Gene expression analysis of pretreatment biopsies predicts the pathological response of esophageal squamous cell carcinomas to neo-chemoradiotherapy

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Background: Neoadjuvant chemoradiotherapy (neo-CRT) followed by surgery has been shown to improve esophageal squamous cell carcinoma (ESCC) patients’ survival compared with surgery alone. However, the outcomes of CRT are heterogeneous, and no clinical or pathological method can currently predict CRT response. In this study, we aim to identify mRNA markers useful for ESCC CRT-response prediction.

Patients and methods: Gene expression analyses were carried out on pretreated cancer biopsies from 28 ESCCs who received neo-CRT and surgery. Surgical specimens were assessed for pathological response to CRT. The differentially expressed genes identified by expression profiling were validated by real-time quantitative polymerase chain reaction (qPCR), and a classifying model was built from qPCR data using Fisher’s linear discriminant analysis. The predictive power of this model was further assessed in a second set of 32 ESCCs.

Results: The profiling of the 28 ESCCs identified 10 differentially expressed genes with more than a twofold change between patients with pathological complete response (pCR) and less than pCR (<pCR). A prediction model based on the qPCR values of three genes was generated, which provided a predictive accuracy of 86% upon leave-one-out cross-validation. Furthermore, the predictive power of this model was validated in another cohort of 32 ESCCs, among which a predictive accuracy of 81% was achieved. Importantly, the discriminant score was found to be the only independent factor that affected neo-CRT response in both the training (P = 0.015) and validation (P = 0.017) sets, respectively.

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Conclusion: The expression levels of three genes determined by qPCR provide a possible model for ESCC CRT prediction, which will facilitate the individualization of ESCC treatment. Further prospective validation in larger independent cohorts is necessary to fully assess its predictive power.

Key words: esophageal squamous cell carcinoma, gene expression, neoadjuvant chemoradiotherapy, response prediction

Introduction

Esophageal cancer (EC) is one of the most common cancers in the world. It can be classified into esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. In China, ESCC accounts for most ECs where it bears more than half of the global burden [1].

Surgery is a major component of treatment of locally advanced ESCCs. But disease recurrence often ensues after curative resection, and the 5-year survival rate (~25%) has not changed significantly for several decades [2]. Neoadjuvant chemoradiotherapy (neo-CRT) followed by surgery improves survival in comparison with surgery alone and has been recommended in the guidelines for ESCC management [3].

However, the outcomes of neo-CRT are heterogeneous. Superior survival is consistently achieved in patients who obtain pathologic complete response (pCR), but not those nonresponders [4]. In addition, neo-CRT may increase postoperative complications. Therefore, understanding the underlying determinants of CRT response would be valuable for tailoring ESCC treatment. Nevertheless, clinical parameters such as TNM classification, tumor location or tumor differentiation do not effectively predict CRT response [5].

The development of DNA microarray technology allows for high-throughput identification of gene expression profiles in cancers. The identification of molecular biomarkers that predict neo-CRT response would be beneficial to individualize ESCC management. With this purpose, we determined the whole genome expression profiles of a set of endoscopic cancer biopsies from locally advanced ESCC patients before treatment who subsequently received neo-CRT and surgery. The expression levels of some of the differentially expressed genes identified by the arrays were assessed via real-time quantitative polymerase chain reaction (qPCR). A prediction model for CRT response was derived based on the qPCR values and further validated in a second set of ESCC patients receiving the same treatment.

Materials and methods

Patient selection and tissue collection

All patients in this study participated in a phase III clinical trial (ClinicalTrials.gov registration ID: NCT01216527) approved by the Institutional Review Board of Sun Yat-sen University Cancer Center, Guangzhou, China. Additional detail is provided in the supplementary material, available at Annals of Oncology online. Patients in the neo-CRT group of the trial receiving radiotherapy with cisplatin and vinorelbine concurrently were enrolled in this study if they volunteered, and an approved tissue collection protocol was used to obtain tumor material. This study utilized both a training set and a validation set. The training set included patients receiving treatment from September 2007 to March 2012 with fresh pretreatment tissue specimens available. For the validation set, patient tissues were selected retrospectively from July 2007 through March 2013 if formalin-fixed paraffin-embedded (FFPE) blocks of pretreatment biopsies were available, excluding those already utilized in the training set.

Up to 10 cancer biopsy specimens, each ~1.0 mm, were collected from the training set of patients during endoscopy before CRT. Multiple tissue specimens were pooled together, snap frozen in liquid nitrogen and stored at −80°C until RNA isolation. For the validation set, four 10-µm sections were cut from the FFPE blocks of pretreatment biopsies after routine pathologic examination.

For each pretreatment specimen, a tissue biopsy adjacent to the biopsies used in microarray or qPCR analysis was routinely hematoxylin and eosin stained and assessed for the presence of cancer and its histology by pathologists. After neo-CRT and surgery, the presence of cancer cells in esophagectomy specimens was carefully evaluated by pathologists blinded to the clinical information and gene expression results. Resected surgical specimens with no residual cancer cells observed microscopically were classified as pCR, whereas others with the presence of any cancer cell either at the primary tumor site or in any of the resected lymph nodes, were classified as less than pCR (<pCR).

In addition, normal esophageal epithelial samples from 10 stage IIB–III ESCC patients who received surgery alone were collected during surgery, snap frozen and stored at −80°C.

Microarray experiments and analysis

Total RNA of the fresh tissues used for microarray analysis was isolated using a mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA). The GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) was adopted for our microarray analyses. Additional detail is provided in the supplementary material, available at Annals of Oncology online. Microarray data are available at www.ncbi.nlm.nih.gov/geo/ (accession no. GSE45670). Differentially expressed genes were identified if the P value was <0.01 using a t-test for unpaired samples.

qPCR analysis

To validate microarray data, we chose genes with differential expression levels greater than twofold between the pCR and <pCR groups and carried out qPCR analysis of the same aliquot of RNA used for the microarray. For the independent validation cohort, total RNA isolated from the aforementioned FFPE blocks by a RecoverAll Total Nucleic Acid Isolation Kit (Ambion) was used for qPCR. qPCR reactions for each gene were carried out in triplicate. Analysis of relative gene expression data was carried out using the 2−ΔΔCt method. Additional detail is provided in the supplementary material and Table S1, available at Annals of Oncology online.

Discrimination analysis

The potential for the differentially expressed genes to discriminate between the ESCC neo-CRT responders and nonresponders was assessed by Fisher’s linear discriminant analysis (FLDA) using the SPSS 19.0 software package (SPSS, Chicago, IL). The log expression values of genes assessed by qPCR of the 28 ESCCs in microarrays were used to select variants and construct the
The CRT-response prediction model with leave-one-out validation. Additional detail is provided in the supplementary material, available at *Annals of Oncology* online.

The overall prediction accuracy and sensitivity and specificity of our classifier for identifying pCRs from the training and validation sets were calculated.

**Statistical analysis**

Statistical analysis was carried out using SPSS 19.0 software. Comparison between microarray and qPCR expression data were measured using Spearman’s rank correlations. The clinicopathological differences between the training and validation cohorts of patients, and the correlation between clinicopathological parameters or discriminant score and neo-CRT response were analyzed by a $\chi^2$ test or Fisher’s exact test. Mann–Whitney U-test was used to compare the differences of discriminant scores between pCR and <pCR groups. To determine independent factors significantly affecting neo-CRT response, logistic regression analysis was carried out with a forward stepwise procedure and the likelihood ratio test. A significant difference was declared if the $P$ value from a two-tailed test was <0.05. Receiver operating characteristic (ROC) plots for the discriminant scores to discriminate pCRs from <pCRs after neo-CRT, and Box and Whisker plots showing the distributions of discriminant scores were calculated by MedCalc 9.6.2.0 (MedCalc Software, Ostend, Belgium).

**Results**

**Characteristics of patients**

Sixty patients who received the neo-CRT (Table 1 and supplementary Table S2, available at *Annals of Oncology* online) were enrolled in this study. All of the patients presented thoracic ESCCs at stage T2-3N1M0. There was no significant difference between the training and validation cohorts in patients’ gender, age, tumor location, differentiation and stage ($P > 0.05$). Postoperative pathological examination showed that pCR was observed in 40% of the cancers (24 of 60), including 39% (11 of 28) in the training set and 41% (13 of 32) in the validation set. pCRs exhibited a tendency of better disease-specific survival than <pCRs in both training and validation set, although statistical significances could not be achieved (supplementary Figure S1, available at *Annals of Oncology* online).

**Microarray analysis and validation**

Microarrays were adopted to analyze 28 pretreatment biopsies composed of 11 pCRs and 17 <pCRs in the training set and 10 samples of normal esophageal epithelia. Approximately 6956 genes were differentially expressed between the 10 benign epithelial specimens and the 28 malignant tissues with a false discovery rate of 0.01 (supplementary Figure S2, available at *Annals of Oncology* online). The statistical analysis subsequently revealed that 178 genes were differentially expressed between the pCR and <pCR group with $P < 0.01$ among the above 6956 genes (supplementary Figure S3, available at *Annals of Oncology* online). Greater than a twofold difference in expression levels was observed for 10 genes, among which six genes (*LIMCH1*, *SDPR*, *C1orf226*, *SLC9A9*, *GSTM3* and *IGSF10*) were downregulated and four genes (*MMP1*, *MMP9*, *MMP12* and *OASL*) were upregulated in pCRs when compared with <pCRs (supplementary Table S3, available at *Annals of Oncology* online).

qPCR was utilized to evaluate the relative expression levels of these 10 genes. The Spearman’s rank correlation comparing the data obtained from qPCR and microarray studies for each gene was $0.837–0.946$, confirming the validity of the microarray data. The distributions of 10 genes’ expression in pCR and <pCR groups are shown in supplementary Figure S4, available at *Annals of Oncology* online. Take *MMP1* for example, its expression values were lower in <pCR group overall; however, it varied with an overlap between pCR and <pCR groups. Similar results were observed for other differentially expressed genes. Therefore, no single marker was able to discriminate between the two subtypes of ESCCs.

**Class prediction analysis**

The FLDA with stepwise variant-selection results showed a classifying model which is expressed by the equation $Y = -10.388 – 0.343 \times \text{MMP1} + 0.642 \times \text{LIMCH1} + 0.921 \times \text{C1orf226}$ (eigenvalue 2.065, canonical correlation 0.821, $P < 0.001$). Group centroids for microarrays were frozen pretreatment tissue and real-time qPCR validation phase FFPE pretreatment tissue.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Microarrays ( P ) value</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Male</td>
<td>25</td>
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<tr>
<td>Female</td>
<td>3</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>&lt;55</td>
<td>12</td>
</tr>
<tr>
<td>≥55</td>
<td>16</td>
</tr>
<tr>
<td>Tumor location</td>
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<td>4</td>
</tr>
<tr>
<td>Middle</td>
<td>18</td>
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<td>T3</td>
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<td>N0</td>
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<td>N1</td>
<td>28</td>
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<td>M0</td>
<td>28</td>
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* $\chi^2$ test with continuity correction.  
** $\chi^2$ test.  
*Median age.  
*Fisher’s exact test.  
qPCR, quantitative polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded.

**Table 1.** Characteristics of enrolled patients with esophageal squamous cell carcinomas receiving neoadjuvant chemoradiotherapy and surgery

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<p>Crs and pCRs were −1.114 and 1.721, respectively. Next, a cut score halfway between the two centroids was determined: cut score = (−1.114 + 1.721)/2 = 0.607. When the discriminant score Y was calculated to be <0.607, the case was predicted to be a <pCR case; otherwise, the case was classified as a pCR. For the training set of 28 leave-one-out-cross-validated cases, 9 of 11 pCRs (82% sensitivity) and 15 of 17 <pCRs (88% specificity) were correctly classified with an overall accuracy of 86% (24 of 28) and an area under the curve (AUC) of 0.973 [P < 0.001, 95% confidence interval (CI) 0.924–1] (Table 2, Figure 1A and B).

<table>
<thead>
<tr>
<th>Table 2. Distribution of pathological and predicted neoadjuvant chemoradiotherapy response of esophageal squamous cell carcinomas</th>
<th>Predicted results</th>
<th>Training phase</th>
<th>Validation phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pCRs, n (%)</td>
<td>&lt;pCRs, n (%)</td>
<td>Total, n</td>
</tr>
<tr>
<td>True results</td>
<td>pCR, n (%)</td>
<td>9 (82)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (18)</td>
</tr>
<tr>
<td></td>
<td>&lt;pCR, n (%)</td>
<td>2 (12)</td>
<td>15 (88)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total, n</td>
<td>11</td>
<td>17</td>
<td>28</td>
</tr>
</tbody>
</table>

pCRs, pathological complete responders; <pCR, non-pathological complete responders.

<sup>a</sup>Leave-one-out cross-validated grouped cases.

<sup>b,c</sup>The sensitivity<sup>b</sup> and specificity<sup>c</sup> for identifying pCRs were 82% and 88% for the training sets of leave-one-out cross-validated grouped cases, respectively.

<sup>d,e</sup>The sensitivity<sup>d</sup> and specificity<sup>e</sup> for identifying pCRs were 54% and 100% for the validation cases, respectively.

Figure 1. Receiver operating characteristic (ROC) curve analysis of the discriminant model with MMP1, LIMCH1 and C1orf226 for discriminate pathological complete responders (pCRs) from non-pathological complete responders (<pCRs) after neoadjuvant chemoradiotherapy on training (A) and validation (C) samples. Box and Whisker plot showing the distributions of the discriminant scores of pCRs and <pCRs in training (B) and validation (D) samples.
We next applied the model consisting of the three genes (MMP1, LIMCH1 and C1orf226) to the FFPE biopsy specimens from the validation set of 32 patients (13 pCRs and 19 <pCRs) (Table 2). A response prediction for 26 of the 32 tumor samples (81%) with an AUC of 0.818 (P = 0.003, 95% CI 0.658–0.978) was achieved (Table 2, Figure 1C and D). The 19 <pCRs (100% specificity) were all correctly identified, whereas 7 of the 13 pCRs (54% sensitivity) were correctly identified (Table 2).

By Fisher’s exact test, tumor location (P = 0.046) and discriminant score calculated from the three-gene model (P < 0.001), but not other clinicopathological parameters (P > 0.05), were significantly associated with neo-CRT response in the training set. In the validation set, a significant association was observed between the discriminant score and neo-CRT response (P = 0.001). By multivariate analyses, the discriminant score was found to be the only independent factor that affected neo-CRT response in both the training [P = 0.015, odds ratio (OR) 15.967, 95% CI 1.728–147.547] and validation (P = 0.017, OR 1.465, 95% CI 1.070–2.006) sets, respectively (supplementary Table S5, available at Annals of Oncology online).

**discussion**

Advances in molecular biology have recently led to the rapid development of individualized cancer management, which allow the tailoring of therapeutic approaches for genetically defined subgroups of tumor patients. However, there are still no validated useful molecular markers to guide cancer chemo- or radiotherapy at present.

With regards to ESCC, the use of neo-CRT before surgery is increasingly proved to improve patients’ survival and becoming the standard approach to treat locally advanced disease. An interim analysis of our ongoing phase III clinical trial in 2011 also showed that neo-CRT with subsequent surgery increased patient’s survival in comparison with surgery alone [6]. However, nonresponders, which account for more than half of ESCC patients, could not benefit from this course of treatment. Therefore, identification of nonresponders before neo-CRT is administrated would allow physicians to discontinue ineffective treatments and institute alternative ones, thereby avoiding both overtreatment and undertreatment of patients, which is in itself a way of realizing ESCC individualized treatment.

In an attempt to identify gene expression biomarkers that allow us to predict response to neo-CRT, we carried out gene expression analysis on a training set comprised of pretreatment cancer biopsies from 28 ESCC patients all undergoing a neo-CRT. The profiling studies identified 10 genes with more than a twofold changes in expression between <pCRs and pCRs, which were validated by qPCR. More importantly, a prediction model based on the qPCR values of 3 of the 10 genes was generated and provided an accuracy level of 86%, a sensitivity of 82% and a specificity of 88% for identifying pCRs with leave-one-out cross-validation. Furthermore, the predictive power of this model was evaluated in another independent cohort of ESCCs receiving the same regimen. A predictive accuracy of 81% was achieved. In addition, all of the patients who could not reach pCR (100% specificity for identifying pCRs) in the validation group were identified, which would be particularly helpful to reduce the use of unproductive treatments (such as neo-CRT) on these patients. More importantly, discriminant score calculated from the three-gene model (but not clinicopathological factors) was the only independent factor that affected neo-CRT response in both the training and validation sets by logistic regression analyses, suggesting its clinical utility for the individualization of ESCC neo-CRT, which cannot be achieved by examining clinical parameters alone.

Several studies have analyzed pretreatment cancer tissues obtained from EC patients who had received neo-therapy and surgery [7–11]. Luthra et al. [7] identified a combination of three differentially expressed genes allowed for the discrimination between pCR and <pCR with sensitivity and specificity of 85% after profiling pretreatment cancer biopsies from 19 EC patients receiving neo-CRT. In another cDNA microarray study of tumor biopsies from EC patients before neo-CRT, a 32-gene classifier was produced in which 10 of 21 <pCRs could be accurately identified [8]. Mahar et al. [9] developed a model composed of five genes which predicted response to neo-CRT with 95% accuracy in 74% of 27 EC patients. Motoori et al. [11] carried out their gene expression profiling on pretreatment samples of ESCC patients who received chemotherapy, and provided a method for chemotherapy response prediction with 199 genes. Schauer et al. [10] proved Ephrin B3 receptor, one of their identified differentially expressed genes via microarrays, to be related with neoadjuvant chemotherapy response by immuno-histochemistry.

In comparison to the previous microarray studies carried out on ECs treated with CRT, our study has several advantages. First, the specimens used in this study were relatively homogeneous. All were obtained from ESCCs at advanced stages (T2–3N1M0) receiving the same neo-CRT regimen. Secondly, the CRT responses of ESCCs in our study were evaluated pathologically. CRT or chemotherapy response evaluated by radiological examinations might not be as accurate as pathological ones. And the role of radiological evaluation on ESCC survival has not been proven. Furthermore, we established the model based on qPCR values of gene expression. qPCR is the standard way to quantify gene expression and is much less expensive than microarray studies; these advantages give our model a higher potential for wide use in clinical practice. Finally, the identified mRNA panel from our study was validated in an independent ESCC cohort, which provides more creditability for our model.

Ten genes were differentially expressed with more than a twofold difference between pCRs and <pCRs. There was no overlap of differential expressed genes with previous EC CRT-related microarray studies. The reason for the diversity was most likely due to the different patient subsets or the microarray technologies adopted. The number of differential expressed genes is smaller than that of some other reports [7–11]. A possible reason might be the homogeneity of our recruited patients in the perspectives of tumor stage, pathological type and treatment regimen. This might help to narrow down the identified differential genes in our study to be ESCC CRT-response specific. Three of the differentially expressed genes MMP1, LIMCH1 and C1orf226 were adopted to build the CRT-response prediction model. MMP1, as another two differentially expressed genes MMP9 and MMP12, are members of the matrix metalloproteinase (MMP) family, which have been reported to play a role in...
cancer drug response via their regulation of vascular leakage [12]. LIMCH1, a gene encoding proteins with actin binding and zinc ion binding domain, have been reported to be dis-regulated in PIK3CA-mutated breast cancers [13] and smoking-related clear-cell renal cell carcinomas [14]. C1orf226 is an uncharacterized protein with no report on its function. The specific role of these genes in ESCC chemosensitivity has not been reported, which warrants further in-depth study.

Due to a difficulty in obtaining samples of significant size at endoscopy and the inevitable RNA degradation that occurs in FFPE samples, the number of patients in our study is not as large as we had anticipated. RNA extracted from FFPE has been proven to be suitable for qPCR analysis [15]. However, future re-evaluation of the predictive model in a large number of fresh samples may provide more accurate results.

As pathological response after neo-CRT has been proved to be a significant determinant of survival advantage for ESCCs [4], we chose it as the grouping variable to build the prediction model. However, our model might be tried to use for survival prediction, as the correlation between pathological response and survival has been proved. Besides, it is difficult to say whether our model is suitable for radical CRT or CRT with similar or different regimens. Any extended application of it requires further verification.

In conclusion, our study demonstrates that the analysis of a combination of three genes by qPCR can effectively and reproducibly classify ESCCs according to the response or resistance to neo-CRT comprised of cisplatin and vinorelbine. Further prospective validation in larger independent cohorts of neo-adjuvant or radical CRT with similar or different regimens is warranted to fully assess its predictive power. However, the three-gene expression signature model offers a novel tool for CRT-response prediction and could have important clinical implications for the identification of CRT responsive and nonresponsive patients, thus providing a framework for future individualized therapy.

**funding**

This work was supported by Chinese Ministry of Health [grant number 179]; National Natural Science Foundation of China [grant number 81272635] and Sun Yat-sen University [grant number 2007048].

**disclosure**

The authors have declared no conflicts of interest.

**references**