[Frontiers in Bioscience 15, 65-72, January 1, 2010]

EGFR gene alterations as a prognostic biomarker in advanced esophageal squamous cell carcinoma

Kazuhiro Kaneko[1,2], Yosuke Kumekawa[2], Reiko Makino[3], Hisako Nozawa[2], Yuichi Hirayama[2,4], Mari Kogo[5], Kazuo Konishi[2], Atsushi Katagiri[2], Yutaro Kubota[2], Takashi Muramoto[2], Miki Kushima[4], Tohru Ohmori[6], Tsunehiro Oyama[7], Norio Kagawa[8], Atsushi Ohtsu[1], Michio Imawari[2]

[1] Division of Digestive Endoscopy/Gastrointestinal Oncology, National Cancer Center Hospital East, Chiba, 277-8577, Japan,[2] Department of Gastroenterology, Showa University School of Medicine, Tokyo, 142-8666, Japan, [3] Clinical Research Laboratory, Showa University School of Medicine, Tokyo, 142-8666, Japan, [4] Division of Pathology, Showa University School of Medicine, Tokyo, 142-8666, Japan, [5] Promotion Center of Pharmaceutical Education, School of Pharmaceutical Sciences, Showa University, Tokyo, 142-8666, Japan,[6] Institute of Molecular Oncology, Showa University School of Medicine, Tokyo, 142-8666, Japan,[7] Department of Environmental Health, University of Occupational and Environmental Health, Kitakyushu, 807-8555, Japan, [8] Global COE, Nagoya University School of Medicine, Nagoya, 466-8550, Japan

TABLE OF CONTENTS

1. ABSTRACT

Esophageal squamous cell carcinoma (ESCC) exhibits abnormalities in epidermal growth factor receptor (EGFR) gene. To identify a prognostic marker, the overexpression of EGFR protein, mutations in *EGFR* and *p53* mutations were analyzed in pretreatment biopsy specimens removed from T3-4 and/or M1 LYM ESCC patients who received chemoradiotherapy. A silent mutation comprised of a single nucleotide polymorphism (SNP) at codon 787 of exon 20 of the *EGFR* gene was found in 19 patients (33%). In multivariate analysis, a significant difference was seen in the overall survival (odds ratio; 2.347, 95% confidence interval; 1.183-4.656, p=0.015) between patients with and without the *EGFR* heterozygous genotype. Among the 57 eligible patients, 3-year survival rates was 21%, while in patients with *EGFR* heterozygous genotype the rate were 0%. However, neither overexpression of EGFR nor *p53* mutations was associated with the overall survival. These results suggest that the *EGFR* SNP at codon 787 of exon 20 determined in pretreatment biopsy specimens may be a clinically useful biomarker for predicting the prognosis of ESCC patients.

2. INTRODUCTION

In 2000, esophageal cancer was estimated to cause 338,000 deaths worldwide (1). The number of patients with esophageal adenocarcinoma has been increasing in Western countries, while the great majority of these lesions are esophageal squamous cell carcinomas (ESCC) (1). In Japanese patients, 95% of esophageal cancers are squamous cell carcinomas (2). Despite recent advances in the surgical treatment of ESCC, surgical outcome remains unsatisfactory with overall 5-year survival rates from various countries ranging between 17-39% (2-7). In contrast, recent reports on chemoradiotherapy (CRT) as a definitive and preoperative treatment have indicated various advantages in managing esophageal carcinoma (8-12). Standard CRT alone has curative potential for locally advanced ESCC, such as T4 and/or M1 lymph node (LYM) cases, while the 3-year survival rate of patients with T4 and/or M1 LYM disease ranged from 17-23% (11,12). One strategy to improve the outcome of patients treated with CRT is to select treatment responders or long-term survivors for directed therapy, which may lead to further improvements in outcomes when

it is coupled with greater understanding of the molecular basis of ESCC. In particular, molecular biomarkers of tumor behavior are potentially powerful tools for predicting outcome and identifying targets for directed therapy.

Molecular biological studies have confirmed that carcinomas develop when genetic and epigenetic alterations of multiple genes accumulate in human cells. Some of these genes are likely to play crucial roles in the acquisition of resistance to chemotherapeutic agents and radiation. However, the results of many reports are controversial. The tumor suppressor gene, *p53*, which induces cell-cycle arrest or triggers apoptosis in response to DNA damage, is mutated in various cancers. A significant correlation between *p53* mutation and response to chemotherapy and radiation therapy has been demonstrated *in vivo* and *in vitro* (13,14). However, association between overall survival and presence of *p53* gene mutation is controversial. One putative biomarker is epidermal growth factor receptor (*EGFR*), a transmembrane protein with intrinsic tyrosine kinase (TK) activity (15,16). Binding of specific ligands, such as epidermal growth factor and transforming growth factor-α, results in the homodimerization of *EGFR* or in heterodimerization with another member of the *EGFR* family of receptors. In turn, this homo- or heterodimerization stimulates phosphorylation of the intracellular TK on the receptor, activating various downstream signal-transduction pathways that ultimately regulate cell proliferation, migration, adhesion, differentiation, and survival (17). In 2004, three groups demonstrated the mutations in *EGFR* TK domain among patients with non-small cell lung cancer and showed a striking correlation between the gefitinib sensitivity and TK domain mutations (18-20). Subsequently, many reports demonstrated that the mutations of *EGFR* gene are detected in two specific regions such as an in-frame deletion in exon 19 and a missense mutation at the second nucleotide of codon 858 in exon 21 (18-24). Protein overexpression or gene amplification of *EGFR* has been reported in a subset of various tumors (25-29). Increased EGFR expression has been associated with advanced disease, development of metastasis, and poor prognosis.

This study was designed to identify useful prognostic markers in patients with T3-4 and/or M1 LYM disease of ESCC given a combination of 5-fluorouracil (5-FU) and cisplatin (CDDP) with radiotherapy in the same regimen. Because of the previous reports described above, we focused to analyze mutations using polymerase chain reaction (PCR) in the exon 18, 19, 20, and 21 of *EGFR* gene as candidates of prognostic biological markers. The multivariate analysis of the *EGFR* mutation and other factors with patient characteristics suggested a significant association of the polymorphism with poor prognosis of the patients with esophageal squamous cell carcinoma.

3. PATIENTS AND METHODS

3.1. Patients

A total of 70 ESCC patients with T3-4 and/or M1 LYM disease received CRT between May 1997 and March 2002 at Showa University Hospital. Of these, 57 were recruited from our database on the basis of the following criteria and were included in the study: (*a*) sufficient biopsy specimens obtainable before treatment; (*b*) no previous treatment had been received; (*c*) age ≤ 75 years; (*d*) PS on the Eastern Cooperative Oncology Group scale ≤ 2; (*e*) adequate bone marrow, hepatic, and renal functions; and (*f*) stage T3-4, any N, M0-1 on the International Union against Cancer (UICC) tumor-node-metastasis (TNM) classification. The patients with distant organ metastasis, except for M1 LYM, were excluded. Patients were given the same regimen of definitive chemoradiotherapy. Sample collection and gene analysis in this study were approved by the Human Ethics Review Committee of Showa University School of Medicine.

3.2. Treatment Schedule

Chemotherapy consisted of a protracted infusion of 5-FU (400 mg/m^2/day) on days 1–5 and 8–12, combined with CDDP (40 mg/m^2/day) with adequate hydration on days 1 and 8 (11,12). This schedule was repeated twice every 5 weeks. Radiation therapy using 10-megavoltage X-rays was started on day 1 concomitantly with chemotherapy. There was a 2-week break after a dose of 30 Gy. Radiation therapy was restarted on day 36, along with the same chemotherapy schedule used before. For patients who showed an objective response to treatment, additional chemotherapy was administered and consisted of a protracted infusion of 5-FU (800 mg/m^2/day) on days 1–5 and a 2-hour infusion of CDDP (80 mg/m^2/day) on day 1. This treatment was repeated every 4 weeks for two courses. Additional courses of chemotherapy were optional but limited to a total of four courses. No further treatment was administered if no disease progression was observed.

3.3. Evaluation of response to chemoradiotherapy

Tumor status was assessed at baseline, every 4 weeks during treatment, by endoscopy, and neck, chest and abdominal CT scans. Follow-up evaluations after CRT were performed every 3 months for the first 2 years and every 6 months thereafter by endoscopy and CT scan. For measurable lesions (≤ 1 cm), response was assessed using the World Health Organization (WHO) criteria.

For primary tumors, the complete response was defined as disappearance of all visible tumors, including ulceration for at least 4 weeks, confirmed by normal endoscopic biopsy specimens.

3.4. Sample collection

Specimens of both primary tumors and non-tumors were obtained from patients with esophageal squamous cell carcinoma, using biopsy specimens taken at the time of the initial diagnosis. Two types of biopsy specimens were endoscopically taken: tumor sample from the Lugol-unstained portion of the carcinoma and the non-tumor sample from normally stained background epithelium (30). Tumor specimens were obtained from the same point with each specimen divided into two samples, one of which was used for DNA extraction and the other for histologic diagnosis. DNA extraction was not performed in specimens where cancer cells were not histologically confirmed. All samples were stored at -80°C before DNA extraction.

3.5. DNA extraction

For amplification of DNA fragments in frozen tissue, 50 ng of DNA was subjected to 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. To purify and visualize polymerase chain reaction (PCR) products, the PCR products were electrophoresed on 2% agarose gels, and stained with ethidium bromide. DNA was extracted by an ethanol/xylene method (31).

3.6. Analysis of the *EGFR* and *p53* genes

Specimens were mixed with 50 µl of digestion buffer (0.04% proteinase K, 10 mM Tris-HCl at pH 8.0, 1 mM EDTA, and 1% Tween-20) and incubated at 37°C for 18 hours. DNA fragments obtained were subjected to the DNA sequencing analysis for identification of mutations in *EGFR* exons 18, 19, 20, and 21 and in *p53* exons 5, 6, 7, and 8. Primers used for PCR amplification of the *EGFR* gene were as follows: For exon 18, these were 5'-AGCATGGTGAGGGCTGAGGT and 5'-ACCAGACCATGAGAGGCCCT; for exon 19, they were 5'-AGCATGTGGCACCATCTCAC and 5'-AGAGCAGCTGCCAGACATGA; for exon 20, they were 5'-CCATGCGAAGCCACACTGAC and 5'-TGCTATCCCAGGAGCGCAGA; for exon 21, they were 5'-TCTGTCCCTCACAGCAGGGT and 5'-TACAGCTAGTGGGAAGGCAG. Primers used for PCR amplification of the *p53* gene were as follows: For exon 5, these were 5'-TTCACTTGTGCCCTGATTTC and 5'-CTCTCCAGCCCCAGCTGCTC; for exon 6, they were 5'-ATTCCTCACTGATTGCTCC and 5'-TCCTCCCAGAGACCCCAGTT; for exon 7, they were 5'-ACAGGTCCTCCCCAAGGCGCA and 5'-TGTGCAGGGTGGCAAGTGGCT; for exon 8, they were 5'-GTAGGACCTGATTTCCTTACTGCC and 5'-CTTGGTCTCCTCCACCGCTTCTTG. PCR reaction mixtures had a final volume of 20 µl, containing 0.4 µl of 50xAdvantage-HF Polymerase Mix (CLONTECH, Palo Alto, CA), 0.4 µmol of each primer, 2 µl of each 10XHF 2 deoxyribonucleoside triphosphatase, 10xHF 2 PCR buffer (CLONTECH), and 12 µl of PCR-grade H_2O. Extracted DNA was subjected to 40 cycles in a three-step sequence including 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute. For visualization of PCR products, these were electrophoresed at 100 V on 2% agarose gels containing ethidium bromide for 50 minutes. For sequencing, the PCR products were purified using a 10×Ex Taq buffer (Takara, Tokyo, Japan). The PCR products were purified and directly sequenced using a 3100 Sequencing machine (Applied Biosystems, Foster City, CA). Peak patterns were analyzed using Sequencing Analysis Software (Applied Biosystems, Foster City, CA), and mutations and amino acid changes were identified. Sequences with deletion or mutation were verified with both forward and reverse sequencing analyses.

To confirm the *EGFR* SNP, the products of exon 20 were sequenced using another primer, with the forward primer shifted 20 bases upstream: 5'-GTCTTCACCTGGAAGGGGTC and the reverse primer shifted 80 bases upstream: 5'-GCTCCCAGTACCTGCTCAAC.

3.7. Immunofluorescence

For immunofluorescence, consecutive slices of samples were used for histologic diagnosis. Sections were deparaffinized in xylene, followed by treatment with a graded series of alcohol and rehydrated in PBS (pH 7.5). Sections were heated in an autoclave for 20 minutes. A positive reaction for EGFR was visualized by incubating the slides for 1 hour with a 1:250 dilution of anti-human EGFR mouse monoclonal antibody (Novocastra, New Castle, UK). The sections were rinsed three times for 3 minutes each with PBS, before being incubated with a 1:40 dilution of secondary anti-rat IgG antibody (DAKO, Glostrup, Denmark) for 1 hour at room temperature in the dark. Samples were then rinsed again three times with PBS. To confirm the site of staining, immunofluorescence was performed by incubating the slides with stable 3,3'-diaminobenzidine for 20 minutes after incubation with the secondary antibody.

Immunofluorescence staining was assessed by a pathologist (MK) in our hospital. To evaluate the staining of EGFR, a significant staining of more than 10% of tumor cells was considered to be positive. The percentage of cells displaying the same intensity of staining was evaluated.

3.8. Statistical Analysis

Survival time was measured from the initiation of the first course of treatment to the date of death or to the final date of confirmation of survival. The χ^2 test and Fisher's exact test were used to determine any association between any of the clinical covariates. Univariate analysis for survival was assessed according to log-rank tests. The influence of each biological variable on patient survival was assessed by the Cox proportional hazards model. T and M factors were adjusted in comparison with the two groups. P-values of less than 0.05 were considered significant.

4. RESULTS

4.1. Patient Characteristics

Clinicopathological features of the patients in this study are shown in Table 1. In terms of T stage, 20 patients had T3 disease, 37 patients had T4 disease. Clinically involved sites in the 37 cases with T4 disease were thoracic aorta (25 patients), tracheobronchial tree (11 patients), and both sites (1 patient). In terms of M stage, 22 patients had M0 disease, and 25 patients had M1 LYM disease. There were no patients with distant organ metastasis. Five patients had cervical node metastasis, 19 had abdominal nodes, and 1 had metastases in both nodes. Fifty-five patients (96%) completed at least the CRT segment with a total radiation dose of 60 Gy. The remaining 2 patients did not complete chemoradiotherapy; 1 experienced disease progression, and 1 died due to treatment-related esophagoaortic fistula. Eleven patients (19%) received one additional course of only chemotherapy, and 34 patients (60%), 5 patients (9%), and 3 patients (5%) received an additional two, three, and four courses, respectively.

4.2. *EGFR* single nucleotide polymorphism

Of the 57 ESCC patients, *EGFR* mutation wa

Table 1. Patient characteristics

Age (range)		**63 (46-75 years)**
Gender (male/female)		53/4
PS (0/1)		43/10
Location[1]		
	Upper	5
	Middle	30
	Lower	22
Histological type		
	Well differentiated	15
	Moderately differentiated	39
	Poorly differentiated	3
Stage (UICC)		
	T3 M0	15
	T3 M1	5
	T4 M0	17
	T4 M1	20

PS, performance status; [1]Location of the tumor according to the TNM classification; UICC, International Union Against Cancer.

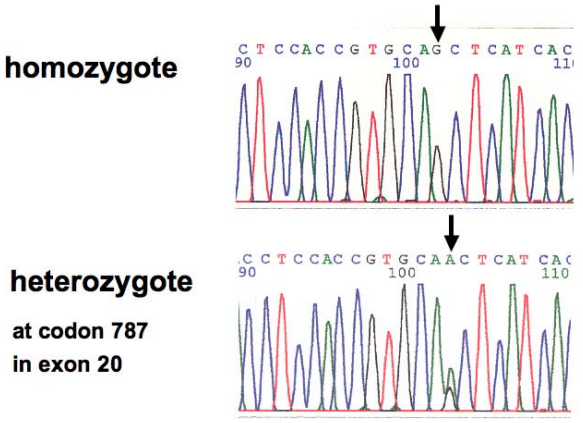

Figure 1. Comparison of sequences between homozygote and heterozygote at codon 787 in exon 20 of the *EGFR* gene. The single nucleotide polymorphism was found at codon 787 in exon 20 (indicated by arrow). The nucleotide alteration, CAG (Gln) to CAA (Gln), was silent mutation.

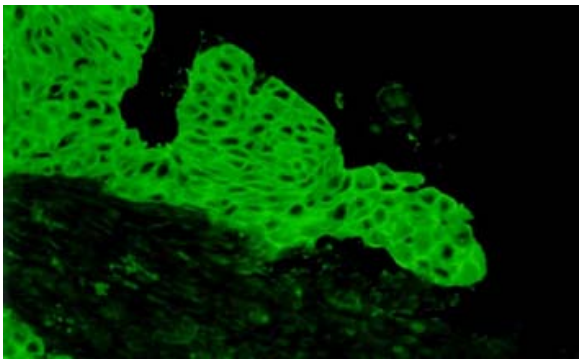

Figure 2. Fluorescence microscopic images of ESCC. The staining of EGFR overexpression was limited to tumor cell membranes. EGFR (green) were immunostained with specific antibodies at a high-power view (x200).

analyzed at exon 18-21 since the most mutations of *EGFR* gene were localized in specific regions, exon 18, 19, and 21 (18-24). A mutation, at codon 787 of exon 20 from G (guanine) to A (adenine), was found in 19 patients (33%) and it was silent, CAG (Gln) to CAA (Gln) (Figure 1). The DNA of the non-tumor sample taken from the same patient were sequenced and also showed the nucleotide substitution, suggesting that the single nucleotide substitution (G to A) is due to the genetic polymorphism. Furthermore, by using another primer, the products of exon 20 were sequenced to confirm the mutation. Based upon the sequencing analysis, genotypes of all 19 cases are heterozygous. This result is consistent with the polymorphism of *EGFR* gene deposited in the NCBI database (32).

4.3. Immunofluorescent analysis of EGFR

Based upon the immunofluorescent analysis, EGFR protein was overexpressed in 39 (68%) of 57 tumor biopsy specimens, but not in any non-tumor biopsy specimens. The EGFR protein was localized on membranes (Figure 2) but not in the cytoplasm of tumor cells although the cell membrane of non-tumor cells was not stained. The *EGFR* mutation in exon 20 was found in 16 (41%) of 39 patients with overexpression and in 3 (16%) of 18 patients without overexpression (p=0.069), suggesting that EGFR overexpression is not associated with the mutation in exon 20.

4.4. *p53* mutations in esophageal carcinoma

The *p53* mutation was found in 25 (44%) of 57 patients with esophageal carcinoma. Among these 25 samples, mutations of the *p53* gene were identified as follows: 10 in exon 5; 4 in exon 6; 7 in exon 7; and 4 in exon 8. Of these 25 mutations, 22 (88%) were missense mutations leading to an amino acid substitution while 2 (8%) were nonsense mutations resulting in insertion of a stop codon. One mutation (4%) represented frameshift. Hotspot mutations, which represent protein alterations that provide a selective growth advantage to the cell (33), were found in 9 of 25 patients (36%) with a *p53* mutation. No mutation was found in samples of non-tumor tissues.

4.5. Clinical response and survival

Of the 57 eligible patients, 25 (44%) achieved CR. The median survival time of the 57 patients was 12 months. The survival of the patients has not yet reached the median time. One- and 3-year overall survival rates were 47% (27 of 57) and 21% (12 of 57), respectively (Table 2).

Survival analysis used the Cox proportional hazards model to determine whether any of the clinical covariates or marker data predicted survival. The overall survival rate of patients with the *EGFR* mutation in exon 20 was lower than that of the patient without the mutation (OR, 2.347; 95%CI, 1.183-4.656; p=0.015) (Figure 3, Table 3). No patients with the *EGFR* mutation attained 3-year survival (maximum survival; 27 months), while 32% (12 of 38) of patients without the mutation attained 3-year survival. In contrast, there was no significant difference in survival between patients with or without the *p53* mutations (p=0.187). In addition, the survival rate was not significantly different between patients with and without EGFR overexpression (p=0.743). In the Cox proportional hazard model, the *EGFR* mutation in exon 20 was the only independent predictor for poor survival (p=0.015, Table 3).

Table 2. Univariate analysis for survival

		<3 year (n=45)	3 year≤ (n=12)	p-value
Age				
	65≤	16	7	0.152
	<65	29	5	
Gender				
	Male	41	12	0.284
	Female	4	0	
Immunofluorescence of EGFR				
	Presence	33	6	0.122
	Absence	12	6	
SNP of *EGFR* gene				
	homozygote	26	12	0.004
	heterozygote	19	0	
p53 mutation				
	Presence	20	5	0.860
	Absence	25	7	

EGFR, epidermal growth factor receptor; SNP, single nucleotide polymorphism

Table 3. Multivariate Cox proportional hazard model

Variable	Hazard ratio	95% CI	P value
Age	0.988	0.951 to 1.025	0.516
Gender	1.122	0.386 to 3.262	0.833
IF of EGFR	0.889	0.442 to 1.792	0.743
SNP of *EGFR* gene	2.347	1.183 to 4.656	0.015
p53 mutation	0.647	0.339 to 1.235	0.187

Age, gender, Immunofluorescence of EGFR, SNP of *EGFR* gene, and *p53* mutation were included in a multivariate Cox proportional hazard model. For each variable, < 65 years, male gender, absence of immunofluorescence of EGFR, SNP of heterozygote, and absence of *p53* mutation were set as reference levels. 95% CI, 95% confidence interval; IF, Immunofluorescence; EGFR, epidermal growth factor receptor; SNP, single nucleotide polymorphism.

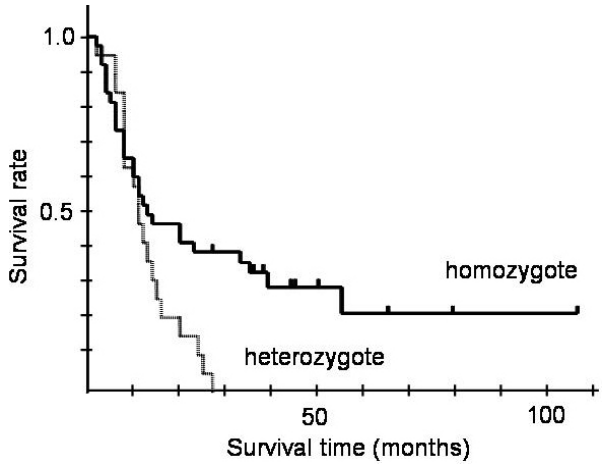

Figure 3. Survival analysis of the homozygote and heterozygote of the EGFR gene. Survival of patients with the homozygote (162093G) or heterozygote (162093G + A) of the EGFR SNP was plotted using the Kaplan-Meier method (p=0.004 by log-rank test).

5. DISCUSSION

In the present study, the usefulness of molecular biomarkers was examined in regard to prognosis in patients with T3-4 and/or M1 LYM ESCC who were treated with definitive CRT. Although mutations of the *EGFR* gene found in lung cancers, such as an in-frame deletion in exon 19 and a missense mutation at the second nucleotide of codon 858 in exon 21, were not found in ESCC patients in our study, a single nucleotide polymorphism (SNP) of the *EGFR* gene was detected at codon 787 in exon 20. This SNP was not associated with the genetic susceptibility of lung cancer (34), however, our results revealed that ESCC patients with the SNP of *EGFR* gene have an increased risk of an unfavorable prognosis. No patients with heterozygous genotype in this SNP attained 3-year survival, while 3-year survival rates were 21% in the eligible 57 patients. In contrast, overexpression of EGFR protein determined immunofluorescent staining and *p53* mutational status were not related to predictive prognostic factors. Results from multivariate analysis emphasize that the SNP of the *EGFR* heterozygous genotype was an independent poor prognostic factor for CRT.

Recent studies have reported the importance of intratumoral genetic mutations within functional domains

of the *EGFR* gene in relation to response to gefitinib (commonly known as Iressa), an EGFR tyrosine kinase inhibitor, in lung cancer (35-37). Most of the responders had a deletion in exon 19 or point mutation in exon 18 or 21 of the *EGFR* gene, which are the coding sequences for the amino acids in the tyrosine kinase domains. In contrast, none of the non-responders had these mutations. It would be of great interest to assemble a more comprehensive picture that includes functional polymorphic variations as well as mutations, and assess their individual and/or collective predictive value to a given treatment. In our present study, a mutation at codon 787 in exon 20 was found in the individual samples of carcinoma tissues as well as non-tumor tissues, suggesting that the mutation is a polymorphism as found in NCBI database. This base change from "G" to "A" causes no alteration of amino-acid (Gln to Gln).

Generally, SNPs are small genetic changes, or variations, that can occur within a person's genome sequence. Some reports have shown that certain SNPs of growth factors are associated with tumorigenesis in malignant melanoma, breast cancer, and prostatic adenocarcinoma (38-41). Papadopoulou *et al.* reported that serum HER-2 could be clinically used as a useful tumor marker for the diagnosis and the progression of breast cancer (42). Furthermore, they provided a clinical evidence that HER-2 Ile655Val SNP does affect serum HER-2 levels and it can be regarded as a predictive biomarker for breast cancer patients with poor prognosis. Since the SNP alters the amino acid from Ile to Val, the SNP may influence the function of the gene product, suggesting that loss of gene function would bring poor prognosis in patients with HER-2 Ile655Val SNP. Among the SNPs in the *EGFR* gene, a SNP 181946C>T (Asp994Asp) was reported that the 181946C homozygous and 181946C + T heterozygous genotypes were associated with a significantly increased risk of lung cancer compared with the 181946T homozygous although the SNP 162093G>A (Gln787Gln) had no association with a risk of lung cancer (34). Intriguingly, our present study strongly suggested that the 162093G + A (Gln787Gln) heterozygous genotype was associated with poor prognosis in ESCC patients compared with the homozygous genotype of the 162093G. The mechanism underlying the association of the 162093G>A polymorphism with ESCC remains to be elucidated. In addition to the involvement of the silent mutation in the cancer risk, it could be also important that the 162093G>A polymorphism was associated with a risk of ESCC but not with a risk of lung cancer.

In contrast, the presence of EGFR overexpression in ESCC patients ranged from 49-68% (27, 43-45). Hanawa *et al*. reported that protein overexpression of ESCC was significantly correlated with the depth of tumor invasion: the frequency of overexpression in T2, 3 and 4 tumors was significantly higher than that in Tis and T1 tumors ($p < 0.0001$). However, univariate analysis revealed no significant difference in survival rates with respect to protein overexpression (43). In our study, there was no significant difference between EGFR overexpression and survival because all patients had T3 or T4 disease. Furthermore, there was no significant difference in incidence between EGFR overexpression and *EGFR* 162093G + A heterozygous genotype ($p=0.069$). Our results suggested that cases with the *EGFR* SNP heterozygous genotype do not result in EGFR overexpression.

In summary, polymorphism of the *EGFR* gene may be associated with treatment efficacy and survival in patients with locally advanced esophageal carcinoma who were treated with definitive CRT. In this context, our results emphasize that SNPs of the *EGFR* gene are potentially useful prognostic markers in patients treated with CRT because the *EGFR* SNP 162093G + A heterozygous genotype was the only independent predictor for poor survival in the Cox proportional hazard model. Furthermore, the prognosis of ESCC patients with the heterozygous genotype was fatal. To our knowledge, this is the first study that shows a relationship between *EGFR* gene polymorphisms and resistance to treatment or unfavorable prognosis in patients with locally advanced ESCC treated with definitive CRT. We believe that assessment of *EGFR* Gln787Gln SNP may aid in the prediction of CRT in ESCC.

6. ACKNOWLEDGMENTS

This study was funded in part by a Research on the association between risk of upper aero-digestive tract cancer and alcohol-metabolizing enzymes, and its clinical significance (to K.K.), grant number 20-10.

7. REFERENCES

1. D.M. Parkin, F.I. Bray & S.S. Devesa. Cancer burden in the year 2000. The global picture. *Eur J Cancer*, 37(supple 8),S4-66 (2001)

2. Registration Committee for Esophageal Cancer (ed). Comprehensive registry of esophageal cancer in Japan: The Japan Society for Esophageal Diseases. http://plaza.umin.ac.jp/70~jsed/

3. Y.G. Goan, H.C. Chang, H.K. Hsu & Y.P. Chou. An audit of surgical outcomes of esophageal squamous cell carcinoma. *Eur J Cardio-Thorac*, 31,536-44 (2007)

4. S. Law, D.L. Kwoung, K.F. Kwort, K.H. Wong, K.M. Chu, J.S. Sham & J. Wong. Improvement in treatment results and long-term survival of patients with esophageal cancer: impact of chemoradiation and change in treatment strategy. *Ann Surg*, 238,339-47 (2003)

5. M.B. Orringer, B. Marshal & M.D. Iannettoni. Transhiatal esophagectomy: clinical experience and refinement. *Ann Surg*, 230,392-400 (1999)

6. N. Ando, S. Ozawa, Y. Kitagawa, Y. Shinozawa & M. Kitajima. Improvement in the results of surgical treatment of advanced squamous esophageal carcinoma during 15 consecutive years. *Ann Surg*, 232,225-32 (2000)

7. A. Peracchia, L. Bonavina, A. Roul & H. Stein. Esophageal cancer: a European perspective Recent Results. Cancer Res 2000;155:119-22.

8. L.R. Coia. Chemoradioation as primary management of esophageal cancer. *Semin Oncol*, 21,483-92 (1994)

9. A.A. Forastiere, M.B. Orringer, C. Perez-Tamayo, S.G. Urba & M. Zahurak. Preoperative chemoradiation followed by trans-hiatal esophagectomy for carcinoma of the esophagus: final report. *J Clin Oncol*, 11,1118-23 (1993)

10. A. Ohtsu, S. Yoshida, N. Boku, T. Fujii, Y. Miyata, K. Hosokawa, I. Koba, W. Shimizu & T. Ogino. Concurrent chemotherapy and radiation therapy for locally advanced carcinoma of the esophagus. *Jpn J Clin Oncol*, 25,261-6 (1995)

11. A. Ohtsu, N. Boku, K. Muro, K. Chin, M. Muto, S. Yoshida, M. Satake, S. Ishikura, T. Ogino, Y. Miyata, S. Seki, K. Kaneko & A. Nakamura. Definitive chemoradiotherapy for T4 and/or M1 lymph node squamous cell carcinoma of the esophagus. *J Clin Oncol*, 17,2915-21 (1999)

12. K. Kaneko, H. Ito, K. Konishi, T. Kurahashi, T. Ito, A. Katagiri, T. Yamamoto, T. Kitahara, Y. Mizutani, A. Ohtsu & K. Mitamura. Definitive chemoradiotherapy for patients with malignant stricture due to T3 or T4 squamous cell carcinoma of the oesophagus. *Br J Cancer*, 88,18-24 (2003)

13. A.J. Levine. p53, cellular gatekeeper for growth and divison. *Cell*, 88,323-31 (1997)

14. M. Waller. Predicting response to cancer chemotherapy: the role of *p53*. *Cell Tissue Res*, 292,435-45 (1998)

15. G. Carpenter & S. Cohen. Epidermal growth factor. *J Biol Chem*, 265,7709-12 (1990)

16. F.X. Real, W.J. Rettig, P.G. Chesa, M.R. Melamed, L.J. Old & J. Mendelsohn. Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation. *Cancer Res*, 46,4726-31 (1986)

17. F. Ciardiello & G. Tortora. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res*, 7,2958-70 (2001)

18. T.J. Lynch, D.W. Bell, R. Sordella, S. Gurubhagavatula, R.A. Okimoto, B.W. Brannigan, P.L. Harris, S.M. Haserlat, J.G. Supko, F.G. Haluska, D.N. Louis, D.C. Christiani, J. Settleman & D.A. Haber. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*, 350,2129-39 (2004)

19. J.G. Paez, P.A. Janne, J.C. Lee, S. Tracy, H. Greulich, S. Gabriel, P. Herman, F.J. Kaye, N. Lindeman, T.J. Boggon, K. Naoki, H. Sasaki, Y. Fujii, M.J. Eck, W.R. Sellers, B.E. Johnson & M. Meyerson. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*, 304,1497-500 (2004)

20. W. Pao, V. Miller, M. Zakowski, J. Doherty, K. Politi, I. Sarkaria, B. Singh, R. Heelan, V. Rusch, L. Fulton, E. Mardis, D. Kupfer, R. Wilson, M. Kris & H. Varmus. EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA*, 101,13306-11 (2004)

21. S.F. Huang, H.P. Liu, L.H. Li, Y.C. Ku, Y.N. Fu, H.Y. Tsai, Y.T. Chen, Y.F. Lin, W.C. Chang, H.P. Kuo, Y.C. Wu, Y.R. Chen & S.F. Tsai. High frequency of epidermal growth factor receptor mutations with complex patterns in non-small cell lung cancers related to gefitinib responsiveness in Taiwan. *Clin Cancer Res*, 10,8195-203 (2004)

22. T. Kosaka, Y. Yatabe, H. Endoh, H. Kuwano, T. Takahashi & T. Mitsudomi. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res*, 64,8919-23 (2004)

23. H. Shigematsu, L. Lin, T. Takahashi, M. Nomura, M. Suzuki, I.I. Wistuba, K.M. Fong, H. Lee, S. Toyooka, N. Shimizu, T. Fujisawa, Z. Feng, J.A. Roth, J. Herz, J.D. Minna & A.F. Gazdar. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst*, 97,339-46 (2005)

24. M. Tokumo, S. Toyooka, K. Kiura, H. Shigematsu, K. Tomii, M. Aoe, K. Ichimura, T. Tsuda, M. Yano, K. Tsukuda, M. Tabata, H. Ueoka, M. Tanimoto, H. Date, A.F. Gazdar & N. Shimizu. The relationship between epidermal growth factor receptor mutations and clinicopathologic features in nonsmall cell lung cancers. *Clin Cancer Res*, 11,1167-73 (2005)

25. E.A. Perez. The role of adjuvant monoclonal antibody therapy for breast cancer: rationale and new studies. *Curr Oncol Rep*, 3,516–22 (2001)

26. A.W. Hemming, N.L. Davis, A. Kluftinger, B. Robinson, N.F. Quenville, B. Liseman & J. LeRiche. Prognostic markers of colorectal cancer: an evaluation of DNA content, epidermal growth factor receptor, and Ki-67. *J Surg Oncol*, 51,147-52 (1992)

27. L. Gibault, J.P. Metges, V. Conan-Charlet, P. Lozac'h, M. Robaszkiewicz, C. Bessaguet, N. Lagarde & A. Volant. Diffuse EGFR staining is associated with reduced overall survival in locally advanced oesophageal squamous cell cancer. *Br J Cancer*, 93,107-15 (2005)

28. R.I. Nicholson, I.R. Hutcheson & M.E. Harper. Modulation of epidermal growth factor receptor in endocrine-resistant, oestrogen receptor-positive breast cancer. *Endocr Relat Cancer*, 8,175-82 (2001)

29. Y. Itakura, H. Sasano, C. Shiga, Y. Furukawa, K. Shiga, S. Mori & H. Nagura. Epidermal growth factor receptor overexpression in esophageal carcinoma. An

immunohistochemical study correlated with clinicopathologic findings and DNA amplification. *Cancer*, 74,795-804 (1994)

30. A. Katagiri, K. Kaneko, K. Konishi, H. Ito, M. Kushima & K. Mitamura. Lugol staining pattern in background epithelium of patients with esophageal squamous cell carcinoma. *Hepatogastroenterol*, 51,713-7 (2004)

31. S.E. Goelz, S.R. Hamilton & B. Vogelstein. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochem Biophys Res Commun,* 130,118-26 (1985)

32. Single Nucleotide Polymorphism: SNP linked to Gene EGFR (geneID:1956) Via Contig Annotation. http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=1956

33. M.S. Greenblatt, W.P. Bennett, M. Hollstein & C.C. Harris. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*, 54,4855-78 (1994)

34. J.E. Choi, S.H. Park, K.M. Kim, W.K. Lee, S. Kam, S.I. Cha, C.H. Kim, Y.M. Kang, Y.C. Kim, S.B. Han, T.H. Jung & J.Y. Park. Polymorphisms in the epidermal growth factor receptor gene and the risk of primary lung cancer: a case-control study. *BMC Cancer*, 7,199 (2007)

35. J.G. Paez, P.A. Janne, J.C. Lee, T. Sean, G. Heidi, S. Gabriel, P. Herman, F.J. Kaye, N. Lindeman, T.J. Boggon, K. Naoki, H. Sasaki, Y. Fujii, M.J. Eck, W.R. Sellers, B.E. Johnson & M. Meyerson. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*, 304,1497-500 (2004)

36. T.J. Lynch, D.W. Bell, R. Sordella, S. Gurubhagavatula, R.A. Okimoto, B.W. Brannigan, P.L. Harris, S.M. Haserlat, J.G. Supko, F.G. Haluska, D.N. Louis, D.C. Christiani, J. Settleman & D.A. Haber. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*, 350,2129-39 (2004)

37. K.S. Kim, J.Y. Jeong, Y.C. Kim, K.J. Na, Y.H. Kim, S.J. Ahn, S.M. Baek, C.S. Park, C.M. Park, Y.I. Kim, S.C. Lim & K.O. Park. Predictors of the Response to Gefitinib in Refractory Non-Small Cell Lung Cancer. *Clin Cancer Res*, 11,2244-51 (2005)

38. M. Shahbazi, V. Pravica, N. Nasreen, N. Fakhoury, A. Fryer, R. Strange, P. Hutchinson, J. Osborne, J. Lear & A. Smith. Association between functional polymorphism in EGF gene and malignant melanoma. *Lancet*, 359,397-401 (2002)

39. J. Bange, D. Prechtl, Y. Cheburkin, K. Specht, N. Harbeck, M. Schmitt, T. Knyazeva, S. Müller, S. Gärtner, I. Sures, H. Wang, E. Imyanitov, H.U. Häring, P. Knayzev, S. Iacobelli, H. Höfler & A. Ullrich. Cancer progression and tumor cell motility are associated with the FGFR4 Arg(388) allele. *Cancer Res*, 62,840-7 (2002)

40. R. McKean-Cowdin, L.N. Kolonel, M.F. Press, M.C. Pike & B.E. Henderson. Germ-line HER-2 variant and breast cancer risk by stage of disease. *Cancer Res*, 61,8393-4 (2001)

41. P. Iughetti, O. Suzuki, P.H. Godoi, V. A. Alves, A. L. Sertié, T. Zorick, F. Soares, A. Camargo, E.S. Moreira, C. di Loreto, C.A. Moreira-Filho, A. Simpson, G. Oliva & M.R. Passos-Bueno. A polymorphism in endostatin, an angiogenesis inhibitor, predisposes for the development of prostatic adenocarcinoma. *Cancer Res*, 61,7375-8 (2001)

42. E. Papadopoulou, G. Tripsianis, K. Anagnostopoulos, I. Tentes, S. Kakolyris, G. Galazios, E. Sivridis, K. Simopoulos & A. Kortsaris. The influence of serum HER-2 levels and HER-2 codon 655 polymorphism on breast cancer outcome. *Neoplasma*, 55,113-21 (2008)

43. M. Hanawa, S. Suzuki, Y. Dobashi, T. Yamane, K. Kono, N. Enomoto & A. Ooi. EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. *Int J Cancer*, 118,1173-80 (2006)

44. P. Sunpaweravong, S. Sunpaweravong, P. Puttawibul, W. Mitarnun, C. Zeng, A.E. Baro´n, W. Franklin, S. Said & M. Varella-Garcia. Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol*, 131,111-9 (2005)

45. Q. Wei, L. Chen, L. Sheng, H. Nordgren, K. Wester & J. Carlsson. EGFR, HER2 and HER3 expression in esophageal primary tumours and corresponding metastasis. *Int J Oncol*, 31,493-9 (2007)

Abbreviations: cisplatin (CDDP); complete response (CR); 95% confidence interval (CI); esophageal squamous cell carcinoma (ESCC); chemoradiotherapy (CRT); epidermal growth factor receptor (EGFR); 5 fluorouracil (5-FU); International Union against Cancer (UICC); lymph node (LYM); odds ratio (OR); polymerase chain reaction (PCR); single nucleotide polymorphism (SNP); tumor-node-metastasis (TNM); tyrosine kinase (TK); World Health Organization (WHO)

Key Words: *EGFR,* SNP, Esophageal Squamous Cell Carcinoma, Prognosis

Send correspondence to: Kazuhiro Kaneko, Division of Digestive Endoscopy/Gastrointestinal Oncology, National Cancer Center Hospital East, Kashiwanoha 6-5-1, Kashiwa, Chiba 277-8577, Japan, Tel: 81-4-7133-1111, Fax: 81-4-7134-6928, E-mail: kkaneko@east.ncc.go.jp

http://www.bioscience.org/current/vol15.htm