Biomarker analysis in stage III–IV (M0) gastric cancer patients who received curative surgery followed by adjuvant 5-fluorouracil and cisplatin chemotherapy: epidermal growth factor receptor (EGFR) associated with favourable survival

The aim of this study was to analyse the impact of epidermal growth factor receptor (EGFR), thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), aurora kinase (ARX) A/B, and excision repair cross-complementing gene 1 (ERCC1) on the efficacy of adjuvant chemotherapy with 5-fluorouracil and cisplatin (FP) after curative gastric resection. Normal and cancer tissue were separately obtained from gastrectomy samples of 153 patients with AJCC stage III–IV (M0) who subsequently treated with adjuvant FP chemotherapy. Normal and cancer tissue were separately obtained from gastrectomy samples of 153 patients with AJCC stage III–IV (M0) who subsequently treated with adjuvant FP chemotherapy. Immunohistochemistry (IHC), EGFR expression was investigated using a standardized IHC with the EGFR PharmDx assay. Amplification of EGFR gene was analysed using fluorescent in situ hybridisation (FISH). In multivariate analysis, stage, ratio of positive to removed lymph nodes, and EGFR expression were significant prognostic factors for overall survival. Patients with higher EGFR expression had better overall survival than those with lower expression (relative risk: 0.475 (95% confidence interval, 0.282–0.791, \( P = 0.005 \)). Low EGFR expression might be a predictive marker for relapse in curative resected stage III–IV (M0) gastric cancer patients who received adjuvant FP chemotherapy.

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The prognostic and predictive roles of epidermal growth factor receptor (EGFR) expression in gastric cancer remain controversial, and the reported frequencies of EGFR expression are varied in gastric cancer (Gamboa-Dominguez et al, 2004; Matsubara et al, 2008b). In the past, high levels of EGFR were reported as a poor prognostic factor for overall survival (OS) in resectable gastric cancer patients who did not receive chemotherapy (Garcia et al, 2003; Galizia et al, 2007). In contrast, high levels of EGFR were reported as a positive prognostic factor in patient group who received 5-fluorouracil (FU)-containing chemotherapy (Al-Batran et al, 2008a; Matsubara et al, 2008a). Thymidylate synthase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD) are known key enzymes in the metabolism of 5-FU and play a role in resistance to fluoropyrimidines. Thymidylate synthase expression level is presumed to influence response to 5-FU-containing chemotherapy, although TS is not unanimously recognised as a determinant of 5-FU sensitivity (Danenberg, 2004; Park and Lenz, 2006). Thymidine phosphorylase catalyses the reversible phosphorylation of thymidine to thymine 2-deoxyribose-1-phosphate, and increases the conversion of 5-FU to its active metabolites, which play an important role in the inhibition of TS (Tahara et al, 2004). Dihydropyrimidine dehydrogenase is the initial and rate-limiting enzyme in the catabolism of 5-FU. Although the role of DPD levels in tumours have not been firmly established as a prognostic factor for clinical responsiveness, there is ample evidence that a DPD deficiency is associated with severe toxicity after 5-FU administration (van Kuilenburg, 2004). Expression of the excision repair cross-complementing gene 1 (ERCC1) may play a role in human tumours because it is essential for nucleotide excision repair and influences genomic instability (Chen et al, 2000). For example, low gene expression levels of ERCC1 were associated with a superior response to 5-FU and cisplatin chemotherapy (FP) in primary gastric cancer (Metzger et al, 1998), and ERCC1 protein expression levels were found to be inversely associated with response. Excision repair cross-complementing gene 1 may possibly have a role in the clinical resistance
to platinum compounds in gastric cancer patients (Kwon et al., 2007). The Aurora kinases, a family of mitotic regulators, have received much attention as potential targets of new drugs (Warner et al., 2006) and in their association with chemoresistance to platinum agents (Yang et al., 2006). However, none of these markers have previously been evaluated in an adjuvant setting in high-risk gastric cancer patients undergoing potentially curative surgery.

MATERIALS AND METHODS

Study population

From the database of Seoul National University Hospital, we identified a total of 5387 patients who underwent gastrectomy between November 1995 and November 2003. Patients with a diagnosis of histologically proven gastric cancer, who received a curative gastrectomy with D2 dissection and adjuvant chemotherapy consisting of 5-FU and cisplatin were identified. Cisplatin (60 mg/m$^2$ as 15 min i.v. infusion) followed by 5-FU (1200 mg/m$^2$ as 12 h continuous i.v. infusion for 4 days) was given in 21-day cycles. The following eligibility criteria were used for patients’ enrollment: age < 75 years; free from distant metastatic disease; stages IIIa, IIIb, and IV (only non-metastatic cases, T4 N1-3 and T1-3 N3; AJCC Cancer Staging, 6th edition); no prior chemotherapy or radiotherapy; World Health Organisation (WHO) performance status ≤ 2; adequate baseline organ function, defined as WBC count ≥ 4000 cells per ml, platelet count ≥ 100,000 cells per ml, serum bilirubin level ≤ 1.5 mg/dl, serum creatinine level ≤ 2.0 mg/100 ml, serum albumin level ≥ 3.0 mg/100 ml, no severe uncontrolled comorbidities (e.g., myocardial infarction in the last 12 months); no second malignancies; and informed consent.

Patient follow-up

In the absence of symptoms, physical examination was performed every 3–4 months for 5 consecutive years. Follow-up assessment consisted of physical examination, a complete blood count, liver function test, chest radiography, and abdominal ultrasound or CT scan, every 3–6 months for 5 years. Toxicities were graded according to NCI-CTC version 2. The site and date of the first relapse and the date of death, if the patient died, were recorded. Survival was calculated from beginning of surgery until the last follow-up or death from any cause; patients who were alive at the last follow-up or death from any cause; patients who were alive at the last follow-up were censored at that time. Patients who were taken off study or who died before progression were censored at the time when they were taken off from the study. Survival data were confirmed either by medical records or by the death reports from the Korea Central Cancer Registry.

Tissue sampling

Cancerous and adjacent normal tissues were obtained from the surgically resected and paraffin-embedded primary gastric cancer specimens of the patients. Samples were examined histologically for the presence of tumour cells.

Tissue microarray methods

Core tissue biopsy specimens (2 mm in diameter) were obtained from individual paraffin-embedded gastric tumours (donor blocks) and arranged in a new recipient paraffin block (tissue
array block) using a trephine apparatus (SuperBiochips Laboratories, Seoul, Korea). Each tissue array block contained up to 60 cases, allowing a total of 153 pairs to be contained in six array blocks. An adequate case was defined as one with a tumour occupying more than 10% of the core area. Each block contained an internal control consisting of non-neoplastic gastric mucosa from adjacent tissue. Sections of 4 μm were cut from each tissue array block, deparaffinised, and dehydrated. As shown earlier, staining results obtained from different intratumoural areas in various cancers correspond with each other (Lee et al, 2007), and a core was sampled in each case.

Immunohistochemistry (IHC)

Immunohistochemical staining for TS, TP, and ERCC1 was performed using an ABC method (labelled biotin) after a microwave antigen retrieval process. Mouse monoclonal antibodies of TS, TP, and ERCC1 were obtained from Neomarkers, Fremont, CA, USA. Staining intensity and stained tumour cell percentages were measured. Stained cell percentage was multiplied by the staining intensity (0–3+), which resulted in an IHC score ranging from 0 to 300 for each cell type, as described previously.

**Table 1** Characteristics of patients and tumours

| Characteristics          | Number | %  |
|--------------------------|--------|----|
| All patients             | 153    |    |
| Age (years)              |        |    |
| Median                   | 52     |    |
| Range                    | 15–72  |    |
| Sex                      |        |    |
| Male                     | 105    | 68.6|
| Female                   | 48     | 31.4|
| ECOG performance status |        |    |
| 0–1                      | 132    | 91.5|
| 2                        | 13     | 8.5 |
| Operation                |        |    |
| Subtotal gastrectomy     | 61     | 39.9|
| Total gastrectomy        | 92     | 60.1|
| Location                 |        |    |
| Proximal                 | 30     | 19.6|
| Distal                   | 123    | 80.4|
| Pathology                |        |    |
| Adenocarcinoma           | 135    | 88.2|
| Signet ring cell carcinoma | 18   | 11.8|
| Lauren classification    |        |    |
| Intestinal               | 44     | 28.8|
| Diffuse                  | 86     | 56.2|
| Mixed                    | 19     | 12.4|
| Borrmann type            |        |    |
| 1                        | 1      | 0.7 |
| 2                        | 13     | 8.5 |
| 3                        | 95     | 62.1|
| 4                        | 44     | 28.8|
| Stage*                   |        |    |
| IIIa                     | 51     | 33.3|
| IIIb                     | 32     | 20.9|
| IV                       | 70     | 45.8|

Abbreviation: ECOG = Eastern Cooperative Oncology Group. *American Joint Committee on Cancer Staging manual, 6th edition.

EGFR fluorescent in situ hybridization (FISH) analysis

EGFR (7p12) gene amplification was determined using a DNA probe set (LSI EGFR/CEP 7; Vysis, Downers Grove, IL, USA) consisting of a SpectrumOrange-labelled EGFR (locus)-specific probe and a SpectrumGreen-labelled probe that hybridises to the centromeric region of chromosome 7 according to protocols described elsewhere (Mitsuji et al, 2007).

Statistical analysis

Statistical analysis was performed on a personal computer using SPSS 12.0K for windows (SPSS Inc. Chicago, IL, USA). The survival rate was calculated using the Kaplan–Meier method, and a statistical analysis was performed using log-rank test. Multi-variable analysis of prognostic factors was conducted by Cox proportional hazards model; P<0.05 was considered statistically significant.

RESULTS

Between 1 November 1995 and 30 November 2003, a total of 153 patients were eligible for the study. The demographic characteristics are outlined in Table 1. The group consisted of 108 men (70.6%) and 48 women (29.4%) with a median age of 52.0 (range: 15–72) years. Median follow-up duration was 72.9 months (range: 2.0–135.0 months). Demographic and clinical data are described in our earlier report in detail.

Expression of EGFR, TS, TP, DPD, and ERCC1 proteins

Epidermal growth factor receptor expression using the PharmDx kit revealed a score of 0 in 29 patients (19.3%), 1+ in 56 patients (37.3%), 2+ in 56 patients (37.3%), and 3+ in 9 patients (6.0%). Epidermal growth factor receptor expression had no significant association with clinicopathologic variables, such as age, gender, stage, PS, Lauren’s classification, and Borrmann type and differentiation. Fluorescent in situ hybridization analysis of EGFR was evaluable in a total of 135 patients, which showed high polysomy in three patients (2.2%) and amplification in four patients (3.0%). Epidermal growth factor receptor expression had no significant correlation with FISH positivity. The expression levels of TS, TP, and ERCC1, as determined by IHC, in cancer tissue were not significantly different in relapsed and non-relapsed patients. These expression levels were not different between stages. However, expression of TP and ERCC1 was significantly higher in cancerous tissue than in normal tissue (P<0.0001). No significant correlation between TP and ERCC1 levels was found in cancerous and normal tissues. Dihydropyrimidine dehydrogenase expression levels in cancerous tissues were not significantly different in relapsed and non-relapsed cases; however, DPD levels were
Table 2  Univariate analyses of clinical prognostic factors (P-values)

| Factors            | Number of patients | Disease-free survival | Overall survival |
|--------------------|--------------------|-----------------------|------------------|
|                    |                    | RR of relapse and 95% CI | P-value | RR of dying and 95% CI | P-value |
| TS                 |                    |                       |                   |
| <25                | 77                 | 0.712                 | 0.153            |
| ≥25                | 74                 | 0.725 (0.467–1.127)   | 0.043            |
| TP                 |                    |                       |                   |
| <25                | 66                 | 0.10                  | 0.051            |
| ≥25                | 85                 | 0.638 (0.413–0.986)   | 0.017            |
| ERCC1              |                    |                       |                   |
| <1.75              | 65                 | 0.060                 | 0.045            |
| ≥1.75              | 86                 | 0.644 (0.414–1.001)   | 0.328            |
| EGFR PhormDx       |                    |                       |                   |
| Negative           | 29                 | 0.115                 | 0.297            |
| 1+ to 3+           | 113                | 0.605 (0.370–0.988)   | 0.017            |
| ARK1               |                    |                       |                   |
| Negative           | 45                 | 0.139                 | 0.067            |
| Positive           | 96                 | 0.791 (0.509–1.229)   | 0.322            |
| ARK2               |                    |                       |                   |
| Negative           | 46                 | 0.101                 | 0.440            |
| Positive           | 104                | 0.678 (0.448–1.028)   | 0.901            |
| EGFR FISH          |                    |                       |                   |
| Negative           | 111                | 0.775                 | 0.322            |
| Positive           | 18                 | 0.779 (0.413–1.469)   | 0.901            |

Abbreviations: ARK = aurora kinase; CI = confidence interval; EGFR = epidermal growth factor receptor; ERCC1 = excision repair cross-complementing gene 1; TP = thymidine phosphorylase; TS = thymidylate synthase; RR = relative risk. Relative risk adjusted for stage. If the relative risk is >1, the relative risk can be thought as the average increased risk of relapse or dying compared with the reference group. The group with the ratio equal to 1 is the reference group.

Discussions

Epidermal growth factor receptor expression was a prognostic factor in our study, but the prognostic role of EGFR in gastric cancer needs to be further elucidated. Some reports showed that high levels of EGFR expression are associated with more distant metastasis, more advanced stage, and poorer OS (Garcia et al, 2003; Gamboa-Dominguez et al, 2004; Galizia et al, 2007). In advanced gastric cancer patients treated mainly with 5-FU or cisplatin-based chemotherapy, lower expression of EGFR mRNA than the cutoff value was a strong predictor of poor survival by multivariate analysis (Matsubara et al, 2008a). Interestingly, the authors also showed that high DPD and ERCC1 expressions were significant predictors of poor survival. Another recent report showed that EGFR expression was a positive prognostic factor in patients with AGC (Al-Batran et al, 2008a), which was a retrospective analysis from the phase III trial comparing 5-FU, folinic acid plus either oxaliplatin vs cisplatin (Al-Batran et al, 2008b). There has also been evidence suggesting that cytotoxic chemotherapy is more effective among patients with high EGFR expression than in those with low EGFR expression (Ceppi et al, 2006; Vallbohmer et al, 2006). The prognostic and predictive roles of EGFR expression in gastric cancer thus remain controversial. Differences in EGFR expression among these studies may also be attributed to the lack of an established immunohistochemical scoring system commonly used to evaluate gastric cancer. Other prognostic variables, such as insulin-like growth factor type 1 receptor (Matsubara et al, 2008c) and class I histone deacetylase expression (Weichert et al, 2008) might also have confounding interactions.

In gastric cancer patients, higher TP expression was reported in cancerous tissues compared with the adjacent normal tissues (Maeda et al, 1996; Tanigawa et al, 1996; Kakeji et al, 1999) by IHC.
and ELISA. These studies indicated that TP expression is closely correlated with cancer invasion, haematogenous metastasis, lymph node metastasis, venous invasion, lymphatic invasion, and microvascular invasion. In our study, IHC scores from cancerous tissue were significantly higher than those of normal tissue. Higher TP expression might be a prognostic marker for OS, but the role of TP expression in gastric cancer also needs to be further elucidated.

Many reports have indicated that DPD activities are unaltered in gastric cancer (Ishikawa et al, 1999; Terashima et al, 2002). However, it was also reported that gastric carcinomas have significantly higher DPD activities than normal mucosa (Nakata et al, 2004). Another report showed that DPD expression in cancer cells, but not in stromal cells, could predict the efficacy of 5-FU chemotherapy in patients with T3 gastric carcinoma (Hisamitsu et al, 2004). In our study, the IHC scores of cancerous tissues were significantly lower than those of normal tissues, and no significant difference was observed between relapsed and non-relapsed patients in terms of IHC scores.

Decreased ERCC1 expression was associated with a superior response to 5-FU/cisplatin in primary intact gastric cancer patients (Metzger et al, 1998). The degree of ERCC1 protein expression was found to be inversely associated with this response, which is potentially relevant to clinical resistance to platinum compounds (Kwon et al, 2007). In patients with curatively resected gastric cancer, it was shown that increased ERCC1 expression was correlated with improved outcome (Baek et al, 2006). In patients with completely resected non-small-cell lung cancer, ERCC1-negative tumours showed greater benefit from cisplatin-based adjuvant chemotherapy compared with ERCC1-positive tumours (Olaussen et al, 2006). Reports concerning ERCC1 in resected lung cancer suggest that ERCC1 overexpression may improve treatment outcome by reducing DNA mutations during cancer progression (Simon et al, 2005; Lee et al, 2008).

In this study, low ERCC1 expression was associated with poor survival, but ERCC1 expression level was also closely associated with EGRF expression level. After multivariate analysis, the impact of ERCC1 on survival disappeared. To our knowledge, the ERCC1 protein expression between cancer and normal tissues has never been compared, although a report suggested that no significant difference exists in ERCC1 mRNA expression (Warnecke-Eberz et al, 2004). Further prospective studies will be needed to resolve this issue.

Table 3 Multivariate analyses of clinical prognostic factors

| Factors                        | Number of patients | Disease-free survival RR of relapse and 95% CI | P-value  | Overall survival RR of dying and 95% CI | P-value |
|--------------------------------|--------------------|-----------------------------------------------|----------|----------------------------------------|---------|
| Positive LN/resected LN        |                    |                                               |          |                                        |         |
| <0.3                           | 45                 | 0.941 (0.538–1.646)                            | 0.041    | 0.858 (0.470–1.566)                    | 0.029   |
| 0.3–0.7                        | 75                 | 1.857 (0.925–3.728)                            |          | 1.780 (0.865–3.663)                    |         |
| >0.7                           | 33                 |                                               |          |                                        |         |
|Stage                           |                    |                                               |          |                                        |         |
| IIIa                           | 51                 | 1.599 (0.890–2.873)                            | 0.072    | 1.669 (0.885–3.145)                    | 0.016   |
| IIIg                           | 32                 | 1.874 (1.060–3.314)                            |          | 2.452 (1.331–4.519)                    |         |
| IV                             | 70                 |                                               |          |                                        |         |
|EGFR PharmDx                    |                    |                                               |          |                                        |         |
| 0                              | 29                 | 0.609 (0.370–1.002)                            | 0.051    | 0.475 (0.282–0.791)                    | 0.005   |
| 1+ to 3+                       | 113                |                                               |          |                                        |         |
| TP expression                  |                    |                                               |          |                                        | 0.077   |
| <25                            | 66                 |                                               |          |                                        |         |
| ≥25                            | 85                 | 0.681 (0.445–1.042)                            |          |                                        |         |

Abbreviations: RR = relative risk, CI = confidence interval. A backward likelihood ratio approach was used to select factors for multivariate analysis. If the RR is > 1, the relative risk can be thought as the average increased risk of relapse or dying compared with the reference group. The group with the ratio equal to 1 is the reference group. P-value is based on log-rank test.
Earlier studies evaluated the expression of pharmacogenomic markers as a group. For example, patients with low TS, TP, and DPD gene expression showed prolonged survival over patients expressing high levels of these genes (Salonga et al., 2000). The main differences between this and the current study are (1) analysis of mRNA vs protein, (2) colorectal cancer vs gastric cancer, and (3) a metastatic or disseminated setting vs an adjuvant setting. No study has been conducted on the evolution of these pharmacogenomic markers during cancer progression in an adjuvant setting. Therefore, further pharmacogenomic studies on adjuvant treatments are required.

In conclusion, high expression of EGFR might be a good predictive marker of relapse and survival in curatively resected stage III–IV (M0) gastric cancer patients who received adjuvant 5-FU and cisplatin chemotherapy. Both EGFR expression, the coupled expression of TP and EGFR, and the LN ratio might be useful predictive markers for patient survival. On the other hand, there was no relationship in this study between clinical outcome and the pharmacogenetic markers reported in earlier studies, such as TS, DPD, and ERCC1. This suggests that these markers might not correlate with chemosensitivity to the FP treatment in gastric cancer patients. Further investigation is necessary, using prospective analysis of a larger cohort in a randomised controlled trial of adjuvant chemotherapy consisting of fluoropyrimidine and platinum agents.

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