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Genetic Alterations in Patients With Esophageal Cancer With Short- and Long-Term Survival Rates After Curative Esophagectomy

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Objective
The objective of this study was to ascertain the exact relation between specific oncogenes and long- and short-term survival rates in patients with esophageal cancer.

Summary Background Data
Recent developments in molecular biology have shown that several oncogenes and suppressor genes are involved in the development of esophageal cancer. However, the role of these genes still is unknown.

Methods
The clinical outcome of 84 cases of R0-resected esophageal carcinomas (from 1986–1993) and the molecular and biologic characteristics of these tumors were studied. The patients studied were divided into three groups, which were designated as follows: shortest term survivors (up to 6 months), short-term survivors (7–12 months), and long-term survivors (>5 years). These groups included 23, 17, and 44 subjects, respectively. For the genomic analysis, CyclinD1, int-2, murine double minute 2 (MDM2), retinoblastoma, p53, adenomatous polyposis coli (APC), deleted in colorectal carcinoma (DCC), and human papillomavirus were studied in these patients. The regrowth capability of primary cultures and the clinicopathologic characteristics of these patients also were analyzed.

Results
The CyclinD1 and int-2 genes, which are located in the 11q13 chromosome, and the MDM2 gene were related to short survival. However, the p53 mutation and human papillomavirus infection were not related to short-term survival. The average ratio of genomic abnormalities to genes examined was higher in the shortest and short-term survival groups than in the long-term survival group. Regrowth capability in primary cultures also was related to short-term survival. Among the long-term survival patients, 7 (16%) of 44 cases suffered further cancer after esophagectomy.

Conclusions

These results suggest that the 11q13 amplicon and MDM2 may play an important role in the progression of esophageal cancer, and an accumulation of genomic abnormalities may result in poor prognosis. Careful follow-up testing for double cancer is needed in long-term survivors of esophageal cancer.

Although surgical techniques and perioperative management have progressed, the prognosis for patients with esophageal cancer remains poor. Various factors (epidermal growth factor receptor,[1] transforming growth factor-α,[2] DNA ploidy,[3] argyrophilic nucleolar organizer regions,[4] E-cadherin, and α-catenin[5]) have been proposed as indicators for esophageal cancer. Genomic analysis also has indicated that an amplification of the chromosome 11q13 region (int-2/hst-1/CyclinD1 gene), a loss of heterozygosity (LOH) in the multiple tumor suppressor genes, an inactivation of p53, and mutations of the cyclin-dependent kinase inhibitor genes occur in esophageal carcinomas.[6]

Although various oncogenes, oncogene products, and suppressor genes have been identified in human esophageal carcinomas, their relation to disease outcome remains controversial. Whether an aggressive, multidisciplinary therapy or a more palliative treatment is required also is controversial.

We previously analyzed LOH of chromosomes, LOH of the retinoblastoma (RB) gene, LOH of the APC gene, LOH of the deleted in colorectal carcinoma (DCC) gene, p53 mutation, murine double minute 2 (MDM2) amplification, human papillomavirus (HPV) infection, int-2 amplification, and CyclinD1 amplification in esophageal carcinomas.[7–11] We also analyzed the growth capability of primary cultures of esophageal cancer cells.[12,13]

To clarify what factors contributed to the differences between the short-term survival patients and long-term survival patients with esophageal cancer, we postoperatively investigated these molecular and the clinicopathologic characteristics of surgically resected specimens.

MATERIALS AND METHODS

Patients With Esophageal Cancer

One hundred fifty-nine patients with esophageal carcinoma who underwent curative esophagectomy, which was performed by the same surgeon (second author) in Kyoto University from 1981 to 1992, were studied (R0 resection according to the International Union Against Cancer tumor node metastasis classification system). All patients had pathologic squamous cell carcinomas, and each patient's clinical status was classified as stage I, II, III, or IV according to the pathologic tumor node metastasis classification system.[14] The standard surgical method that was used has been described previously.[15] In brief, esophagectomy with lymph node dissection was performed by means of a right thoracotomy, and subsequent reconstruction was done by esophagogastrostomy using a gastric tube through the retrosternal route.

Tumor Samples

The tumor tissue was divided into three samples in the operating room. The first tumor sample was immediately frozen in liquid nitrogen for molecular analysis and stored until examination. The second tumor sample was cultured for biologic analysis, and the third and last tumor sample was processed for pathologic examination. The normal mucosal tissue also was stored.

For genomic analysis, p53 mutation, LOH of the RB gene, LOH of the APC gene locus, LOH of the DCC gene locus, MDM2 amplification, CyclinD1 amplification, int-2 amplification, and HPV infection were studied in 64 samples of the 159 cases. High molecular weight DNA was extracted from the tissue as described previously.[16] Some samples were not available for a complete genetic analysis because of tumor size and lack of stored sample. A brief description of the gene analyses follows.

Polymerase Chain Reaction–Single-Strand Conformation Polymorphism and Direct Sequencing Analysis of the p53 Gene

Identification of mutation in the p53 gene was performed by PCR–SSCP (polymerase chain reaction–single-strand conformation polymorphism) from exon 2 to 11 and by direct sequencing as described previously.[7,11]

Analysis of CyclinD1, int-2, and MDM2 Genes

The human MDM2 probe was kindly provided by Dr. B. Vogelstein, and a 969-bp *Taq* 1 fragment was sub-

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Table 1. PATIENT CLINICOPATHOLOGIC DATA

	Shortest-Term Survival (<6 mo)	Short-Term Survival (7–12 mo)	Long-Term Survival (>5 yr)	p
Total no. of cases	23	17	44	
M:F	21:2	15:2	37:7	0.6964
T1	0	3	11	
T2	3	5	11	0.0002
T3	9	7	19	
T4	11	2	3	
N0	0	4	30	<0.0001
N1	23	13	14	
No. of lymph node metastases	10.6 ± 12.3	4.9 ± 4.6	0.9 ± 1.8	<0.0001

cloned by us.[11] The int-2 gene probe (SS6) was obtained from Japanese Resources for Cancer Study (Gene Bank). For the CyclinD1 gene probe, we used an EcoR1 1.3-kbp fragment of pcBZO5.4.[9,17]

To determine the degree of amplification of these genes, Southern blot analysis was performed using these probes, and the density of the bands was determined by scanning the autoradiographs with a densitometer. A relative increase in signal intensity of more than three times that of the normalized constitutional DNA signal was considered to indicate gene amplification.

Analysis of Human Papillomavirus

The integration of HPV DNA was screened by PCR with consensus primers (sense:5′-TGTCAAAAACCG-TTGTGTCC-3′; antisense: 5′-GAGCTGTCGCTTAAT-TGCTC-3′).[11] Hela cells were used as a positive control specimen. The HPV infection also was analyzed by Southern blot hybridization. The HPV-16 and -18 probes were obtained from the Japanese Resources for Cancer Study (Gene Bank).

Analysis of RB, APC, and DCC Genes

To examine LOH in the APC locus, RB locus, and DCC locus, the tumors and their corresponding normal tissues were analyzed by PCR–RFLP (restriction fragment length polymorphism) as reported previously.[10] The probes that we used were Pi225 for the APC locus, 68RS2.0 for the RB locus, and JOSH4.4, p15-65 for the DCC locus.

Cell Culture

To determine whether any of the cellular biologic characteristics were related to prognosis, we checked the monolayer epithelial growth capability of the tumor samples. The resected esophageal samples were cultured in Petri dishes, and culture patterning was checked under phase contrast microscopy as described previously.[12,13,18] The growth pattern in which tumor cells migrated out from scattered tumor samples was designated as monolayer epithelial growth.

Statistical Analysis

Statistical differences between the groups were analyzed by Kruskal–Wallis testing and chi square testing.

RESULTS

Patient Clinicopathologic Data

In this study, 84 of the 159 cases of resected esophageal carcinoma were studied. These patients consisted of 23 shortest term survivors (within 6 months), 17 short-term survivors (between 7–12 months), and 44 long-term survivors (more than 5 years). Table 1 summarizes the clinicopathologic data for these three groups. There were statistically significant differences among the three groups in the number of lymph node metastases ($p < 0.0001$), regional lymph node metastasis ($p < 0.0001$), and the depth of tumor invasion ($p = 0.0002$). All of the patients in the shortest term survival group had lymph node metastasis. Fourteen (31.8%) of 44 long-term survivors also had lymph node metastasis. However, the number of lymph node metastases was less than 3 (0.9 ± 1.8).

There were no T1 tumors among the shortest term survivors. Conversely, T3 and T4 tumors were observed in 22 (50%) of 44 patients with long-term survival rates. To clarify whether any specific gene was related to poor prognosis, we analyzed the genomic abnormalities of the patients.

Table 2. GENOMIC ANALYSIS AND CELL CULTURE STATUS OF THE PATIENTS [No. (%)]

	Shortest-Term Survival (<6 mo)	Short-Term Survival (7–12 mo)	Long-Term Survival (>5 yr)	p
Monolayer growth	12/16 (75)	13/17 (76)	8/19 (42)	0.0524
Cyclin D1 amplification	7/14 (50)	1/9 (11)	2/15 (13)	0.0402
int-2 amplification	6/7 (86)	3/8 (38)	3/13 (30)	0.0181
MDM2 amplification	4/12 (33)	2/6 (33)	0/13 (0)	0.0681
p53 mutation	5/12 (42)	4/6 (67)	3/13 (23)	0.1864
Rb loss	2/5 (40)	1/3 (33)	2/7 (29)	0.9179
APC loss	4/5 (80)	2/2 (100)	1/5 (20)	0.1097
HPV infection	1/12 (8)	1/6 (17)	3/13 (23)	0.6052

APC = adenomatous polyposis coli; HPV = human papillomavirus.

Genomic Analysis and Cell Culture Status of the Patient

The results of genomic analysis and the cell culture status of the patients are listed in Table 2. Because of insufficient material, it was not always possible to analyze each gene in every patient. Amplification of the CyclinD1 gene and the int-2 gene was observed most frequently in the shortest term survivors (<6 months) of esophageal cancer, whereas regrowth capability of primary tissue samples, p53 mutation, and MDM2 gene amplification were observed most frequently among short-term survivors (<12 months). Incidence of HPV infection was not significantly different among the three groups.

The same samples were checked for LOH in the RB, APC, and DCC, gene loci, but almost half of these analyses were not informative because of methodologic factors. As a result, these three gene analyses did not yield enough univariate data.

Regarding the relation between pathologic data and genetic abnormalities in long-term survivors, 7 (63.6%) of 11 patients with T3 or T4 tumors and 5 (62.5%) of 8 patients with lymph node metastasis had genetic abnormalities.

Accumulation of Gene Abnormalities

As listed in Table 3, patients with a poor prognosis tended to have a high incidence of gene abnormalities. To ascertain whether the accumulation of gene abnormalities was related to patient prognosis, we checked the ratio of abnormal genes among the genes examined. This ratio was higher in the shortest and short-term survival groups (0.5 and 0.3, respectively) than in the long-term survival group (0.21).

Type of Recurrence After Curative Esophagectomy

Hematogenous recurrence (*e.g.*, lung, liver, bone) was a major cause of death in the patients who died within 12 months after surgery (Table 4). However, there was no specific gene that indicated hematogenous or lymph node recurrence. Metachronous double cancer was observed in 7 (15.9%) of 44 patients with long-term survival rates (Table 5).

DISCUSSION

The patients with p53 mutation had the worse prognosis, although there were differences (not statistically significant)

Table 3. ACCUMULATION OF GENE ABNORMALITIES

	Shortest-Term Survival (<6 mo)	Short-Term Survival (7–12 mo)	Long-Term Survival (>5 yr)
Total number of patients	23	17	44
Patients investigated genetically	16	9	15
Average number of genetic investigations per person	5.2	5.1	5.7
Patients with genetic abnormalities	14	7	9
Average number of genetic abnormalities per person	2.2	1.8	1.2
Average ratio of genetic abnormalities to genes examined	0.5	0.3	0.21

Table 4. TYPE OF RECURRENCE AFTER CURATIVE ESOPHAGECTOMY

	Shortest-Term Survival (<6 mo)	Short-Term Survival (7–12 mo)	Long-Term Survival (>5 Yr)	p
Total number of patients	23	17	44	
Total recurrence	18 (78%)	14 (82%)	6 (14%)	
Hematogenous	12	6	3	0.0002
Lymph node recurrence	9	9	5	0.0017
Pleura recurrence	2	1	0	
Local recurrence	2	1	0	

in the backgrounds of the patients.[18] The p53 mutation plays an important role in carcinogenesis, but its role in the invasion and metastasis of esophageal carcinomas remains unclear. Indeed, many reports concerning the prognostic value of the p53 mutation in various tumors are already in existence. However, these articles contain contradictory data.[19–22] Recent reports have shown that tumors possessing the p53 mutation have poor sensitivity to anticancer drugs.[23,24] These facts necessitate a reanalysis of the preoperative and postoperative response to chemotherapy or radiation therapy in patients with a p53 mutation.

The MDM2 gene amplification, which originally was thought to be related to sarcomatous tumors,[25] was associated with the worse prognosis. The MDM2 gene also regulates the p53 gene, and the role of this gene was thought to be unimportant in esophageal carcinoma. The recent revelation that the MDM2 gene also regulates the RB gene[26,27] gives rise to the possibility that the MDM2 gene may be the main contributing gene in esophageal carcinoma.

The int-2 and hst-1 genes initially were thought to be genes contributing to esophageal carcinoma.[8,28] However, there was no expression of mRNA for either of these genes. Thus, the CyclinD1 gene, which is located at the int-2 and hst-1 locus (11q13), is thought to be the gene expressed most frequently in esophageal cancers.[9,29–31] The CyclinD1 gene encodes a cell-regulatory protein that is expressed at high levels during the G1 phase of the cell cycle. The D cyclin binds to the cyclin-dependent kinases (CDK2 and CDK4) and to proliferating cell nuclear antigen, and formation of these complexes has been implicated in the control of cell proliferation.[32] Antisense mRNA for CyclinD1 inhibits the growth of tumor cells,[33] and transfection of the CyclinD1 gene results in the overexpression of other adjacent genes.[34] It is therefore likely that an amplification and overexpression of the CyclinD1 gene could lead to uncontrollable cell growth and the proliferation of tumor cells. These facts suggest that CyclinD1 is the main contributing gene in esophageal carcinomas. Furthermore, our results suggest that the CyclinD1 gene may play an important role in tumor invasion and in the metastasis of esophageal carcinomas.

The RB gene, a gene contributing to retinoblastoma, also plays an important role in the cell cycle.[35] A recent report

Table 5. DOUBLE CANCER IN PATIENTS WITH ESOPHAGEAL CANCER

	Patients	Double Cancers	Patients	Time	Gene Abnormality
Shortest-term survival (<6 mo)	2	Rectal	1	5 yr before EX	int-2
		Laryngeal	1	7 yr before EX	APC, int-2, Cyclin D1
Short-term survival (7–12 mo)	4	Gastric	3	Synchronous	int-2, Cyclin D1
		Thyroid	1	Synchronous	int-2
Long-term survival (>5 yr)	9	Gastric	1	Synchronous	
		Thyroid	1	Synchronous	
		Osteosarcoma	1	5 yr after EX (breast, 17 yr before EX)	
		Oral floor	1	5 yr after EX	int-2, Cyclin D1, RB
		Tonsil	1	9 yr after EX	
		Liver	2	3 yr after EX	HPV, DCC
				3 yr after EX	
		Gastric	2	6 yr after EX	
				11 yr after EX	

EX = esophagectomy; HPV = human papillomavirus; DCC = deleted in colorectal carcinoma.

showed that the RB gene also is related to the carcinogenesis of esophageal cancer,[36] but the RB gene has not been shown to be related to prognosis. Our data similarly did not show a relation between the RB gene and patient prognosis.

Although the number of cases analyzed was insufficient to confirm this, our results suggested that concomitant expression of genomic abnormalities resulted in a poor prognosis. An accumulation of genomic abnormalities could lead to uncontrollable cell growth and several types of malignant behaviors in tumor cells. Harpole et al.[37] reported that the survival prognosis of patients with two genomic abnormalities was worse than that of patients with one genomic abnormality. Genomic accumulation analyses and multiparametric analyses have been reported recently to be useful for characterizing malignant tumors.[38,39] Thus, the genomic abnormality ratio, like the FAL (fractional allelic loss) value[40] in colorectal cancer, may be a good prognostic indicator. Conversely, long-term survivors with T3 to T4 tumors and positive lymph node metastasis also had an accumulation of genetic abnormalities. Prognosis in these patients may be related to other factors such as immunologic response.

Regarding the value of molecular analysis, Akslen and Varhaug[41] reported that in papillary thyroid cancer, genomic abnormalities were of significant prognostic importance in univariate analysis, but multivariate analysis showed that only conventional variables such as age and histologic grade were associated independently with decreased survival. This observation suggests that the value of molecular analysis does not always surpass that of conventional variables. Thus, our data need to be analyzed using a multivariate model, and further studies will be necessary to confirm the biologic implications of our findings. For use in clinical applications, we have started to analyze CyclinD1, MDM2, and regrowth capability in primary cultures initiated from biopsy specimens.

The number of lymph node metastases is the best predictive factor for determining prognosis in patients with esophageal cancer,[42–44] and this value differs dramatically between short-term survivors and long-term survivors. Although factors, such as epidermal growth factor receptor and E-cadherin,[5,45] have been proposed to be related to lymph node metastasis, the role of these genes remains controversial. There dose not seem to be a gene specifically related to lymph node metastasis. An accumulation of genomic abnormalities and a loss of host defense may lead to multiple lymph node metastasis.

Monolayer epithelial growth capability in primary cell cultures also has been shown to be related to the malignant potential of esophageal cancer after curative resection, and this growth capability is related strongly to hematogenous recurrence.[12,13,18,42] This method is useful for detecting dynamic biologic tumor characteristics. In this study, tumors from short-term survivors had high monolayer growth capacity. However, even tumors from long-term survivors had relatively high levels of growth capacity. Among the tumors from long-term survivors, monolayer growth capability was observed more frequently than genomic abnormalities. This means that the regrowth capability of primary cultures represents a less aggressive characteristic of tumor cells than the accumulation of genomic abnormalities.

Patients with esophageal cancer are well known to have a high incidence (20%) of double cancers.[46] Our data suggested that although a patient may survive for more than 5 years after radical curative resection of esophageal carcinoma, a strict follow-up procedure is important to detect early metachronous carcinoma. The prognosis for patients with double cancer of the esophagus and other organs is not uniform because it depends on the grade and stage of each tumor. In this study, long-term survivors with double cancer had few gene abnormalities, but the contribution of cell cycle-related genes to double cancer remains unknown because genomic analysis was not performed in all double cancer cases. Our previous research showed that there was no correlation between p53 mutation and double cancer (unpublished data, 1994). Recent research also has suggested that genetic instability and DNA repair genes, which are well known to be causes of multiple cancer in other sites, may not contribute to multiple cancers in patients with esophageal cancer.[47] Therefore, further analysis is needed in this matter.

In conclusion, the CyclinD1 and int-2 genes, which are located in the 11q13 chromosome, and the MDM2 gene play an important role in the progression of esophageal cancer, and the accumulation of genomic abnormalities results in a poor prognosis. Moreover, careful checking for double cancer is needed in long-term survivors of esophageal cancer.

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