Gene amplification of EGFR, HER2, FGFR2 and MET in esophageal squamous cell carcinoma

HIROAKI KATO1, TOKUZO ARAO2, KAZUKO MATSUMOTO2, YOSHIHIKO FUJITA2, HIDEHARU KIMURA2, HIDETOSHI HAYASHI2, KOUHEI NISHIKI1, MITSURU IWAMA1, OSAMU SHIRAISHI1, ATSUSHI YASUDA1, MASAYUKI SHINKAI1, MOTOHIRO IMANO1, HARUHIKO IMAMOTO1, TAKUSHI YASUDA1, KIYOTAKA OKUNO1, HITOSHI SHIOZAKI1 and KAZUTO NISHIO2

Departments of 1Surgery and 2Genome Biology, Kinki University Faculty of Medicine, Osaka 589-8511, Japan

Received July 30, 2012; Accepted September 28, 2012

DOI: 10.3892/ijo.2013.1830

Abstract. Molecular targeted therapy is expected to be a promising therapeutic approach for the treatment of esophageal squamous cell carcinoma (ESCC); however, the gene amplification status of molecular targeted genes in ESCC remains largely unclear. The gene amplification of EGFR, HER2, FGFR2 and MET was examined using a real-time PCR-based copy number assay of 245 ESCC surgical specimens of formalin-fixed, paraffin-embedded samples. Fluorescence in situ hybridization (FISH) and comparative genomic hybridization analyses verified the results of the copy number assay. EGFR mutation was detected using the Scorpions-ARMS method. The EGFR status and drug sensitivity to an EGFR tyrosine kinase inhibitor was then evaluated in vitro. Gene amplification of EGFR and HER2 was observed in 7% (16/244) and 11% (27/245) of the ESCC specimens. A multivariate analysis revealed that HER2 amplification was a significant predictor of a poor prognosis in patients with stage III post-operative ESCC. The L861Q type of EGFR mutation with hypersensitivity to EGFR tyrosine kinase inhibitor was found in one of the eight ESCC cell lines and one del745 type of EGFR mutation was identified in 107 clinical specimens. In addition, we demonstrated for the first time that FGFR2 amplification was observed in 4% (8/196) of the ESCC specimens. MET amplification was observed in 1% (2/196). In conclusion, the frequent gene amplification of EGFR, HER2 and FGFR2 and the presence of active EGFR mutations were observed in ESCC specimens. Our results strongly encourage the development of molecular targeted therapy for ESCC.

Introduction

Despite extensive investigations of therapeutic improvements in surgical techniques, chemotherapy and chemo-radiotherapy, esophageal squamous cell carcinoma (ESCC) remains one of the most aggressive and fatal malignancies and the prognosis of patients with ESCC remains poor (1). Although curative surgical resection can be performed, half of all patients develop recurrences within a few years after surgery and the 5-year survival rate is only approximately 50% (2). Therefore, more effective therapies are urgently needed to improve the prognosis of patients with ESCC.

The overexpression of epidermal growth factor receptor (EGFR) and HER2 can be observed in a variety of human malignancies and the roles of such overexpressions in cancer development, progression and aggressiveness have been widely recognized (3,4). Approximately 50-70% of ESCC tumors express EGFR protein when examined using immunohistochemistry (IHC), while 15-28% of specimens exhibit EGFR gene amplification when examined using fluorescence in situ hybridization (FISH) (5,6). Similarly, HER2 protein overexpression has been observed in 30-41% of specimens examined using IHC, while HER2 gene amplification has been observed in 11-19% of specimens using FISH (7,9). These results indicate that EGFR and HER2 overexpression and gene amplification are frequently observed in ESCC, strongly suggesting that signaling involving these factors may play important biological roles and may be useful molecular targets in ESCC. Somatic mutations of EGFR tyrosine kinase in non-small cell lung cancer (NSCLC) have been shown to increase kinase activity and to be associated with hypersensitivity to gefitinib, a selective EGFR tyrosine kinase inhibitor (EGFR-TKI) (10,11). A recent phase III study demonstrated that first-line gefitinib for patients with advanced NSCLC with EGFR mutations improved progression-free survival, compared with standard chemotherapy (12). Therapeutics targeting EGFR and HER2, such as small-molecule inhibitors or specific monoclonal antibodies, are now under intensive investigation in clinical settings and some of them have achieved clinical success in the treatment of diverse solid cancers (4,13).

Fibroblast growth factor receptor (FGFR) signaling is deregulated in a wide variety of cancers (14). We previously reported that FGFR2 amplification was observed in 4.1% of gastric cancers and that FGFR2 amplification confers hypersensitivity to FGFR inhibitor in gastric cancer cell lines both in vitro and in vivo (15,16), strongly suggesting that FGFR2 amplification...
may be a promising molecular target for gastric cancer treatment. In ESCC, information regarding FGFR2 amplification remains unclear. Additionally, hepatocyte growth factor (HGF)-MET receptor signaling provides important signals for cell survival and migration in cancer cells; thus, these molecules have also emerged as promising molecular targets for cancer therapy (17).

Very limited information is available regarding the gene amplification of EGFR, HER2, FGFR2 and MET and the EGFR mutation status in relation to the prognostic impact for post-curative surgery in ESCC. In an attempt to advance molecular-targeted therapy for ESCC, we retrospectively studied these issues using formalin-fixed, paraffin-embedded (FFPE) samples from patients with ESCC who had undergone surgery.

Materials and methods

Cell culture. KYSE170, KYSE180 and KYSE270 were maintained in a 1:1 mixture of Ham's F12 medium and RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY). T.T. was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) and Ham's F12 medium with 10% FBS. KYSE30 and KYSE50 were maintained in DMEM with 10% FBS. KYSE70 was maintained in DMEM with 2% FBS. KYSE150 was maintained in Ham's F12 with 2% FBS.

Patients. This study was performed retrospectively. The criteria for eligibility were histologically confirmed ESCC, surgery for stage I-III disease, absence of prior radiotherapy or chemotherapy before surgery and the availability of a FFPE sample. Tumor specimens were collected from 246 patients with ESCC who were treated at the Kinki University Faculty of Medicine between 2001 and 2011. One sample was excluded because of poor DNA quality and 245 ESCC samples were finally evaluated. The World Health Organization Classification of Tumors of the University Faculty of Medicine was approved by the institutional review board of the Kinki University Faculty of Medicine.

Isolation of genomic DNA. Macro-dissection of the surgical specimen was performed before deparaffinization to select a region of cancer tissue. Genomic DNA samples were extracted using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA).

Copy number assay. The DNA copy numbers of EGFR, HER2, FGFR2 and MET were determined using commercially available and pre-designed TaqMan Copy Number Assays (Applied Biosystems, Foster City, CA), as described previously (15). The primer IDs used in this study were as follows: EGFR, Hs00997424_cn; HER2, Hs05475431_cn; FGFR2, Hs05182482_cn (introns 14 and 15); and MET, Hs05005660_cn (introns 16 and 17). The TERT locus was used for the internal reference copy number. Human genomic DNA (Takara, Otsu, Japan) and DNA from non-cancer FFPE tissue were used as normal controls. The PCR analysis was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and the results were analyzed using SDS 2.2 and CopyCaller software (Applied Biosystems).

FISH analysis. FISH analysis of EGFR and HER2 amplification was performed using the Vysis EGFR/CEP7 FISH Probe Kit (Abbott Laboratories, Abbott Park, IL) or the PathVysions HER2 DNA Probe Kit (Abbott Laboratories), according to the manufacturer's instructions. Amplification was determined based on a HER2/CEP17 signal ratio of >2.2. A two or more increase in the EGFR gene signal relative to the CEPI7 signal was considered to indicate gene amplification. The FGFR2-FISH method has been previously described (15), as has the MET-FISH method (18).

Detection of EGFR mutations. EGFR mutations (exons 18-21) were detected using the Therascreen RQ PCR kit (Qiagen), which combines Scorpions technology and the amplified refractory mutation system (ARMS) to detect mutations using real-time PCR. This sensitive method can detect 29 types of active mutations in the EGFR gene. All the reactions were performed according to the manufacturer's instructions, as previously described (19).

Cell growth inhibitory assay. To evaluate growth inhibition in the presence of various concentrations of EGFR-TKI AG1478 (Sigma), we used an MTT assay and a previously described method (20). Briefly, the cells were seeded at a density of 2x10^3 cells/well in 96-well plates. Twenty-four hours later, AG1478 was added and the incubation was further continued for 72 h at 37˚C. The assay was conducted in triplicate.

Immunoblotting. A western blot analysis was performed as described previously (21). The following antibodies were used: polyclonal EGFR antibody, polyclonal phospho-EGFR antibody, polyclonal HER2 antibody, monoclonal HER4 antibody, polyclonal Akt antibody, monoclonal phosphor-Akt antibody, polyclonal p44/42 MAPK antibody, polyclonal phospho-p44/42 MAPK antibody, β-actin antibody and HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA); and monoclonal HER3 antibody (Upstate Biotechnology, Lake Placid, NY). The cells were cultured overnight in serum-starved medium and exposed to 0.1-10 µmol/l of AG1478 for 3 h before the addition of EGF (10 ng/ml) for 15 min.

Comparative genomic hybridization (CGH) analysis. The CGH analysis was performed using a SurePrint G3 Human CGH Microarray (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. For the analysis, 0.2 µg of DNA was extracted from each FFPE sample of ESCC and an FGFR2-amplified tumor or a non-cancer tissue were used as a control. The copy number changes were analyzed using Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO).

Statistical analysis. The prognostic analyses of the clinicopathological features and molecular factors were performed using a Cox regression. In the multivariate Cox models, the variable selection was based on the presence of significance (P<0.10) in a univariate analysis; variables that were not significant in
the final model were removed using the stepwise method. The disease-free survival (DFS) and overall survival (OS) curves were constructed using the Kaplan-Meier method and were compared using the log-rank test. Statistical analyses were performed using PAWS Statistics 18 (SPSS Japan Inc., Tokyo, Japan).

**Results**

**Patient results.** Of the 245 patients evaluated in this study, all the patients had undergone surgery for histologically confirmed stage I-III ESCC. The patient characteristics are shown in Table I. The
percentages of the pathological stages were as follows: stage I, 24%; stage II, 27%; and stage III, 49%. Fourteen (6%) patients had residual cancer at the time of surgery and tumor recurrence occurred in 98 (42%) patients. The median follow-up period was 24 months (range 0-126 months).

**Gene amplification of EGFR and HER2 in ESCC.** To determine the gene amplification of EGFR and HER2 in FFPE samples, we used a high-throughput and real-time PCR-based copy number assay, as previously reported (15). Gene amplification was defined as more than four copies. The copy number assay showed that EGFR and HER2 were amplified in 7% (16/244, one not determined; range 0.6-52.8 copies) and 11% (27/245; range 0.4-185.0 copies) of the ESCC specimens, respectively (Fig. 1A). FISH analysis demonstrated that the EGFR/CEP7 signal ratio was increased in EGFR-amplified samples, while the ratio was not increased in a non-amplified sample (Fig. 1B). Similarly, the HER2/CEP17 signal ratio was consistent with the results of a copy number assay for HER2. FISH analysis verified the results of the copy number assays for EGFR and HER2.

**Prognostic impact of clinicopathological and gene amplification in ESCC.** Of the 121 patients with stage III ESCC, 14 were excluded because of residual cancer and three were excluded because of the lack of copy number results; finally, 104 patients with stage III ESCC were evaluated to determine the prognostic impact of post-operative ESCC findings. The correlations between clinicopathological features, including age, sex, pathological tumor stage, pathological lymph node stage, tumor differentiation, lymphatic vessel invasion (Ly), vascular invasion (V) and the gene amplification statuses of EGFR and HER2 and the DFS or OS were evaluated. A univariate analysis showed that the pathological lymph node stage, Ly grade, V grade and HER2 amplification status were significant predictors of a poor DFS (Table II). A multivariate analysis revealed that the pathological lymph node stage (P=0.00003) and HER2 amplification (P=0.021) were significant predictors of a poor DFS. Meanwhile, the pathological lymph node stage, tumor differentiation, Ly grade and V grade were significant predictors of a poor OS. A multivariate analysis demonstrated that the pathological lymph node stage (P=0.004) was a significant predictor of a poor OS. The Kaplan-Meier curves for DFS and OS plotted according to the gene amplification status are shown in Fig. 1C. These results indicated that HER2 amplification, but not EGFR amplification, was a predictor of a poor outcome among post-operative patients with stage III ESCC in the present study.

**Active EGFR mutation in ESCC cell lines and clinical samples.** We next examined the growth inhibitory effect of the EGFR-TKI AG1478 against eight ESCC cell lines to evaluate the effect of EGFR-TKI treatment on ESCC. Notably, the KYSE270 cells were hypersensitive to AG1478 at a sub-micro molar level of
IC_{50} (0.45 µM), which is similar to the hypersensitivity of lung cancer cells harboring an EGFR mutation (Fig. 2A). The possible presence of 29 types of EGFR mutations in the eight ESCC cell lines was examined using the Scorpion-ARMS method. The KYSE270 cells, which exhibited hypersensitivity to AG1478, harbored the L861Q type of EGFR mutation, whereas the other cell lines did not exhibit any EGFR mutations. (C) The TaqMan copy number assay was used to determine the copy numbers of EGFR and HER2 in ESCC cell lines. (D and E) Western blot analysis for EGFR, HER2, HER3, HER4 and phospho-EGFR expression in ESCC cell lines. β-actin was used as an internal control. Marker, molecular marker. Western blot analysis for expression levels of EGFR, phospho-EGFR, MAPK, phospho-MAPK, AKT and phospho-AKT in KYSE270 cells (L861Q) and KYSE170 cells (EGFR wild-type). The cells were exposed to AG1478 at the indicated concentrations for 3 h and then were stimulated with 10 ng/ml of EGF. β-actin was used as an internal control. M, molecular marker. (F) Among the 107 clinical ESCC samples that were evaluated, one (no. 117) carried a del745-750 type EGFR mutation.

Figure 2. EGFR mutation in esophageal squamous cell carcinoma (ESCC) cell lines and clinical samples. (A) Growth inhibition in response to the EGFR tyrosine kinase inhibitor AG1478 was evaluated at the indicated concentrations using an MTT assay. (B) The status of the 29 types of EGFR mutation determined using the Scorpion-ARMS method in eight ESCC cell lines. Notably, KYSE270 cells, which were hypersensitive to AG1478, harbored the L861Q type of EGFR mutation, whereas the other cell lines did not exhibit any EGFR mutations. (C) The TaqMan copy number assay was used to determine the copy numbers of EGFR and HER2 in ESCC cell lines. (D and E) Western blot analysis for EGFR, HER2, HER3, HER4 and phospho-EGFR expression in ESCC cell lines. β-actin was used as an internal control. Marker, molecular marker. Western blot analysis for expression levels of EGFR, phospho-EGFR, MAPK, phospho-MAPK, AKT and phospho-AKT in KYSE270 cells (L861Q) and KYSE170 cells (EGFR wild-type). The cells were exposed to AG1478 at the indicated concentrations for 3 h and then were stimulated with 10 ng/ml of EGF. β-actin was used as an internal control. M, molecular marker. (F) Among the 107 clinical ESCC samples that were evaluated, one (no. 117) carried a del745-750 type EGFR mutation.

ESCC tumor exhibited a del745-750 type of EGFR mutation (Fig. 2F). Thus, although the frequency of EGFR mutation was not high compared with NSCLC, we did find a mutation in a cell line and in a clinical sample of ESCC.

Gene amplification of FGFR2 and MET in ESCC. To gain insight into molecular therapy targeting FGFR2 or MET amplification in ESCC, we evaluated the amplifications of these genes. FGFR2 and MET were amplified in 4% (8/196; range 0.4-13.8 copies) and 1% (2/196; range 0.4-7.7 copies) of the ESCC specimens, respectively (Fig. 3A). A FISH analysis confirmed the FGFR2 and MET amplification (Fig. 3B). A CGH analysis showed that the FGFR2 locus was amplified in FGFR2-amplified ESCC and that the amplicon seemed to consist of a relatively narrow region (Fig. 3C). The clinical features of FGFR2-amplified or MET-amplified ESCC are shown in Table III. Although the numbers of amplified cases were relatively small and, accordingly, definitive evidence could not be obtained, patients who had tumors with FGFR2 or MET amplification seemed to have no significant trends regarding clinicopathological factors, including patient outcome. Collectively, these findings indicate...
that FGFR2 amplification is present but that MET amplification is rare in ESCC.

Discussion

FGFR2 is frequently amplified in gastric cancer cell lines, especially in poorly differentiated type cells and amplification confers hypersensitivity to FGFR inhibitors (16,22). Regarding the mutation of FGFR2, somatic mutations of FGFR2 have been found in 12% (15/122) of endometrial carcinomas and these FGFR2 mutations have an oncogenic property that confers hypersensitivity to FGFR inhibitors (23). We demonstrated, for the first time, that FGFR2 amplification was observed in ESCC. Our findings provide novel insight into FGFR-targeting therapy and further prospective studies evaluating FGFR2 amplification in ESCC are needed. Meanwhile, recent study has demonstrated that 2% of patients (10/489) with esophagogastric adenocarcinoma harbored MET amplification and two of four patients with MET-amplified tumors treated with a MET inhibitor experienced tumor shrinkage (24). Although MET amplification is rare in ESCC, MET-targeted therapy may be a useful therapeutic approach in some cases.

The presence of active EGFR mutations or drug sensitivity to EGFR-TKI in ESCC cells remains unknown (25). We found that one out of eight ESCC cell lines harbored an L861Q mutation with hypersensitivity to EGFR-TKI and 1% (1/107) of clinical ESCC samples had a del745-750 type of mutation when examined using a highly sensitive detection method. In an EGFR-vIII-based method of overexpression, L861Q mutation reportedly enhances EGFR kinase activity and transforms activity without an increase in sensitivity to EGFR-TKI but with an increase in sensitivity to irreversible second-generation EGFR-TKI (26). Our results indicate that L861Q is an active mutation to EGFR-TKIs in ESCC cell lines; however, the 1% frequency of EGFR mutation in ESCC makes it difficult to stratify patients who may benefit from EGFR-TKI treatment, compared with NSCLC. For HER2-positive advanced gastric or gastroesophageal junction cancer, recent advances in the clinical development of molecular targeted therapy have enabled the use of trastuzumab as a standard therapy (27); however, similar regimens for the treatment of ESCC remain elusive. EGFR family-targeted therapy is considered to be the most promising approach to date, because EGFR and HER2 overexpression and amplification are frequently observed in ESCC. The anti-EGFR antibody cetuximab used in combination with radiotherapy or chemotherapy exhibited a significant clinical benefit when used against head and neck squamous cell carcinoma (28,29), leading to an ongoing intensive clinical trial using cetuximab for the treatment of ESCC. We detected EGFR and HER2 amplification in 7 and 11% of ESCC specimens and our results support a rationale for introducing anti-EGFR and anti-HER2 antibody therapies to the treatment of patients with ESCC.

In conclusion, we determined the frequency of EGFR, HER2, FGFR2 and MET amplification in ESCC and the pres-
AZD2171 shows potent Epidermal growth factor receptor (EGFR) inhibition in ESCC cell lines and clinical samples. Our results warrant serious consideration of the development of EGFR family inhibitors and FGFR-targeted therapies for ESCC exhibiting gene amplification.

**Acknowledgements**

We thank Ms. Tomoko Kitayama and Ms. Fusako Kamada for their technical assistance. This study was supported by the Third-Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare.

**References**