., Hiddemann, W., Schoch, C., and Haherlach, T. (2004) Gene expression profiling as a diagnostic tool in acute myeloid leukemia. *Am. J. Pharmacogenomics* **4**, 225–237
13. Ferrando, A.A. and Look, A.T. (2004) DNA microarrays in the diagnosis and management of acute lymphoblastic leukemia. *Int. J. Hematol.* **80**, 395–400
14. Smith, L., Underhill, P., Pritchard, C., Tymowska-Lalanne, Z., Abdul-Hussein, S., Hilton, H., Winchester, L., Williams, D., Freeman, T., Webb, S., and Greenfield, A. (2003) Single primer amplification (SPA) of cDNA for microarray expression analysis. *Nucleic Acids Res.* **31**, e9
15. Spirin, K.S., Ljubimov, A.V., Castellon, R., Wiedoeft, O., Marano, M., Sheppard, D., Kennet, M.C., and Brown, D.J. (1999) Analysis of gene expression in human bullous keratopathy corneas containing limiting amounts of RNA. *Invest. Ophthalmol. Vis. Sci.* **4**, 3108–3115
16. Wang, E., Miller, L.D., Ohnmacht, G.A., Liu, E.T., and Marincola, F.M. (2000) High-fidelity mRNA amplification for gene profiling. *Nature Biotechnol.* **18**, 457–459
17. Pabon, C., Modrusan, Z., Ruvolo, M.V., Coleman, I.M., Daniel, S., Yue, H., and Arnold, L.J., Jr. (2001) Optimized T7 amplification system for microarray analysis. *Biotechniques* **31**, 874–879
18. Andrej, N.S., Nadine, M., and Richard, I. (2003) Amplified RNA degradation in T7-amplification methods results in biased microarray hybridizations. *BMC Genomics* **4**, 44–57
19. Al-Mulla, F., Al-Tamini, R., and Bitar, M.S. (2004) Comparison of two probe preparation methods using long oligonucleotide microarrays. *Biotechniques* **37**, 827–833
20. Peterlinz, K.A., Georgiadis, R.M., Herne, T.M., and Tarlov, M.J. (1997) Observation of hybridization and dehybridization of Thiol-tethered DNA using two-color surface plasmon resonance spectroscopy. *J. Am. Chem. Soc.* **119**, 3401–3402

21. Shippy, R., Sendera, T., Lockner, R., Palaniappan, C., Kaysser-Kranich, T., Watt, G., and Alsobrook, J. (2004) Performance evaluation of commercial short-oligonucleotide microarrays and the impact of noise in making cross-platform correlations. *BMC Genomics* **5**, 61
22. Henry, A.C., Tutt, T.J., Galloway, M., Davidson, Y.Y., McWhorter, C.S., Soper, S.A., and McCarley, R.L. (2000) Surface modification of poly(methyl methacrylate) used in the fabrication of microanalytical devices. *Anal. Chem.* **72**, 5331–533
23. Waddell, E., Wang, Y., Stryjewski, W., McWhorter, S., Henry, A.C., Evans, D., McCarley, R.L., and Soper, S.A. (2000) High-resolution near-infrared imaging of DNA microarrays with time-resolved acquisition of fluorescence lifetimes. *Anal. Chem.* **72**, 5907–5917
24. Ichijima, H., Kobayashi, M., and Ikada, Y.J. (1992) In vitro evaluation of biocompatibility of surface-modified poly(methyl methacrylate) plate with rabbit lens epithelial cells. *Cataract Refract. Surg.* **18**, 395–401
25. Cheng, J.Y., Cheng, W.W., Hsu, K.H., and Young, H.Y. (2004) Direct-write laser micromachining and universal surface modification of PMMA for device development. *Sensors and Actuators B* **99**, 186–196
26. Hermsndon, T. (1996) *Bioconjugate Techniques*, pp. 90–94, Academic Press, San Diego

High throughput comparative genomic hybridization array analysis of multifocal urothelial cancers

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The purpose of this study was to examine genetic alterations occur during synchronous or metachronous multifocal development of urothelial cancers on the whole genome using a comparative genomic hybridization (CGH) array. We used 10 tumor pairs (2 tumors for each patient), in which we had previously defined a clonal relationship by microsatellite analysis. For CGH array analysis, Vysis GenoSensor Array 300 kit was used. An unsupervised hierarchical cluster analysis revealed that the tumors from one patient were clustered together independent of the tumors of all other patients. On the other hand, many genetic divergences among multifocal urothelial cancers were newly found by a CGH array analysis. The concordant genetic alteration patterns of the chromosomal arm in tumor pairs were most frequently observed in 9p, 9q, 8p, 7p, 7q and 11q, while discordant patterns were most frequently found in 15q, 20q, 2q, 10p and 11q. Investigation using a CGH array showed that genetically stable multifocal tumors were less frequent, and that a large percentage of urothelial cancers accumulate genetic alterations during multifocal development by clonal evolution. We might have to consider these genetic accumulations during multifocal development when designing strategies for prevention and detection of recurrent multifocal urothelial cancers. CGH array can be a powerful tool for genetic analysis of multifocal urothelial cancer. (*Cancer Sci* 2006; 97: 746–752)

Urothelial cancers have two clinically important features, multifocality and recurrence. Around 30% of urothelial cancers are found as multiple tumors at the time of diagnosis.[1] Urothelial cancers occur most often (70%) as superficial cancer that can be treated by endoscopic treatment, and 60–80% of patients present one or more recurrences after initial treatment. This multifocal nature of superficial urothelial cancer has been a good material to trace accumulation of genetic alterations in multifocal development of tumor and provided insights into relationship between genetic alterations and carcinogenesis.

Many studies using molecular analysis have suggested a monoclonal origin for multifocal urothelial cancer as appears from X chromosome inactivation studies and genetic and cytogenetic analyzes,[2–5] while other studies have shown an independent origin.[6,7] In our previous LOH analysis of multifocal urothelial cancer using 20 microsatellite markers, we demonstrated that genetic alterations detected were stable in 9 (64%) of the 13 patients with multiple metachronous tumors with a possible identical clonal origin.[5] Because urothelial cancer is characterized by highly complex chromosomal changes affecting numerous chromosomal loci, genome-wide screening may identify genetic divergence among multifocal urothelial cancers that have been missed by the methods applied to date.

Detection of recurrent tumors by non-invasive technique has been an important issue in clinical management of superficial urothelial cancer. Genetic analysis of urine sediments in urothelial cancer patients has been used for diagnosis and follow-up of urothelial cancers, such as FISH, microsatellite analysis and SNP array.[8–11] However, there have been few reports describing the genetic alterations of metachronous multifocal tumors in increased resolution of current technique of DNA analysis.

In this study, to investigate genetic alterations occur during multifocal development of superficial urothelial cancers throughout the genome, we used a Vysis Genosensor CGH array kit with 287 target clone DNAs to analyze 20 tumor specimens from 10 patients in which a clonal relationship had been defined in the previous study using microsatellite analysis.

Materials and Methods

Patients and tumor samples

Topologically distinct urothelial cancers of the bladder, ureter, and renal pelvis in 10 patients were included in this study (2 tumors for each patient). Tumor samples were snap-frozen and stored −80°C until DNA extraction. The present study did not include tumor materials which contaminated more than 20% of

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Abbreviations: BAC, bacterial artificial chromosome; CGH, comparative genomic hybridization; DAPI, 4,6,-diamidino-2-phenylindole; dCTP, 2'-deoxycytidine 5'-triphosphate; FISH, fluorescence *in situ* hybridization; LOH, loss of heterozygosity; PAC, P1-derived artificial chromosome; PCR, Polymerase Chain Reaction; SNP, single nucleotide polymorphisms; WHO, world health organization; SSC, sodium chrolide-sodium citrate.

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Table 1. Characteristics of 20 multifocal urothelial tumors of 10 patients

Patient		Tumor	Interval (month)	Site†	Grade	pT
Group I	9	2		B2	1	a
		8	38	B4	1	a
	26	1		B5	2	1
		2	0	B2	2	1
	2	2		B3	1 > 2	a
		8	3	P	1,2	a
	18	1		B2	1	a
		2	12	B2	1	a
	10	1		P	2	1
		2	7	B2	2	1
	15	1		B2	2	1
		2	37	B2	2	1
Group II	21	1		B1	2	1
		5	8	B2	2 > 1	a
	16	1		U	2	a
		4	7	B2	2	NE‡
	23	1		P	2	3
		2	18	B4	2	a
	30	1		B2	2	a
		2	8	B5	2	a

Patients were divided into two groups by the previous microsatellite analysis.[5] Group I patients had concordant LOH patterns alone. Group II patients had both discordant and concordant LOH patterns. †U, ureteral tumor; P, renal pelvic tumor; B, bladder tumor [the locations of the bladder tumor were as follows: B1, trigone; B2, posterior wall; B3, right wall; B4, left wall, B5, dome]; ‡NE, not examined.

normal interstitial cells in hematoxylin and eosin (HE) staining. Normal reference DNA was obtained from the peripheral blood of each patient. Tumor and reference DNAs were prepared by proteinase K digestion and phenol/chloroform extraction. The tumor stage and grade were classified according to the tumor-node-metastasis system and WHO criteria, respectively,[12,13] by pathologists who were unaware of the aims of this study. All tumors were urothelial carcinomas, and brief clinical and pathological data are presented in Table 1.

These specimens were analyzed in our previous LOH study and a clonal relationship had already been defined.[5] Using microsatellite markers, we examined genetic alterations at 20 loci on eight chromosome arms (2q, 4p, 4q, 8p, 9p, 9q, 11p, and 17p). The markers we used D2S206 and D2S336 on chromosome 2q; D4S404 and D4S1546 on 4p; D4S426 and D4S171 on 4q; D8S261 and D8S520 on 8p, D9S171, D9S126, D9S1749 and D9S736 on 9p; D9S66, D9S1848, D9S1793 and GSN on 9q, D11S907 and D11S922 on 11p; and D17S796 and D17S1176 on 17p. Completely identical LOH patterns were detected in six patients (No. 9, 26, 2, 18, 10, and 15), while discordant patterns together with concordant patterns were observed in the remaining four patients (No. 21, 16, 23, and 30). In patient 30, no clonal relationships could be defined in the previous microsatellite study. All tumor pairs in other patients were considered to be monoclonal based on the results of microsatellite analysis. Written informed consent was obtained from each patient before surgery, according to the ethical guidelines of our university.

Array-based CGH (Array CGH)

Array-based CGH was performed using a GenoSensor Array 300 kit (Vysis, Downers Grove, IL), which contained 287 target clone DNAs (P1, PAC or BAC clones) representing regions that are important in cytogenetics and oncology. Each DNA clone was arrayed on the slide in three spots. Tumor and reference DNAs (100 ng) were labeled with Cy3-dCTP or Cy5-dCTP (Perkin Elmer, Wellesley, MA), respectively, by random priming reaction. Labeled DNAs were mixed with a microarray hybridization buffer that contained a high concentration of Cot-1 DNA (Vysis). The probe mixture was denatured at 80°C for 10 min and incubated at 37°C for 1 h before being transferred to the microarray. After an overnight hybridization at 37°C, the microarrays were washed three times with 50% formamide/2XSSC at 40°C for 10 min each. Thereafter, the microarrays were subjected to four washes with 1XSSC at room temperature for 5 min each. The microarrays were counterstained with the DAPI IV mounting solution (Vysis).

Images of fluorescence signals were captured and analyzed with the GenoSensor Reader System and GenoSensor Reader software (Vysis) according to the manufacturer's instructions. The mean normalized test/reference (T/R) ratio was calculated from each set of T/R ratio of three spots. Gain and loss of DNA copy number were judged by T/R ratios >1.2 and <0.8, respectively. Two different sets of labeling and hybridization tests were performed on two tumors from one patient (Patient No. 15) to validate the reproducibility of the GenoSensor Array 300 kit. Spots on which the difference in the ratios was greater 0.2 were 3 (1%) of 286 evaluated spots in tumor 1 and 1 of 286 (0.3%) in tumor 2.

Data analysis of array CGH Results

We performed an unsupervised hierarchical cluster analysis on log base 2 transformed data obtained for 287 target clones to weight how the prevalence of genomic changes is similar or different. The Ward linkage and cosine coefficient metric were used. The results were visualized in the software program R.

In order to assess global chromosomal aberrations in multifocal urothelial cancers, our subsequent analysis focused to 'the pattern of chromosomal aberrations between tumor pairs in each patient'. On one clone of the array, an identical genetic alteration of two tumors in one patient was defined when a loss or gain was detected and the result was identical. Different genetic alterations were defined as follows: 1. Loss or gain was detected; 2. The result was different in the two tumors; 3. The difference in the ratio of the two tumors was greater than 0.2. Then, the pattern of chromosomal aberrations compared between tumor pairs was evaluated on each chromosomal arm. The pattern of chromosomal aberrations was defined as concordant or discordant if identical or different gains or losses were detected in 2 or more clones on the chromosomal arm. If both discordant and concordant patterns coexisted, we defined the chromosomal arm as discordant. A chromosomal arm which had only one clone with a gain or loss was neglected.

Quantitative real-time PCR

Quantitative real-time PCR analyzes were performed on the same set of urothelial cancers to detect copy number changes for four genes, *BIN1* (2q14), *CDKN2A (p16)* (9p21), *CCND1*

(11q13), and *MAP2K5* (15q23), which were included in the CGH array target DNAs. The relative gene copy number was evaluated by the comparative C_T Method as described by Ginzinger *et al.*[14] on the ABI Prism 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The *PFKL* gene served as an internal control, located on 21q22.3, which is rarely altered numerically in urothelial cancers.[15] Twenty normal peripheral blood samples were examined to determine the reference range.

Results

Overall results

Of 287 clones examined in 20 tumors (5740 in total) gain and loss were observed in 437 (7.6%) and 512 (8.9%), respectively. On average, one tumor had gains in 21.9 (4–42) clones and losses in 25.6 (1–53) clones. The identical alterations between tumor pairs were most frequently observed in CDKN2A (p16) (9p21), MTAP (9p21.3), and AFM137XA11 (9p11.2) (6 patients), CTSB (8p22), D8S596 (8p Tel.), D8S504 (8p Tel.), AF170276 (9p Tel.) and D9S913 (9p Tel.) (5 patients). On the other hand, the different alterations between tumor pairs were frequently identified in SNRPN (15q12), UBE3A, D15S10 (15q11-q13) and STK6 (20q13.2-q13.3) (4 patients), MYBL2 (20q13.1) and PTPN1 (20q13.1-q13.2) (3 patients). These genes/loci may be susceptible to genetic alterations during multifocal development of urothelial cancers.

Hierarchical clustering analysis

Fig. 1 shows the cluster dendrogram of 20 tumors of 10 patients based on the similarity of genetic alterations detected by CGH array analysis. Tumor pairs from one patient were clustered together. One tumor was more related to the second tumor from the same patient than tumors from all other patients, suggesting that these tumor pairs were clonally related.

The pattern of chromosomal aberrations on the CGH array

On average, concordant patterns of chromosomal aberrations were detected in 5.8 chromosomal arms (range 1–13) in one patient; while discordant patterns were detected in 4.2 chromosomal arms (range 0–12). Fig. 2 summarizes the patterns of chromosomal aberrations.

In 8 of 10 patients, both concordant and discordant patterns were observed. In patient No. 9, concordant patterns were found in chromosomal arms 8q (gain), 9p (loss), 11p (partial loss), 12q (partial gain) and 22q (partial loss), while discordant patterns were found in 1p, 2p, 8p, 9q, 18p and 18q (Fig. 3a,b). In 2 of

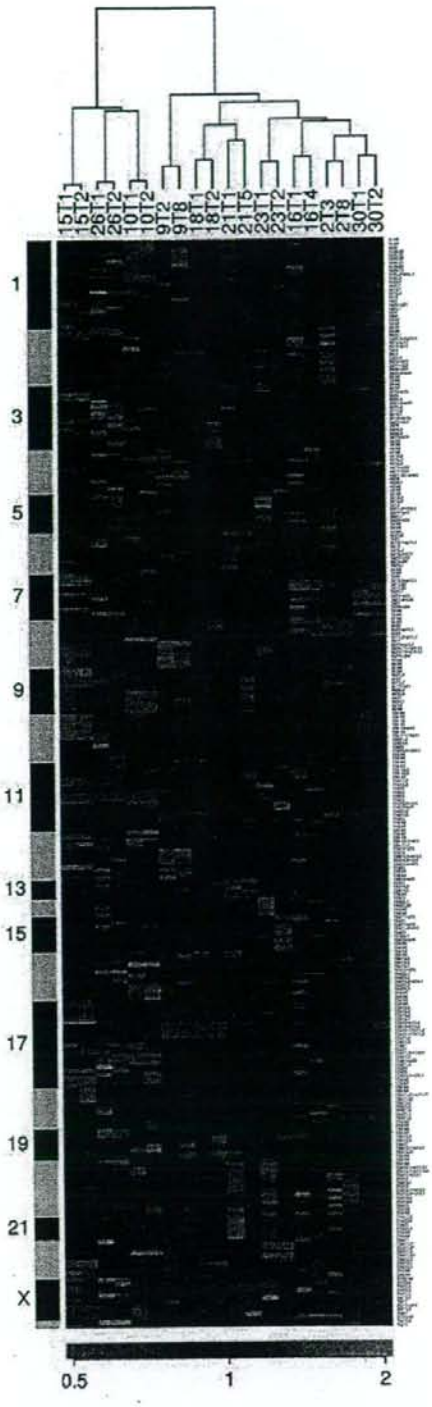

Fig. 1. Hierarchical clustering of data from 287 clones on the GenoSensor array 300 in 20 tumors. The data are presented in a matrix format. A column corresponds to a single tumor, and each row corresponds to a single clone ordered by mapping position. Gain or loss of a clone is represented by the color of the corresponding cell in the matrix. Green indicates gain; black, no change; and red, loss. Color saturation is proportional to the magnitude of the difference. The sidebars to the left of the matrix format represent chromosome cluster, ordered from chromosome 1 to Y. The horizontal dendrogram shows the association between the tumors and the length of the branches of the dendrogram reflects the similarity between tumors. Note that one tumor is more related to the second tumor from the same patient than tumors from all other patients.

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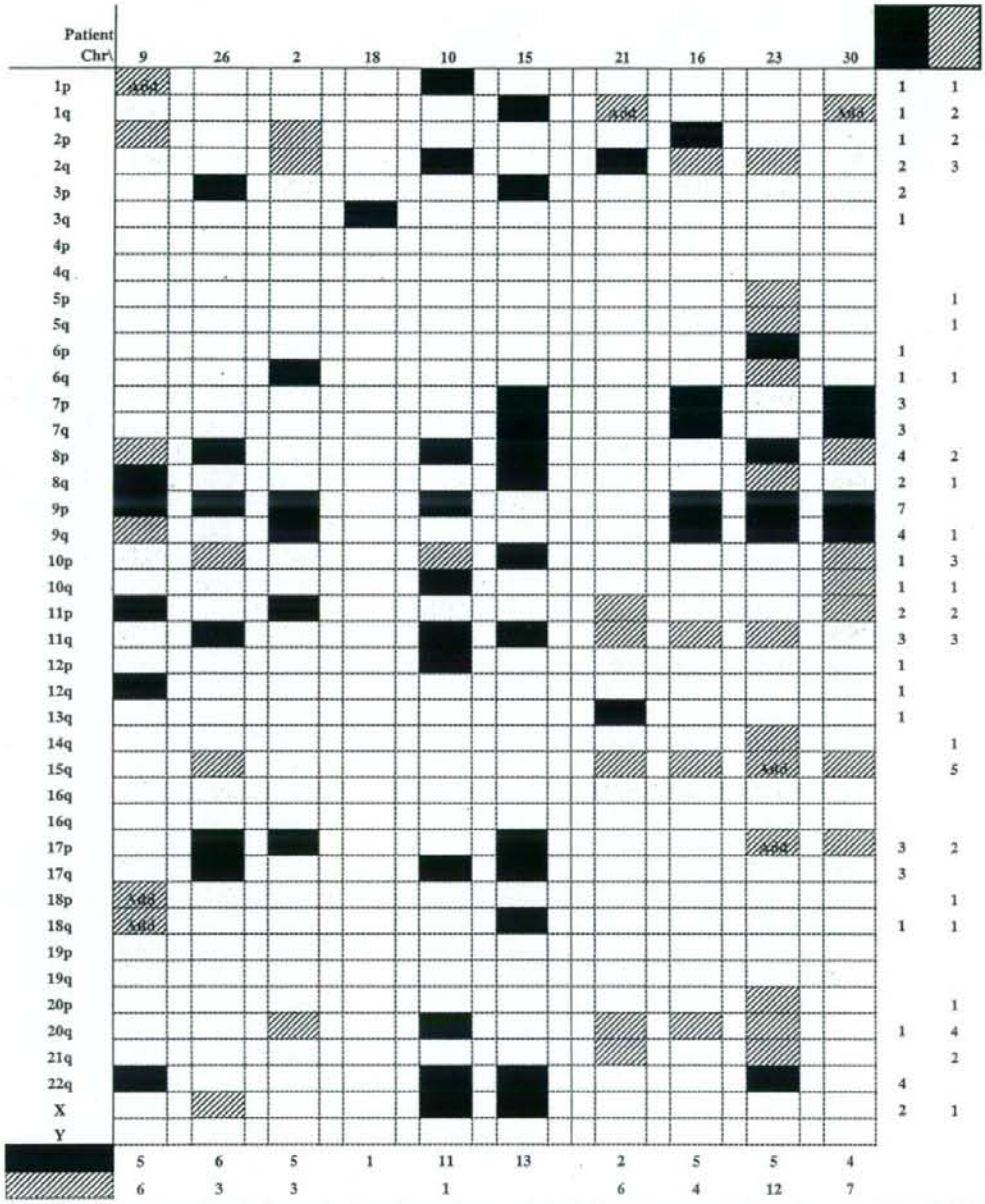

Dark boxes represent identical genetic alteration patterns and shaded boxes represent discordant ones. The shaded boxes with 'Add' represent simple addition of genetic alteration, which means latter tumor had additional genetic alteration to that of former tumor. The shaded boxes without 'Add' represent genetic diversity, which means that former tumor has discordant genetic alterations which latter tumor does not.

Fig. 2. Summary of genetic alteration patterns of chromosomal arms.

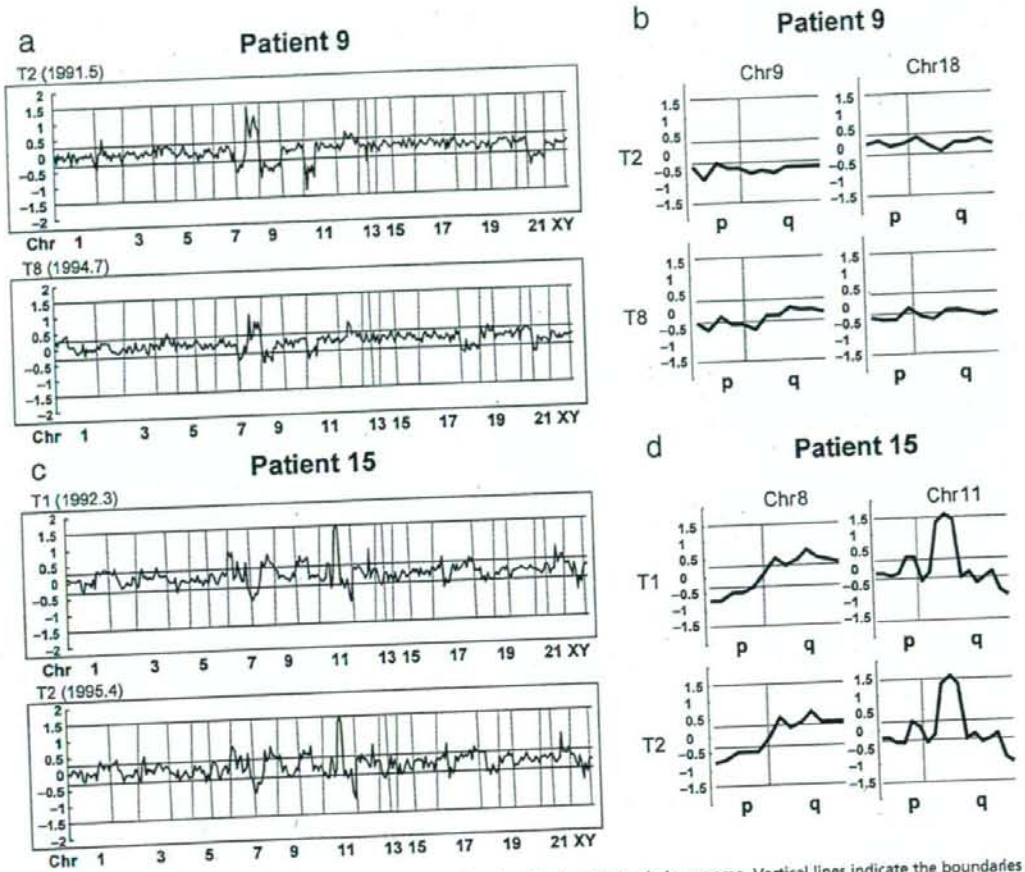

Fig. 3. (a,c) Graphical representation of CGH array analysis of patient No. 9 and 15, whole genome. Vertical lines indicate the boundaries of chromosomes. Both concordant and discordant patterns of chromosomal aberrations were observed in patient No. 9, while the patterns of chromosomal aberrations were completely identical in patient No. 15. (b,d) Representative chromosome arms. In patient No. 9, a concordant pattern was found in 9p (loss), while discordant patterns were found in 9q, 18p and 18q. In patient No. 15, loss of 8q, gain of 8q, and a partial loss and gain of 11q were observed in both tumors. Vertical lines indicate boundaries of chromosome arms. Average $\log_2$ ratios were plotted for all clones on chromosome position. Thresholds for gain or loss are shown within $\log_2$ ratios of 0.2 and −0.2, respectively. T; tumor, Chr; chromosome.

10 patients, the patterns of chromosomal aberrations were identical. The results of patient No. 15 are shown in Fig. 3c,d. Of note, these two tumors developed with a 3 years interval and the patient had 4 tumor recurrences between the two tumors. These data suggest that in part of superficial bladder cancers, the whole genome is genetically stable during development of metachronous cancers.

Concordant patterns of chromosomal aberrations between tumor pairs were most frequently observed in 9p (7 patients), 8p (5 patients), 9q, 22q (4 patients), 7p, 7q, 11q, 17p and 17q (3 patients). Among concordant patterns, aberrations on 9p, 9q, 8p and 17p were losses of the chromosomal arm, and on 7p, 7q and 17q were gains. Discordant patterns were most frequently found in 15q (5 patients), 20q (4 patients), 2q, 10p, and 11q (3 patients). Among the total 42 discordant patterns, simple addition of genetic alterations to latter tumor was found in only 7, which suggests that the accumulation of genetic alterations does not parallel the chronological order of tumor occurrence.

Comparison of CGH array and PCR-based microsatellite analysis

Exactly same loci to 20 microsatellite markers used in the previous study were not included in the CGH array target DNAs. Of 20 microsatellite markers used in the previous study, 6 markers for which the distance to the nearest spot on the CGH array was within 1 Mb length were selected and the results of the 2 methods were compared. Of 104 informative microsatellite analyzes using 6 markers, D8S520, D9S126, D9S1749, D9S1848, D9S66 and D11S922, 61 showed

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LOH. Overall, of 104 informative microsatellite analyzes, 59 (56.7%) matched the results of the CGH array. Among the 61 LOH, 36 (59%) were detected as gains or losses by the CGH array. The results of the CGH array analysis and microsatellite analysis were not closely matched, probably it is because the loci of most microsatellite markers were not exactly same as those of the spot on the CGH array. In addition, the difference in the result may be caused by the difference of the assay platform; microsatellite involves amplification step while the CGH array does not.

Validation of CGH array analysis by quantitative Real-time PCR

Quantitative real-time PCR was performed on all tumor samples using four genes. We calculated the Pearson correlation coefficient (r) between the results of the CGH analysis and quantitative real-time PCR analysis. We found a strong correlation BINI ($r = 0.75$), CDKN2A ($r = 0.70$), CCND1 ($r = 0.96$), and MAP2K5 ($r = 0.71$) between them. Both methods matched in 87.5% (70 of 80) when the copy numbers of each gene/locus were decided by using their respective cutoff criteria. The discordant results between them were found only in the case which the genes/loci had the subtle copy number gain/loss. The discordance of the results using both methods may be inevitable to some degree in cases of subtle copy number gain/loss. In addition, the difference in the result is probably caused by the difference of assay methodology, or the low resolution of the CGH array. Among 7 tumor pairs showing a discordant pattern of chromosomal aberrations in the CGH array, we also obtained matched results in 4 pairs in quantitative real-time PCR analysis.

Discussion

In the present study, using commercially available CGH array kits, we investigated global chromosomal aberrations in multifocal urothelial cancers. The multiloci survey by the CGH array of the whole genome enabled us to detect genetic alterations on the loci that we did not detect in the previous study. In the previous report, 64% of tumor pairs showed completely identical LOH patterns and we concluded that a high percentage of superficial urothelial cancers are genetically stable. However, among tumor pairs of 6 patients with completely concordant LOH patterns on microsatellite analysis, discordant patterns of chromosomal aberrations between tumor pairs were newly found in 4 patients using the CGH array. The current results showed that such genetically stable multifocal cancers are rather infrequent and that a large number of superficial urothelial cancers accumulate genetic alterations during multifocal development. On the other hand, in patients 18 and 15, only concordant chromosomal aberration patterns were observed even by genome-wide CGH array analysis, which indicates that some multifocal urothelial cancers are genetically stable during multifocal development.

Since the introduction of molecular analysis into clonal analysis, the majority of multifocal urothelial cancers have been suggested to be monoclonal or have a common clonal origin.[4,16,17] In multistep carcinogenesis model, an original transformed cell grows out and sheds cells into the lumen of the bladder. Some of these cells will have required additional genetic alterations. Interestingly, among the 42 discordant patterns, simple addition of genetic alterations to latter tumor was found in only 7. In these tumor pairs, the latter tumor is not directly derived from the former tumor. van Tilborg *et al.* performed the LOH analysis of metachronous multifocal bladder cancer using 48 microsatellite markers,[18] and reported similar findings to our data. To develop a strategy to prevent and detect recurrent multifocal urothelial cancers, it should be remembered that the chronology of tumor appearance does not always run in parallel with the accumulation of genetic alterations during the process of intraepithelial spread or intraluminal seeding. The commercially available CGH array used in this study is a relatively low-resolution scan, and much higher resolution scans of the bladder cancer genome have been reported.[19] By using a CGH array with high resolution and high throughput for analysis of a larger number of samples, we might be able to draw more detailed genetic trees and pedigrees of multifocal urothelial cancers and trace genetic alterations accumulated during multifocal development.

The development and progression of urothelial cancer is the result of a series of genome instability occurring over the lifetime of a tumor. The number of chromosomal alterations was reported to be higher in high grade, advanced stage tumors.[20,21] Discordant chromosomal aberrations between multifocal tumors might reflect genome instability as well. It would be useful to know whether the degree of discordant chromosomal aberrations has an association with recurrence, progression, tumor grade and stage. Considering that most tumors we analyzed were low grade superficial type, it is likely that many of the chromosomal aberrations we detected were not related to differences in grade or stage. With accumulation of data on the chromosomal aberrations of multifocal tumors, it might become possible to identify target chromosomal regions and target genes which play an important role in recurrence, progression and invasion of urothelial cancers.

A number of studies have reported the detection of tumor cells in urine. Halling *et al.* reported high sensitivity (81%) of multicolor FISH analysis consisting of probes for chromosome 3, 7 and 17, and the 9p21 band in detecting urothelial cancer.[8] Several investigators showed that 85% or greater sensitivity was achieved using 13–20 highly informative microsatellite markers to detect genetic alterations in urinary exfoliated cells.[9,10] Furthermore, Hoque *et al.* used a SNP array to detect urothelial cancer and reported a 100% detection rate.[11] Our data suggest that we should be careful when we use these genetic methods for monitoring urothelial cancer recurrence, because the genetic alterations we detect in primary tumors may not be present in recurrent tumors. In the current study, concordant patterns of chromosomal aberrations were frequently detected on 9p, 8p, 9q, 22q, 7p, 7q, 11q, 17p and 17q. The loci on these chromosomal arms may be good candidates for probes for efficient monitoring of urothelial cancer recurrence. Furthermore, our data is consistent with the view that genetic alterations on chromosome 9 occur early in the development of multifocal urothelial cancer and remain stable during clonal progression. Accumulation of data regarding chromosomal aberrations among multifocal urothelial cancers would be necessary to improve efficiency and reduce the cost of the genetic analysis to detect tumor cells in urinary exfoliated cells.

References

1 Kiemeney LA, Witjes JA, Heijbroek RP, Verbeek AL, Debruyne FM. Predictability of recurrent and progressive disease in individual patients with primary superficial bladder cancer. *J Urol* 1993; **150**: 60–4.

2 Sidransky D, Frost P, Von Eschenbach A, Oyasu R, Preisinger AC, Vogelstein B. Clonal origin bladder cancer. *N Engl J Med* 1992; **326**: 737–40.

3 Habuchi T, Takahashi R, Yamada H, Kakehi Y, Sugiyama T, Yoshida O. Metachronous multifocal development of urothelial cancers by intraluminal seeding. *Lancet* 1993; **342**: 1087–8.

4 Dalbagni G, Ren ZP, Herr H, Cordon-Cardo C, Reuter V. Genetic alterations in tp53 in recurrent urothelial cancer: a longitudinal study. *Clin Cancer Res* 2001; **7**: 2797–801.

5 Takahashi T, Habuchi T, Kakehi Y *et al.* Clonal and chronological genetic analysis of multifocal cancers of the bladder and upper urinary tract. *Cancer Res* 1998; **58**: 5835–41.

6 Miyake H, Hara I, Kamidono S, Eto H. Multifocal transitional cell carcinoma of the bladder and upper urinary tract: molecular screening of clonal origin by characterizing CD44 alternative splicing patterns. *J Urol* 2004; **172**: 1127–9.

7 Jones TD, Wang M, Eble JN *et al.* Molecular evidence supporting field effect in urothelial carcinogenesis. *Clin Cancer Res* 2005; **11**: 6512–19.

8 Halling KC, King W, Sokolova IA *et al.* A comparison of BTA stat, hemoglobin dipstick, telomerase and Vysis UroVysion assays for the detection of urothelial carcinoma in urine. *J Urol* 2002; **167**: 2001–6.

9 Steiner G, Schoenberg MP, Linn JF, Mao L, Sidransky D. Detection of bladder cancer recurrence by microsatellite analysis of urine. *Nat Med* 1997; **3**: 621–4.

10 Schneider A, Borgnat S, Lang H *et al.* Evaluation of microsatellite analysis in urine sediment for diagnosis of bladder cancer. *Cancer Res* 2000; **60**: 4617–22.

11 Hoque MO, Lee J, Begum S *et al.* High-throughput molecular analysis of urine sediment for the detection of bladder cancer by high-density single-nucleotide polymorphism array. *Cancer Res* 2003; **63**: 5723–6.

12 Sobin LH, Wittekind Ch. *UICC TNM Classification of Malignant Tumours*, 5th edn. New York: John Wiley & Sons, Inc. 1997.

13 Fleming ID, Cooper JS. *Henson DE et al. American Joint Committee on Cancer on Cancer Staging Manual*, 5th edn. Philadelphia: JB Lippincott 1997.

14 Ginzinger DG, Godfrey TE, Nigro J *et al.* Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis. *Cancer Res* 2000; **60**: 5405–9.

15 Aveyard JS, Knowles MA. Measurement of relative copy number of CDKN2A/ARF and CDKN2B in bladder cancer by real-time quantitative PCR and multiplex ligation-dependent probe amplification. *J Mol Diagn* 2004; **6**: 356–65.

16 Hafner C, Knuechel R, Stoehr R, Hartmann A. Clonality of multifocal urothelial carcinomas: 10 years of molecular genetic studies. *Int J Cancer* 2002; **101**: 1–6.

17 Simon R, Eltze E, Schafer KL *et al.* Cytogenetic analysis of multifocal bladder cancer supports a monoclonal origin and intraepithelial spread of tumor cells. *Cancer Res* 2001; **61**: 355–62.

18 van Tilborg AA, de Vries A, de Bont M, Groenfeld LE, van der Kwast TH, Zwarthoff EC. Molecular evolution of multiple recurrent cancers of the bladder. *Hum Mol Genet* 2000; **9**: 2973–80.

19 Veltman JA, Fridlyand J, Pejavar S *et al.* Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors. *Cancer Res* 2003; **63**: 2872–80.

20 Primdahl H, Wikman FP, von der Maase H, Zhou XG, Wolf H, Orntoft TF. Allelic imbalances in human bladder cancer: genome-wide detection with high-density single-nucleotide polymorphism arrays. *J Natl Cancer Inst* 2002; **94**: 216–23.

21 Hoque MO, Lee CC, Cairns P, Schoenberg M, Sidransky D. Genome-wide genetic characterization of bladder cancer. a comparison of high-density single-nucleotide polymorphism arrays and PCR-based microsatellite analysis. *Cancer Res* 2003; **63**: 2216–22.

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Review

Clinical Aspects of Multimodality Therapy for Resectable Locoregional Esophageal Cancer

Masayuki Shinoda, MD, Shunzo Hatooka, MD, Shoichi Mori, MD, and Tetsuya Mitsudomi, MD

Radical resection has been considered the only possible way to save the lives of patients with esophageal cancer. Therefore, tremendous efforts have been made in order to improve the surgical results for resectable locoregional esophageal cancer. Various surgical approaches have been developed. Combination therapies such as neoadjuvant, adjuvant chemotherapy, and neoadjuvant chemoradiation have been extensively investigated in numerous randomized clinical trials. Due to insufficient surgical results and high postoperative mortality rates, definitive chemoradiation has been studied as alternative treatment in selected patients, based on the concept that combined-modality therapy allows simultaneous treatment of locoregional disease and systemic micrometastases. Chemoradiation has shown survival rates equivalent to surgery in some non-randomized comparative studies. Presently, concerns appear to be shifting to the question of whether definitive chemoradiation could be an alternative to surgery in the primary treatment of resectable locoregional esophageal cancer. Recently, 2 randomized trials, comparing definitive chemoradiation with chemoradiation and surgery were published. These trials seem to show at first glance that definitive chemoradiation can achieve results comparable to surgery with neoadjuvant chemoradiation. More sophisticated trials should be conducted as treatment modalities used in these trials are far from routine. (Ann Thorac Cardiovasc Surg 2006; 12: 234–41)

Key words: esophageal cancer, neoadjuvant therapy, definitive chemoradiation, salvage esophagectomy

Introduction

Esophageal cancer is an increasingly common malignancy. This neoplasm is devastating because of its aggressive clinical course and high mortality rate. The long-term survival rates of under 10% are disappointing in part, due to the high incidence of advanced and/or metastatic disease at the time of diagnosis. Over 2 decades, several treatment modalities have been developed to improve the survival of patients with esophageal cancer. Among these, surgical resection remains the preferred choice. It currently provides the best palliation for dysphagia and local control and the best chance for cure when compared with other therapeutic options. However, as definitive chemoradiation has been gradually improving, the boundaries of treatment strategies have become blurred in the patients with resectable locoregional esophageal cancer.

This overview will examine the available data on current treatment modalities and discuss the future direction clinical research and treatment.

Preoperative Chemotherapy

Since surgery alone cannot achieve a good survival rate, trials evaluating the efficacy of preoperative chemotherapy followed by surgery for resectable esophageal cancer have been conducted since the 1980s. The rationale of preoperative chemotherapy includes down-staging of the tu-

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Table 1. Phase III trials to investigate the impact of neoadjuvant chemotherapy in patients with resectable esophageal carcinoma

Author/ ref. no.	Year	Protocol	Histology	No. of patients	R0 (%)	Mortality (%)	Median survival (mos)	2-year survival (%)	*P*
Kelsen et al.[1]	1998	Surgery	SCC/AC	227	59	6	16.1	35	ns
		CDDP/5-FU		213	62	7	14.9	37	
MRCOCWP[2]	2002	Surgery	SCC/AC	400	54	10	13.3	34	0.004
		CDDP/5-FU		402	60	10	16.8	43	

R0, microscopically complete resection; mos, months; CDDP, cisplatin; 5-FU, 5-fluorouracil; SCC, squamous cell carcinoma; AC, adenocarcinoma; MRCOCWP, Medical Research Council Oesophageal Cancer Working Party; ns, not significant.

mor and elimination of micrometastatic disease.

Two recent randomized trials evaluating the utility of preoperative chemotherapy for esophageal cancer are summarized in Table 1. They are reported from the U.S. Intergroup trial 0113[1] and the Medical Research Council Oesophageal Cancer Working Party (MRCOCWP).[2]

In the U.S. Intergroup trial, 440 patients with operable esophageal cancer, histologically squamous cell carcinoma (SCC) or adenocarcinoma (AC), stage I to III, were eligible and follow-up data was adequate. A total of 213 patients were assigned to receive chemotherapy consisting of 3 cycles of 5-fluorouracil (5-FU: 1,000 mg/m^2/24 hours for 5 days) and cisplatin (CDDP: 100 mg/m^2/bolus day 1) followed by surgery. Two additional cycles of the same chemotherapy were administered to these patients after curative resection. The remaining 227 patients were assigned to undergo immediate surgery. There were no significant differences between the 2 groups in the median survival of 14.9 months for the patients who received preoperative chemotherapy and 16.1 months for those who underwent immediate surgery (p=0.53). Among the patients whose resection was curative, median survival in each group was over 2 years. At 2 years, the survival rate was 35% and 37% respectively and at 3 years, the survival rate was 23% and 26% respectively. Furthermore, no statistically significant differences were observed between the 2 groups in terms of curative resection rate (59% vs. 62%) and treatment mortality rate (6% vs. 7%). The pattern of failure was almost identical for the 2 groups. The histology of the tumor did not have an effect on response to treatment. Complete responses as assessed by pathological examination were achieved in 2.5% of patients who had received at least 1 cycle of chemotherapy. Preoperative chemotherapy with a combination of 5-FU and CDDP did not demonstrate a survival benefit in the patients with SCC and AC of the esophagus. This trial concluded that surgery alone remains the standard treatment for patients with localized esophageal cancer.

In contrast, in the MRCOCWP trial, 802 previously untreated patients with resectable esophageal cancer were accrued regardless of histologic cell type, 31% with SCC and 69% with AC or undifferentiated carcinoma. Eligible patients were randomly allocated to receive chemotherapy consisting of 2 cycles of 5-FU (1,000 mg/m^2/24 hours for 4 days) and CDDP (80 mg/m^2/bolus day 1) with an interval of 3 weeks between the first day of each cycle followed by surgery 3 weeks apart or to undergo immediate surgery. In this trial, the curative resection rate was 59% in the preoperative chemotherapy group versus 62% in the resection alone group. The treatment effect was similar for the patients with SCC and those with AC. There were no differences in the postoperative mortality rate of 10% in both groups. The median survival of 16.8 months in the preoperative chemotherapy group was better than 13.3 months in the resection group, and the survival rate at 3 years was 43% and 34% respectively. The MRCOCWP trial suggested that the preoperative chemotherapy regimen used in the study should be considered for patients with resectable cancer of the oesophagus.

It is difficult to explain the difference in survival benefits between these 2 trials. This conflicting difference might be dependent upon the resectability rate of only 80% in the Intergroup and 92% in the MRCOCWP. Although the MRCOCWP trial is not included in this analysis, recently published meta-analysis of 11 randomized controlled trials that compared neoadjuvant chemotherapy and surgery with surgery alone for esophageal cancer did not demonstrate a survival benefit for the treated patients.[3] In the papers on randomized trials including esophagectomy, the type of procedures performed and there distribution into groups should be clearly mentioned. The efficacy of neoadjuvant chemotherapy remains controversial but, in any case, seems to be limited.

Review

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Table 2. Phase III trials to investigate the impact of adjuvant chemotherapy in patients with resectable esophageal carcinoma

Author/ ref. no.	Year	Protocol	Histology	No. of patients	5-year survival (%)	*P*
Ando et al.[4]	1997	Surgery	SCC	100	45.1	ns
		CDDP/VDS		105	48.3	
Ando et al.[5]	2003	Surgery	SCC	122	52	ns
		CDDP/5-FU		120	61	

CDDP, cisplatin; VDS, vindesine; 5-FU, 5-fluorouracil; SCC, squamous cell carcinoma; ns, not significant.

Postoperative Chemotherapy

The use of chemotherapy in the adjuvant setting could prevent undesired delays in definitive surgery. However, due to the high postoperative mortality and morbidity that accompany complicated surgical procedures, there has been little impetus to promote clinical trials with adjuvant chemotherapy in esophageal cancer, postoperative chemotherapy has not been widely studied in randomized trials.

Postoperative chemotherapy has not been widely studied in randomized trials. However, 2 Japanese randomized trials, involving patients with SCC compared it with surgery alone without preoperative therapy[4,5](Table 2). In the first trial, chemotherapy consisted of 2 cycles of vindesine (VDS: 3 mg/m^2/bolus day 1) and CDDP (70 mg/m^2/2 hours day 1). In the second trial, chemotherapy consisted of 5-FU (800 mg/m^2/24 hours for 5 days) and CDDP (80 mg/m^2/2 hours day 1) with an interval of 3 weeks between the first day of each cycle. Chemotherapy was well tolerated in the postoperative setting. Though the first trial did not demonstrate the benefit of adjuvant chemotherapy, the second trial showed a trend toward improved 5-year disease-free survival.

In the second trial with 5-FU and cisplatin, both groups showed no remarkable difference in overall survival. The 5-year survival rate of the surgery alone group was 52% while that for the postoperative chemotherapy group was 61% (p=0.13). In contrast, the 5-year disease-free survival rate of 55% in patients in the surgery plus chemotherapy group was significantly better than the 45% achieved by patients in the surgery alone group (p=0.037). Furthermore in subgroup analyses, the subgroup with lymph node metastasis showed risk reduction of 52% in the adjuvant group versus 38% in the surgery alone group which was also statistically significant.

The efficacy of postoperative chemotherapy is currently unclear because of the small number of trials. However, a potential benefit might exist for certain patient subgroups. As long as postoperative mortality and morbidity rates are not decreased, postoperative chemotherapy cannot be easily adapted to the context of clinical trials.

Preoperative Chemoradiation

Another common treatment strategy is preoperative chemoradiation. Neoadjuvant chemoradiation followed by surgery has been extensively studied over the past decade as a result of the pattern of both local and distant failure associated with surgery alone. The rationale of preoperative chemoradiation includes down-staging of the tumor and eradication of micrometastases. Moreover, most chemotherapeutic agents that have an effect on esophageal cancer simultaneously act as radiosensitizers. Although 8 randomized controlled trials have been reported that evaluate the benefit of preoperative chemoradiotherapy in patients with esophageal cancer,[6-13] a sufficient number of patients to allow statistically meaningful results have been accrued in only 2 of these trials[10,13](Table 3). Bosset et al.[10] reported the results of a randomized trial of preoperative combined modality therapy from the European Organization for Research and Treatment of Cancer (EORTC). A total of 282 patients with clinically resectable esophageal cancer, histologically SCC and stage I to II, were randomized to receive either preoperative combined modality therapy or surgery alone. The preoperative radiotherapy was unconventional in design and consisted of 5 daily fractions of 3.7 Gy each followed by a 2-week rest and another 3.7 Gy for 5 days. Also unusual was the chemotherapy which consisted of a single use of CDDP given at a dose of 80 mg/m^2 on day 0–2 before starting radiotherapy. Though the 3-year disease-free survival rate of 40% in patients who received preoperative combined modality therapy was a signifi-

Table 3. Phase III trials to investigate the impact of neoadjuvant chemoradiation in patients with resectable esophageal carcinoma

Author/ ref. no.	Year	Protocol	Histology	No. of patients	R0 (%)	Mortality (%)	Median survival (mos)	3-year survival (%)	*P*
Nygaard et al.[6]	1992	Surgery	SCC	41	37	13	7.5	9	ns
		CDDP/BLM+35 Gy		47	55	24	7.5	17	
Le Prise et al.[7]	1994	Surgery	SCC	45	84	7	10	14	ns
		CDDP/5-FU+20 Gy		41	85	8.5	10	19	
Apinop et al.[8]	1994	Surgery	SCC	34	na	15	7.4	20	ns
		CDDP/5-FU+40 Gy		35		14	9.7	26	
Walsh et al.[9]	1996	Surgery	AC	55	na	8	11	6*	0.01
		CDDP/5-FU+40 Gy		58		4	16	32	
Bosset et al.[10]	1997	Surgery	SCC	139	na	3.6	18.6	37	ns
		CDDP+37 Gy		143		12.3	18.6	39	
Law et al.[11]	1998	Surgery	SCC	30	42		27		ns
		CDDP/5-FU+40 Gy		30	80	na	26	na	
Urba et al.[12]	2001	Surgery	SCC/AC	50	na	2	17.6	16	ns
		CDDP/VBL/5-FU+45 Gy		50		16.9	30	30	
Burmeister et al.[13]	2002	Surgery	SCC/AC	128	na	4.6*	18.5	na	ns
		CDDP/5-FU+35 Gy		128			21.7		

R0, microscopically complete resection; mos, months; CDDP, cisplatin; BLM, bleomycin; 5-FU, 5-fluorouracil; VBL, vinblastine; SCC, squamous cell carcinoma; AC, adenocarcinoma; na, not available; ns, not significant; *, overall treatment related mortality.

cantly better than the 28% achieved by patients treated with surgery alone, there was no significant difference in the 3-year overall survival rate (37% and 39% respectively) and median survival of 18.6 months in both groups.

A recent randomized trial of 256 patients who received either neoadjuvant chemoradiation consisting of 1 cycle of 5-FU and CDDP given with radiotherapy at a dose of 35 Gy in 15 fractions or surgery alone was reported in abstract form by Burmeister et al.[13] The results did not show a survival benefit that could be attributed to the added chemoradiation. In a subgroup analysis, patients with SCC had an increased disease-free survival, but not overall survival. The administration of single agent chemotherapy is not effective enough to eradicate micrometastatic disease.

Given the above data and, despite the widespread use of preoperative chemoradiation, randomized trials have raised significant methodological concerns and have yielded conflicting outcomes. There are several unanswered questions, i.e., optimal radiation dose, adequate radiation field, chemotherapeutic agents and administration schedule, histological distribution, etc. Moreover, preoperative chemoradiation tends to increase postoperative mortality; this may be most important factor swaying the results of trials.[6,10,12]

Chemoradiation without Surgery

Treatment with chemotherapy and radiotherapy, has been shown to be superior to radiation alone.

In 1985, the Radiation Therapy Oncology Group (RTOG) started RTOG 85-01, a prospective randomized controlled trial. They evaluated the hypothesis that concurrent chemoradiation could achieve a higher overall survival rate in patients who had localized carcinoma of the thoracic esophagus than was possible with radiation alone.[14] In this study 121 patients were randomly assigned to receive either combined-modality therapy or radiation therapy alone. The chemoradiation therapy consisted of protracted infusion of 5-FU (1,000 mg/m^2/24 hours for 4 days) and CDDP (75 mg/m^2/bolus day 1) on weeks 1, 5, 8 and 11 with 50 Gy of radiation therapy, while the radiation alone comprised 64 Gy. In this study, patients who received chemoradiation showed a significant improvement in median survival of 12.5 months compared with 8.9 months for patients who received radiation alone. With a 2-year survival rate of 38% and 10% respectively. Histologic type did not have a significant affect on the outcomes. The protocol was closed early because of positive results at the interim analysis. An additional 69 patients treated with the same combined-modality therapy after the closure of the randomization confirmed the results of

Table 4. Phase III trials to investigate the impact of neoadjuvant chemoradiation vs definitive chemoradiation in patients with resectable esophageal carcinoma

Author/ ref. no.	Year	Protocol	Histology	No. of patients	Mortality (%)	Median survival (mos)	2-year survival (%)	*P*
Bedenne et al.[18]	2002	I-CRT → Surgery	SCC/AC	259	9*	17.7	34	0.56
		I-CRT → CRTx			1*	19.3	40	
Stahl et al.[19]	2005	I-CT+CRT → Surgery	SCC	86	12.8	16.4	39.9	ns
		I-CT+CRT → CRTx		86	3.5	14.9	35.4	

mos, months; I-CRT, induction chemoradiotherapy; I-CT+CRT, induction chemotherapy + chemoradiotherapy; SCC, squamous cell carcinoma; AC, adenocarcinoma; ns, not significant; *, the death rate within 3 months after starting induction treatment.

chemoradiation with a median survival of 17.2 months and 3-year survival rate of 30%.[15,16] Based on this positive result, concurrent chemoradiotherapy has become the standard therapy for patients with localized carcinoma of the thoracic esophagus selected for nonsurgical treatment. Although chemoradiation was associated with a higher incidence of toxicity, this problem has led to studies aimed at improving the efficacy of radiotherapy, especially in determining the appropriate radiation dosage a part of combined modality therapy.

In the intergroup INT 0123 (RTOG 94-05),[17] 236 patients with esophageal cancer, histologically SCC or AC, clinical stage T1 to 4, N0/1, M0 were allocated to receive either combined-modality therapy consisting of 4 monthly cycles of 5-FU (1,000 mg/m^2/24 hours for 4 days) and CDDP (100 mg/m^2/bolus day 1) with concurrent 64.8 Gy radiation or the same chemotherapy dose and schedule but with 50.4 Gy. Since the data revealed that it was highly unlikely there would be any advantages to using the high dose radiation compared with the standard dose, this trial was closed at the time of the planned interim analysis. For the 218 eligible patients, there was actually no significant difference in median survival of 13.0 months for the high dose arm and 18.1 months for the standard dose arm. The survival rate at 2 years for the 2 arms was 31% and 40%, and local persistence or failure, respectively. Although 11 treatment related deaths occurred in the high dose arm compared with 2 in the standard dose arm, 7 of the 11 deaths occurred in patients who had received 50.4 Gy or less. It is unlikely that the increase in treatment related deaths in the high dose arm was related to a higher dose of radiation. Thus the standard radiation dose for patients treated with 5-FU and CDDP chemotherapy is 50.4 Gy.

This randomized trial data suggest the clear superiority of chemoradiation over radiotherapy alone. Definitive chemoradiation is thus widely recognized as the standard of nonsurgical treatment for locoregional operable esophageal cancer.

Necessity for Surgery after Chemoradiation

Chemotherapy and radiotherapy without surgery has not been compared with surgery alone in prospective clinical trials. Recently 2 trials have been reported from the Federation Francaise de Cancerologie Digestive (FFCD) and the German Oesophageal Cancer Study Group (GOCSG)[18,19] that indicate the necessity of surgery after radiation (Table 4).

In the FFCD trial (FFCD 9102), 445 patients with operable thoracic esophageal cancer, histologically SCC, stage T3-4N0-1M0 were eligible. Induction therapy consists of 2 cycles of 5-FU and CDDP (days 1–5 and 22–26) plus concurrent protracted (46 Gy in 4.5 weeks) or split-course (15 Gy in days 1–5 and 22–26) radiation. The 259 patients who had at least a partial response were randomly allocated to receive additional chemoradiation or perform surgery. The additional chemoradiation consisted of 3 cycles of the same chemotherapy and concurrent protracted (20 Gy) or split-course (15 Gy) radiation. The 2-year survival rate was 34% in the surgery group versus 40% in the chemoradiation group, which was not significant (p=0.56). Furthermore, median survival was 17.7 months versus 19.3 months respectively. Thus, the FFCD 9102 trial concluded that additional chemoradiation is an alternative to surgery in patients with locally advanced resectable esophageal cancer who respond to initial chemoradiation.

The GOCSG compared preoperative chemoradiation followed by surgery with chemoradiation alone. In this trial, 172 patients with locally advanced SCC of the esophagus, stage T3-4N0-1M0 were randomized to ei-

ther induction chemotherapy followed by chemoradiation (40 Gy) followed by surgery, or the same induction chemotherapy followed by definitive chemoradiation (at least 65 Gy) without surgery. Induction chemotherapy consists of 3 cycles of bolus 5-FU, leucovorin, etoposide, and CDDP on days 1–3 every 3 weeks. This was followed by concomitant chemoradiation with CDDP and etoposide. In the surgery arm, tansthoracic esophagectomy was performed 3–4 weeks after the end of irradiation. In the definite chemoradiation arm, the same combined chemoradiation was administered with a radiation dose of 40 Gy. Afterwards, the radiation dose was increased to at least 65 Gy with hyperfractionated external-beam radiotherapy or high dose-rate afterloading brachytherapy. Although the local progression-free survival rate at 2 years (64.3% in the surgery group and 40.7% in the chemoradiation group) was significantly different, (p=0.003), overall survival in both treatment groups, was roughly equal (39.9% at 2 years and 35.4%, 31.3% and 24.4% at 3 years). Median survival of each group also showed no difference. As a result, the GOCSG concluded that chemoradiation followed by surgery can no longer be recommended as routine treatment in patients with good tumor response to induction therapy. However, surgery is recommended in limited cases to provide survival benefit in patients defined as nonresponders.

In these 2 trials, definite chemoradiation appears to be an alternative to surgery in the initial treatment of locoregional advanced esophageal cancer. However, several issues should be raised, e.g., 1) since each trials include clinical T4 which could lead to non-curative resection (R1–R2), the patients with this stage of tumor should be excluded because prognoses were extremely poor in patients whose resection was incomplete; 2) treatment schedule in radiotherapy should be integrated into 1 regimen, and there are significant methodological concerns, including radiation field, dose, fraction and split; 3) even now, a combination of 5-FU and cisplatin remains the standard regimen for esophageal cancer. Since the protocol used for chemotherapy in the German study is not the common protocol, the validity of the study's conclusion is not easily acceptable; and 4) of the randomized trials comparing concurrent chemoradiation followed by surgery with surgery alone, only one trial reported by Walsh et al.,[9] in which only adenocarcinoma patients were eligible, demonstrated a significantly better median survival and 3-year survival rate. This result means histology could have an important influence on outcome. Thus randomized controlled trials of non-surgical approaches ought to be planned for patients with different histologies.

Salvage Esophagectomy after Definitive Chemoradiation Therapy

Isolated persistence or local failure of the disease to respond is not uncommon after definitive chemoradiation. Although salvage esophagectomy is one of the strategies for selected patients, it is a far riskier operation from the standpoint of mortality and morbidity than planned esophagectomy with or without neoadjuvant therapy. In general, anastomotic leakage and pulmonary complications are more common when esophagectomy is performed after definitive chemoradiation. Swisher et al.[20] reported anastomotic leak rates of 39% in patients who underwent salvage esophagectomy. This was significantly higher than the 7% leak rate experienced by those who received planned esophagectomy (p=0.005) and the average hospital stay (29.4 days) for the former was significantly longer than that for the latter (18.4 days) (p=0.03). Postoperative mortality rates for salvage esophagectomy patients trended upward (15% vs. 6%, p=0.2). Swisher et al.[20] also described how salvage esophagectomy increased the complexity or difficulty of resecting the relapsed tumors. Meunier et al.[21] also stressed the difficulty of surgery.

Thus surgeons who perform salvage esophagectomy face a difficult challenge in trying to reduce concomitant postoperative mortality and morbidity.

Conclusion

The view that surgery might not be essential and that the apparent advantage of chemoradiation alone arises from the avoidance of perioperative mortality. The treatment modality of resectable locoregional esophageal cancer seems to be evolving from surgery alone to definitive chemoradiation and preoperative chemoradiation. Though the survival rates for definitive chemoradiation and surgery appear similar, surgery-related death rates might be most important factors swaying the results of studies. On the other hand, local failure occurs more frequently in the patients treated with definitive chemoradiation alone. Patients with local recurrence or residual disease after chemoradiation should be referred for surgery by a medical oncologist. Considering the high postoperative mortality and morbidity rates, salvage esophagectomy could be considered a difficult challenge for a surgeon. If de-

finitive chemoradiation is carried out on the assumption that local failure can be salvaged by surgery, it is difficult to regard it as a valid treatment strategy. Moreover, if a method of predicting results before and during treatment can be developed, definitive chemoradiation could become acceptable as a separate treatment option. There are no widely accepted clinical trials contrasting definitive chemoradiation with surgery using standard and appropriate protocols. These issues can only be resolved by carefully designed, randomized, controlled trials. At present, such trials have yet to be carried out. It should be noted that neoadjuvant therapy which is mainly performed in Western countries might increase the postoperative mortality rate. The possibility cannot be denied that surgical treatment might prove to be superior as long as concomitant mortality can be reduced.

The survival advantage over surgery in all neoadjuvant and adjuvant settings remains unclear. A breakthrough seems impossible unless more promising chemotherapeutic agents are developed, as the efficacy of both definitive chemoradiation and surgical results have reached their limit. Thus one must conclude that surgery remains the gold standard for resectable locoregional esophageal cancer with which other treatment options must be compared.

References

1. Kelsen DP, Ginsberg R, Pajak TF, et al. Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. *N Engl J Med* 1998; **339**: 1979–84.
2. Medical Research Council Oesophageal Cancer Working Party. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. *Lancet* 2002; **359**: 1727–33.
3. Urschel JD, Vasan H, Blewett CJ. A meta-analysis of randomized controlled trials that compared neoadjuvant chemotherapy and surgery to surgery alone for resectable esophageal cancer. *Am J Surg* 2002; **183**: 274–9.
4. Ando N, Iizuka T, Kakegawa T, et al. A randomized trial of surgery with and without chemotherapy for localized squamous carcinoma of the thoracic esophagus: the Japan Clinical Oncology Group Study. *J Thorac Cardiovasc Surg* 1997; **114**: 205–9.
5. Ando N, Iizuka T, Ide H, et al. Surgery plus chemotherapy compared with surgery alone for localized squamous cell carcinoma of the thoracic esophagus: a Japan Clinical Oncology Group Study—JCOG9204. *J Clin Oncol* 2003; **21**: 4592–6.
6. Nygaard K, Hagen S, Hansen HS, et al. Pre-operative radiotherapy prolongs survival in operable esophageal carcinoma: a randomized, multicenter study of preoperative radiotherapy and chemotherapy. The second Scandinavian trial in esophageal cancer. *World J Surg* 1992; **16**: 1104–10.
7. Le Prise E, Etienne PL, Meunier B, et al. A randomized study of chemotherapy, radiation therapy, and surgery versus surgery for localized squamous cell carcinoma of the esophagus. *Cancer* 1994; **73**: 1779–84.
8. Apinop C, Puttisak P, Preecha N. A prospective study of combined therapy in esophageal cancer. *Hepatogastroenterology* 1994; **41**: 391–3.
9. Walsh TN, Noonan N, Hollywood D, et al. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. *N Engl J Med* 1996; **335**: 462–7.
10. Bosset JF, Gignoux M, Triboulet JP, et al. Chemoradiotherapy followed by surgery compared with surgery alone in squamous-cell cancer of the esophagus. *N Engl J Med* 1997; **337**: 161–7.
11. Law S, Kwong DLW, Tung HM, et al. Preoperative chemoradiation for squamous cell esophageal cancer: a prospective randomized trial (abstr.). *Can J Gastroenterol* 1998; **12** (Suppl B): 56B.
12. Urba SG, Orringer MB, Turrisi A, et al. Randomized trial of preoperative chemoradiation versus surgery alone in patients with locoregional esophageal carcinoma. *J Clin Oncol* 2001; **19**: 305–13.
13. Burmeister BH, Smithers BM, Fitzgerald L, et al. A randomized phase IJI trial of preoperative chemoradiation followed by surgery (CR-S) versus surgery alone (S) for localized respectable cancer of the esophagus (abstr.). *Prog Proc Am Soc Clin Oncol* 2002; **21**: 130a.
14. Herskovic A, Martz K, al-Sarraf M, et al. Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. *N Engl J Med* 1992; **326**: 1593–8.
15. al-Sarraf M, Martz K, Herskovic A, et al. Progress report of combined chemoradiotherapy versus radiotherapy alone in patients with esophageal cancer: an intergroup study. *J Clin Oncol* 1997; **15**: 277–84.
16. Cooper JS, Guo MD, Herskovic A, et al. Chemoradiotherapy of locally advanced esophageal cancer: long-term follow-up of a prospective randomized trial (RTOG 85-01). Radiation Therapy Oncology Group. *JAMA* 1999; **281**: 1623–7.
17. Minsky BD, Pajak TF, Ginsberg RJ, et al. INT 0123 (Radiation Therapy Oncology Group 94-05) phase III trial of combined-modality therapy for esophageal cancer: high-dose versus standard-dose radiation therapy. *J Clin Oncol* 2002; **20**: 1167–74.
18. Bedenne L, Michel P, Bouche O, et al. Randomized phase III trial in locally advanced esophageal cancer: radiochemotherapy followed by surgery versus radiochemotherapy alone (FFCD 9102). *Proc Am Soc Clin Oncol* 2002 (abstr 519); **21**: 130a.
19. Stahl M, Stuschke M, Lehmann N, et al. Chemoradiation with and without surgery in patients with locally advanced squamous cell carcinoma of the esophagus. *J Clin Oncol* 2005; **23**: 2310–7.

20. Swisher SG, Wynn P, Putnam JB, et al. Salvage esophagectomy for recurrent tumors after definitive chemotherapy and radiotherapy. *J Thorac Cardiovasc Surg* 2002; **123**: 175–83.

21. Meunier B, Raoul J, Le Prise E, et al. Salvage esophagectomy after unsuccessful curative chemoradiotherapy for squamous cell cancer of the esophagus. *Dig Surg* 1998; **15**: 224–6.

特集 知っておきたいがん放射線治療の知識

放射線治療の実際 食道癌

Radiation therapy for esophageal cancer

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食道癌に対する放射線治療は食道温存を目指した根治的治療法として広く用いられるようになってきている．ほぼすべての病期に対して用いられるが，化学放射線療法の導入により治療成績は病期によっては手術成績と有意な差がみられなくなっている．しかしながら，食道癌の治療成績は満足に足るものではなく，さらなる治療成績の向上には集学的な試みが必要であろう．

はじめに

食道癌に対する放射線治療は，抗癌剤同時併用の導入による生存率の向上から食道温存を目指す根治的治療法として近年ますます重要になってきている．その適応は，表在癌からII期III期の切除可能ステージ，また切除不能T4N0,1M0症例や鎖骨上窩リンパ節転移を有するIV期症例まで根治的照射の適応となる．

また進行症例や再発症例に対しては，症状緩和においても放射線治療は有効である．放射線治療計画ガイドライン2008や食道学会の食道癌診断・治療ガイドライン2007にて治療対象や治療方法の標準化が進められているが，化学療法の併用方法や予防リンパ節領域の設定，照射線量など一定のコンセンサスにまだ達していない部分もある．

食道癌に対する放射線治療の現状について述べる．

I. 放射線線量について

米国 Radiation Therapy Oncology Group (RTOG)での放射線単独療法(64 Gy)と化学放射線療法(シスプラチン+5-FU+放射線50 Gy)のランダム化比較試験(RTOG8501)では，それぞれの5年生存率が，0%，27%と有意に化学放射線療法の成績が良好であったため，現在の標準療法は化学放射線療法である．線量増加の効果をみるためにその後施行された標準量50.4 Gy と高用量64.8Gy の化学放射線療法同士を比較したランダム化比較試験(RTOG9405/INT0123)では，生存期間中央値，2年生存率，局所制御率のいずれに

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