S100A9 and EGFR gene signatures predict disease progression in muscle invasive bladder cancer patients after chemotherapy

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Background: In our previous gene expression profile analysis, IL1B, S100A8, S100A9, and EGFR were shown to be important mediators of muscle invasive bladder cancer (MIBC) progression. The aim of the present study was to investigate the ability of these gene signatures to predict disease progression after chemotherapy in patients with locally recurrent or metastatic MIBC.

Patients and methods: Patients with locally advanced MIBC who received chemotherapy were enrolled. The expression signatures of four genes were measured and carried out further functional analysis to confirm our findings.

Results: Two of the four genes, S100A9 and EGFR, were determined to significantly influence disease progression (P = 0.023, 0.045, respectively). Based on a receiver operating characteristic curve, a cut-off value for disease progression was determined. Patients with the good-prognostic signature group had a significantly longer time to progression and cancer-specific survival time than those with the poor-prognostic signature group (P < 0.001, 0.042, respectively). In the multivariate Cox regression analysis, gene signature was the only factor that significantly influenced disease progression [hazard ratio: 4.726, confidence interval: 1.623–13.763, P = 0.004]. In immunohistochemical analysis, S100A9 and EGFR positivity were associated with disease progression after chemotherapy. Protein expression of S100A9/EGFR showed modest correlation with gene expression of S100A9/EGFR (r = 0.395, P = 0.014 and r = 0.453, P = 0.004). Our functional analysis provided the evidence demonstrating that expression of S100A9 and EGFR closely associated chemoresistance, and that inhibition of S100A9 and EGFR may sensitize bladder tumor cells to the cisplatin-based chemotherapy.

Conclusions: The S100A9/EGFR level is a novel prognostic marker to predict the chemoresponsiveness of patients with locally recurrent or metastatic MIBC.

Key words: urinary bladder neoplasms, drug resistance, receptor, epidermal growth factor, urinary bladder

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Introduction

Although radical cystectomy is the standard treatment for muscle invasive bladder cancer (MIBC), ~50% of these patients develop metastases within 2 years, and the 5-year survival rate after surgery alone is ~50% [1, 2]. Systemic cisplatin-based combination chemotherapy is the first-line treatment modality for patients with metastatic bladder cancer; however, despite the initial high response rates of 40–70% reported in patients with advanced disease, chemotherapy is usually not curative and the overall 5-year survival is only 5–15% [3, 4]. Accordingly, there is growing interest in the role played by genes in the chemotherapeutic response of patients with MIBC and the predictive power of this relationship in an individual patient.

Thus far, information that would allow the response to chemotherapy to be predicted in an individual patient is lacking in the case of MIBC as well as many other cancers. Consequently, some patients suffer the adverse side-effects of these highly toxic drugs without the benefit of their intended action. Perhaps even more important is that, as their physical condition worsens, some of these unnecessarily treated patients may be deprived of additional therapy.

In our previous study, a gene expression profile analysis was carried out with the aim of identifying a genetic signature for progression in MIBC patients. Among the 1320 genes thus identified by microarray data analysis, four genes (IL1B, S100A8, S100A9, and EGFR) were determined to be important in predicting disease progression [5]. In the present study, we asked whether this four-gene signature could be used to predict disease progression after chemotherapy in patients with locally recurrent or metastatic MIBC. Additionally, to assess the hypothesis that S100A9 and EGFR have important function in chemoresistance, we have attempted in vitro functional analysis to test whether the altered gene expression of S100A9 or EGFR regulates on chemosensitivity to cisplatin treatment.

Materials and Methods

The Materials and Methods section can be seen in supplementary File (available at Annals of Oncology online).

Results

Baseline Characteristics

The mean age of the 80 patients who received chemotherapy was 65.60 ± 9.28 years, and the mean follow-up period was 41.13 ± 53.78 months. Sixty-five (81.3%) patients had local recurrence without visceral metastasis and 15 (18.7%) had metastases to other organs. Forty-six (57.5%) patients underwent prior radical cystectomy. Other baseline characteristics of the patients are presented in supplementary Table S2, available at Annals of Oncology online.

Identification of Genes Associated with Disease Progression in Locally Recurrent or Metastatic MIBC

The univariate Cox regression analysis of four genes (IL1B, S100A8, S100A9, and EGFR), which were previously determined to play an important role in MIBC progression, was carried out. Two of them, S100A9 and EGFR, significantly correlated to disease progression (P = 0.023, 0.045, respectively). These two genes were then used to calculate a risk score of disease progression after chemotherapy in MIBC patients. The risk score identified two groups of patients. A good-prognostic signature group represented relatively low expression levels of the two genes, while a poor-prognostic signature group had significantly higher expression. A cut-off value (35.251) was determined for disease progression with the highest combined sensitivity (91.1%) and specificity (54.3%) based on the receiver operating characteristic curve [area under the curve = 0.668, confidence interval (CI): 0.543–0.793].

Prognostic Value of the Two-gene Signature for Disease Progression in Locally Recurrent or Metastatic MIBC

A comparison of the two groups showed that the time to progression was significantly longer in the good-prognostic signature group [log-rank P < 0.001; hazards ratio (HR): 5.702, 95% CI: 2.024–16.062, P = 0.001] (Figure 1A, supplementary online).

![Figure 1](image-url). Time to progression (A), cancer-specific (B), and overall (C) survival in patients with recurrent or metastatic muscle invasive bladder cancer who received chemotherapy according to gene signature according to the combined gene signature of S100A9 and EGFR.
Table S4, available at Annals of Oncology online. The univariate Cox regression analysis showed that metastasis, prior cystectomy, and combined gene signature were significant influential factors for disease progression after chemotherapy (Table 1). In the multivariate Cox regression analysis, only combined gene signature was a significant influential factor for disease progression in patients with locally recurrent or metastatic MIBC after chemotherapy (Table 1). The good-prognostic signature group had a significantly longer cancer-specific survival time than the poor-prognostic signature group (log-rank P = 0.006; HR: 3.846, 95% CI: 1.357–10.906, P = 0.011) (Figure 1B, supplementary Table S4, available at Annals of Oncology online). However, the overall survival time was only marginally enhanced in the good-prognostic signature group (log-rank P = 0.060; HR: 1.894, 95% CI: 0.962–3.729, P = 0.065) (Figure 1C, supplementary Table S4, available at Annals of Oncology online).

immunohistochemical staining of S100A9 and EGFR in bladder cancer

To expand our findings suggesting that S100A9/EGFR may be a novel diagnostic marker for MIBC with local recurrence or metastasis after chemotherapy, we assessed the value of S100A9/EGFR protein as a prediction marker. The protein expression levels of the S100A9 and EGFR protein were assessed in 38 bladder cancer samples by immunohistochemical (IHC) analysis. Various intensities (from negative to strong) of S100A9 and EGFR were observed in cancer tissues (supplementary Figure S1, available at Annals of Oncology online). S100A9 were detected in cytoplasm as well as nucleus (supplementary Figure S1A, available at Annals of Oncology online), while EGFR was detected in cytoplasm, nucleus, and plasma membrane (supplementary Figure S1C, available at Annals of Oncology online). EGFR positivity was marginally associated with disease progression after chemotherapy in our MIBC patients (P = 0.106). However, S100A9 positivity was strongly correlated to disease progression (P = 0.047); 65% (15 among 23) of bladder cancer patients with progression after chemotherapy exhibited high S100A9 levels, while only 26% (4 among 15) of patients without progression showed high intensities (supplementary Table S3, available at Annals of Oncology online). Furthermore, the combined approach of S100A9 and EGFR was much significantly associated with progression (P = 0.018): 73.9% (17 among 23) patients with progression showed high S100A9/EGFR staining, and no patient has negative staining of S100A9/EGFR (supplementary Table S3, available at Annals of Oncology online). Notably, we found that protein expression intensities of S100A9 and EGFR showed good correlation with mRNA levels of S100A9 and EGFR (r = 0.395, P = 0.014 and r = 0.453, P = 0.004).

S100A9 overexpression leads to hyper-proliferation and resistance to cisplatin-induced apoptosis

Our two independent expression analysis (qRT–PCR and IHC based) suggest that the S100A9/EGFR as a novel prognostic marker in bladder cancer for disease progression after cisplatin-based chemotherapy. To uncover whether S100A9 has a functional link to chemosensitivity of bladder cancer, to test this hypothesis in vitro, T24 cells were transfected with S100A9 overexpressing construct or control and found that S100A9 involving in migration and proliferation of bladder cancer cells (supplementary Figure S2, available at Annals of Oncology online). Overexpression of S100A9 was confirmed by western blot analysis (supplementary Figure S2A, available at Annals of Oncology online). As shown in supplementary Figure S2B (available at Annals of Oncology online), the wound-healing assay revealed that transfected bladder tumor cells with S100A9 construct moved faster and filled the path earlier than control cells. Enforced S100A9 significantly enhanced proliferation rate of T24 cells in growth medium, compared with control cells (Ctrl, transfected T24 cells with a vector only) (supplementary Figure S2C, available at Annals of Oncology online). The increased S100A9 level allowed T24 cells more viable in the presence of cisplatin. Cell viability of control cells was reduced by ∼20% after 2 days treatment with 10 µM cisplatin. In contrast, S100A9 expressing cells showed ∼60% of viable cells by the same treatment (supplementary Figure S2D, available at Annals of Oncology online). The increased apoptosis by gene silencing of S100A9 was also confirmed in independent bladder
cancer cell line, TCCSUP (supplementary Figure S2E, available at *Annals of Oncology* online).

**EGFR expression level alters response to cisplatin-induced apoptosis**

We further examined the gain and loss of functional studies in T24 bladder cancer cells to assess the biological role of EGFR. Overexpression of EGFR was checked by western blot analysis (supplementary Figure S3A, available at *Annals of Oncology* online), which was able to enhance cell viability in the presence of 10 μM cisplatin in serum-free medium. Control T24 cells transfected with empty vector showed ~50% viability after 18 h cisplatin treatment, while EGFR overexpressing cells did not show significant apoptosis until 24 h treatment (supplementary Figure S3B, available at *Annals of Oncology* online). EGFR was knocked down using RNAi approach, which was confirmed by western blot analysis (supplementary Figure S3C, available at *Annals of Oncology* online). Cell viability assay revealed that silencing of EGFR sensitized T24 cells to cisplatin-induced apoptosis (supplementary Figure S3D, available at *Annals of Oncology* online). Control RNAi (siCtrl) showed ~80% of cell viability 6 h after cisplatin treatment, while two sets of EGFR knockdown (siEGFR-1 and siEGFR-2) both displayed only 30–40% of viability of controls (supplementary Figure S3D, available at *Annals of Oncology* online). Cell apoptosis assay carried out using TCCSUP bladder cancer cells also showed that knockdown of EGFR increased cell apoptosis of TCCSUP bladder cancer cells in the presence of cisplatin (supplementary Figure S3E, available at *Annals of Oncology* online).

**inhibition of both S100A9 and EGFR displayed enhanced effect on sensitization of T24 bladder cancer cells to cisplatin-induced apoptosis**

Experimental data in supplementary Figure S4 (available at *Annals of Oncology* online) suggest that S100A9 and EGFR play roles in response to a chemotherapeutic reagent, cisplatin, in bladder cancer cells. We tested whether down-regulation of EGFR and S100A9 alters levels of cisplatin-induced apoptosis. EGFR kinase activity was down-regulated by Iressa (ZD1839, gefitinib), an EGFR kinase inhibitor. S100A9 was silenced using RNAi approach. Control siRNA was used for a control for non-target effect by siRNA. EGFR inhibiton (with Iressa) significantly reduced viability, and enhanced chemosensitivity to cisplatin (supplementary Figure S4A, available at *Annals of Oncology* online, line 2). Moreover, combined inhibition of S100A9 and EGFR enhanced chemosensitivity of T24 bladder cancer cells (supplementary Figure S4A, available at *Annals of Oncology* online, line 4), suggesting the potential therapeutic strategy overcoming the chemoresistance, which is often observed during cisplatin-based chemotherapy on bladder cancer patients. Additional apoptosis analysis demonstrated that knockdown of EGFR or S100A9 increased cell apoptosis of TCCSUP bladder cancer cells induced by cisplatin treatment (supplementary Figure S4B, available at *Annals of Oncology* online).

**down-regulation of EGFR or S100A9 re-sensitized cisplatin-resistant T24 bladder cancer cell**

Cisplatin-resistant T24 bladder cancer cells (T24-R) were compared with control cells (T24-S) to determine the physiological roles of EGFR and S100A9 in chemoresistance. T24-R cells were resistant to 10 μM cisplatin treatment, while T24-S parent cells, which are sensitive to 10 μM cisplatin treatment, resulting in over 60% of cells reached to apoptosis within 2 days after treatment (supplementary Figure S5, available at *Annals of Oncology* online). Either inhibited EGFR kinase activity or silenced S100A9 could reduce the viability of T24-R cells. Moreover, combined inhibition of EGFR and S100A9 synergistically suppressed the viability of cisplatin-resistant T24 cells, suggesting the potential therapeutic strategy overcoming the chemoresistance often observed during cisplatin-based chemotherapy on bladder cancer patients.

**discussion**

In a previous study, we determined that an expression signature consisting of four genes (IL1B, S100A8, S100A9, and EGFR) was a reliable prognostic indicator of disease progression in patients with MIBC [5]. Here, the expression signatures of two genes (S100A9 and EGFR) were demonstrated to predict disease progression and cancer-specific survival in patients with locally recurrent or metastatic MIBC after systemic chemotherapy.

Poor performance status (PS) and the presence of visceral metastases have been shown to be predictors of poor prognosis in MIBC patients with locally advanced or metastatic disease after chemotherapy [6]. In another study, hemoglobin, serum albumin, PS, and visceral metastasis were determined to be very strong predictors of a poor prognosis, based on the construction of a nomogram [7]. Since in previous reports, poor PS was shown to be a highly influential prognostic factor for locally advanced and metastatic urothelial carcinoma, these patients were excluded from our study in order to minimize confounding effects. However, the univariate Cox regression analysis identified metastasis to visceral organs as an influential factor in disease progression. Hence, visceral metastases may serve as a predictor of poor prognosis in MIBC patients with locally advanced or metastatic disease.

Several molecular markers have been evaluated in terms of predicting disease progression. Several of them, including transforming growth factor β-1, interleukin-6, its soluble receptor, and insulin growth factor binding protein 3, were found to be elevated in invasive bladder cancer and to be associated with metastatic disease as well as increased risk of progression [8–10]. Markers such as ERCC1, JUN, MAP2K6, STAT3, and ICAMI associated with the survival of patients with advanced bladder cancer have also been evaluated [6, 11]. Similarly, in the current study, a two-gene signature (S100A9 and EGFR) was able to predict disease progression after chemotherapy in patients who developed locally recurrent or metastatic disease. Furthermore, the results of the present work confirm those of our previous study regarding the prediction of disease progression in MIBC [5]. However, whereas the earlier findings identified four genes, here only two of them were found to be associated with disease progression after chemotherapy in
locally recurrent or metastatic disease. Whether this was due to the different patient cohorts of the two studies remains to be determined.

The S100A9 or EGFR was identified in this study have been implicated in other types of cancer. S100A9 is up-regulated not only in MIBC but also in gastric, prostate, and colorectal cancers [12–14]. Its expression is often associated with cell proliferation in prostate cancer and metastatic processes in lung cancer [13, 15]. In addition, S100A9 could be used to predict the response to chemotherapy in breast cancer patients [16] and promoted breast cancer cell survival under chemotherapy and associated with resistance to perioperative chemotherapy [17]. In this study, we demonstrated the increased S100A9 level allowed cancer cell more viable against cisplatin in bladder cancer. Similarly, EGFR has also been shown to be a strong prognostic indicator in many different cancer types. In bladder cancer, increased EGFR expression was significantly associated with reduced relapse-free survival [18]. In fact, the effects of conventional chemotherapy were shown to be potentiated by the addition of EGFR inhibitors; as evidenced by a decrease in proliferation and an increase in apoptosis [19]. In this study, we also showed the EGFR overexpression is associated with chemoresistance. Additionally, we approved inhibition of S100A9 or combined inhibition of S100A9 and EGFR could reduce cancer cell viability and suppress viability of cisplatin-resistant cancer cells. Interestingly, this study is the first to examine the relationship between S100A9 or combined inhibition and chemoresponse in bladder cancer.

Generally, patients with advanced MIBC have a lower survival rates after chemotherapy in spite of initial high response rates [3, 4]. Interestingly, the survival rate of the current study was relatively higher than other studies. However, there were several reasons. The most enrolled patients of this study had a higher PS to endure at least four cycles of the cytotoxic chemotherapy. Patients with poor PS and impaired renal function were excluded.

Taken together, these results implicate S100A9 and EGFR in disease progression. In the current study, the expression of both genes was increased in patients with locally recurrent and metastatic MIBC who had a poor outcome. Hence, our results provide further evidence for the utility of S100A9 and EGFR as strong prognostic indicators in patients with invasive bladder cancer. Further studies on the expression of these genes and agents-related to these genes may lead to improved time to progression in patients with locally recurrent or metastatic MIBC.

Although the benefit of systemic chemotherapy as a first-line treatment in advanced or metastatic MIBC is well established, it is unclear which group of patients will respond in terms of disease progression and survival. Collectively, our findings suggest that in patients with advanced or metastatic MIBC who received chemotherapy, both parameters are genetically influenced. Consequently, an evaluation of intratumoral molecular markers could be used to identify patients more likely to respond to chemotherapy and those for whom other forms of treatment may be preferable.

In conclusion, a combined expression signature consisting of two genes, S100A9 and EGFR, was shown to be an independent prognostic determinant for disease progression after chemotherapy in patients with locally recurrent or metastatic MIBC. This two-gene signature could serve as a useful marker for predicting chemoresponse in these patients. Also, this result suggests that combined inhibition of S100A9 and EGFR may increase chemosensitivity for cisplatin-based chemotherapy.

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disclosure

The authors have declared no conflicts of interest.

references

Intensity-modulated radiation therapy leads to survival benefit only in patients with high-risk prostate cancer: a population-based study

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Background: During the last years, there has been a rapid adoption of intensity-modulated radiation therapy (IMRT) in patients with prostate cancer (PCa), despite the lack of randomized trials evaluating its effectiveness. The aim of our study was to evaluate the survival beneﬁt associated with IMRT in patients with PCa.

Patients and methods: Overall, 42,483 patients with PCa treated with IMRT or initial observation between 2001 and 2007 within the Surveillance, Epidemiology, and End Results (SEER)-Medicare were evaluated. Patients in both treatment arms were matched using propensity-score methodology. After propensity-score matching, 19,064 patients remained in our analyses. Eight-year cancer-speciﬁc mortality (CSM) rates were estimated, and the number needed to treat (NNT) was calculated. Competing risks regression analyses tested the relationship between treatment type and CSM.

Results: Overall, the 8-year CSM rates were 3.4% and 4.1% for patients treated with IMRT versus initial observation, respectively (P = 0.7). In patients with low-intermediate-risk disease, IMRT was not associated with lower CSM rates compared with observation (P = 0.7). In patients with high-risk disease, the 8-year CSM rates for IMRT versus observation were 5.8% versus 10.5%, respectively (P < 0.001). The corresponding NNT was 21. When high-risk patients were stratified according to age (<73 versus ≥73), and Charlson comorbidity index (≤1 vs > 1), IMRT was only associated with lower CSM rates in patients aged <73 (P = 0.03; NNT = 11) but not in patients aged ≥73 (P = 0.24; NNT = 53).

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