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ORIGINAL ARTICLE

Molecular Marker Expression Is Highly Heterogeneous in Esophageal Adenocarcinoma and Does Not Predict a Response to Neoadjuvant Therapy

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Abstract A reliable method to identify pathologic complete responders (pCR) or non-responders (NR) to neoadjuvant chemoradiation therapy (NAT) would dramatically improve therapy for esophageal cancer. The purpose of this study is to investigate if a distinct profile of prognostic molecular markers can predict pCR after neoadjuvant therapy. Expression of p53, Her-2/neu, Cox-2, Beta-catenin, E-cadherin, MMP-1, NFkB, and TGF-B was measured by immunohistochemistry in pre-treatment biopsy tissue and graded by an experienced pathologist. A pCR was defined as no evidence of malignancy on final pathology. Molecular profiles comparing responders to non-responders were analyzed using classification and regression tree analysis to investigate response to NAT and overall survival. Nineteen patients were pCRs and 34 were NRs. pCRs were more likely to be alive at follow-up than NRs ($p<0.01$). Thirty-seven distinct profiles were identified. Expression of molecular markers was highly heterogeneous between patients and did not correlate with a response to NAT, survival ($p=0.47$) or clinical stage ($p=0.39$) when evaluated either as individual markers or in combination with other expression patterns. NAT dramatically impacts survival through a mechanism independent of known molecular markers of esophageal cancer, which are expressed in a highly heterogeneous fashion and do not predict response to NAT or survival.

Keywords Esophageal cancer · Oesophageal cancer · Molecular marker · Tumor profile · Neoadjuvant therapy

Introduction

Patients with locoregional esophageal adenocarcinoma (stage II or III) are currently offered neoadjuvant chemoradiotherapy followed by esophagectomy (trimodality therapy), which can carry a very high morbidity and a significant mortality. Of those few patients who qualify for neoadjuvant therapy, 6 % will die prior to resection, 33 % will experience life-threatening complications, and nearly all will experience protracted nausea and vomiting, diarrhea, poor appetite, and susceptibility to infections throughout the course of treatment.[1] Those who subsequently proceed to surgical resection have an average 4 % operative mortality and at least a 30 % morbidity rate.[2] Unfortunately, despite aggressive treatment, overall oncologic results have been disappointing, with very high rates of recurrence and an overall 5-year survival after optimal therapy of no better than 46 %.[3]

Interestingly, 20–30 % of patients are found to have no evidence of tumor in post-operative pathologic review,[4] indicating that they have had a pathologic complete response

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(pCR) to neoadjuvant therapy. This complete response imparts a significant survival benefit. Patients with a complete response enjoy a 5-year overall and disease-free survival that is roughly double that of patients who show no response.[5,6]

A method to determine a patient's likelihood of responding to neoadjuvant therapy would be of tremendous value. Patients determined to be highly likely to have a pCR could avoid undergoing an esophagectomy. Likewise, if a patient were found to be a non-responder prior to undergoing neoadjuvant therapy, they might forego neoadjuvant therapy and instead, proceed immediately to resection. Unfortunately, we lack a reliable means of identifying patients who will mount a pathologic complete response.

There is growing interest in the role of cellular and molecular markers in defining tumor response to neoadjuvant therapy.[5–9] A number of individual molecular markers have been shown to be associated with the development of esophageal cancer, and may also show promise in predicting response to neoadjuvant therapy. Some of these have been reported to be a sufficiently reliable predictor of pCR despite limited clinical applicability.[5,8] Consequently, determining specific expression patterns of these highly predictive markers may better reflect overall tumor biology and the likelihood of pCR. We undertook this study to determine whether the expression profile of several prognostic molecular markers may correlate with response to neoadjuvant therapy prior to esophagectomy for esophageal cancer. Specifically, we hypothesized that altered expression of p53, NF-kB, TGF-B, COX-2, Her-2/neu, B-catenin, E-cadherin, and MMP-1 in pretreatment tumor biopsies would correlate with a higher probability of complete response to neoadjuvant therapy.

Methods

This study was approved by the Oregon Health and Science University (OHSU) Institutional Review Board. The OHSU Esophageal Cancer and Related Diseases database (ECRD) is a prospectively maintained registry of clinical and pathologic information for all patients treated at this institution treated for esophageal cancer. The records of all patients between the ages of 18 and 89 who had undergone esophagectomy between January 2000 and July 2012 were reviewed and patients with sufficient pre-treatment biopsy tissues available for biochemical analysis were selected for the study. All patients who showed a complete response to neoadjuvant therapy on final pathologic review were selected as our pathologic complete responder (pCR) group. One additional patient who was thought to have an indeterminate focus of possible adenocarcinoma in a lymph node of the surgical specimen (listed as ypN1) but no evidence of any adenocarcinoma at the site of the primary tumor and no evidence of adenocarcinoma on subsequent long-term follow-up was included in the pCR group. The patients in the pCR group were matched in a 1:2 ratio with patients who were found to be non-responders (NR) to neoadjuvant therapy. Patients were matched on the basis of gender, age, and clinical tumor staging. Data pertaining to demographics, treatment, surgical procedures and outcomes, clinical and pathologic tumor staging, and survival, were collected from the ECRD for analysis with the tumor marker data.

Tissues were obtained at or near the time of initial cancer diagnosis then formalin fixed and paraffin embedded at the time of collection. Two 10-μm-thick sections were taken from each specimen and mounted on a slide, and a total of 10 slides were produced for each specimen for a total of 20 sections per specimen. One slide from each specimen was stained with hematoxylin and eosin to confirm the presence of malignancy in the specimen. Next, slides were stained with one of the following commercially available antibodies for each of the respective markers; p53 (Ventana clone DO-7), Her-2/neu (Ventana clone 4B5), Cox-2 (Dako clone cx-294), Beta-catenin (Dako clone B-catenin-14), E-cadherin (Cell Marque clone ECH-6), MMP-1 (Epitomics clone EP1247Y), NFkB (Epitomics clone E379), and TGF-B (Santa Cruz Biotechnology clone sc-82). Staining was performed using a Ventana BenchMark XT automated slide staining system (Roche Diagnostics). Available positive and negative controls recommended by the antibody supplier were used for each set of molecular markers.

Slides were scored in a blinded fashion by a single board-certified pathologist who specializes in gastrointestinal malignancies at a high volume center [KMG]. Slides were scored as 0 (no stain), 1 (stained but negative), 2 (stained positively), or 3 (strongly positive). A score of 2 or 3 was considered positive expression. Pathologic complete response was defined as no evidence of malignancy in the surgical specimen at the time of esophagectomy, and all other patients who demonstrated any evidence of malignancy at the site of the primary tumor on final pathologic examination were considered non-responders. All tumors were staged according to AJCC seventh edition criteria.

Pearson's chi squared test was used to correlate clinical and pathological variables, while Kaplan-Meir analysis was used to describe survival. Statistical significance was set at $p<0.05$. For combination analysis, combination and regression tree analysis was used to determine the most predictive combinations of molecular marker expression patterns. All statistical analysis was performed using R (version 2.14.11, R-project, Vienna, Austria).

Results

Of the 440 patients in the database at the time of our query, 53 completed neoadjuvant therapy, had sufficient clinical data,

and had sufficient quality of pre-treatment tissues available to qualify for this study. Nine (17 %) of these were female and 44 (83 %) were male, with an average age of 67 years old. All completed neoadjuvant therapy as prescribed by their care team. Demographics and tumor characteristics are described in Table 1. Overall survival for all patients was 43 % at 5 years, with a median survival of 3.8 years (Fig. 1).

Nineteen of these patients were pCRs and 34 were NRs. pCRs were more likely to be alive at follow-up than NRs ($p<0.01$) and pCRs had a significantly higher survival at 5 years as compared to NRs ($p<0.001$) (Fig. 2).

There was no significant difference in expression of any of the molecular markers between pCR and NRs (Fig. 3). When taken individually, none of the molecular markers correlated with pCR, although there was a trend toward significance for COX-2 and NF-kB ($p=0.09$ and $p=0.08$, respectively). When combined, 37 distinct molecular marker profiles were identified. Expression of molecular markers was highly heterogeneous between patients and did not correlate with survival ($p=0.47$) or clinical stage ($p=0.39$) when evaluated in combination with other expression patterns. The overall number of positively expressed molecular markers for any given tumor did not correlate with response to neoadjuvant therapy ($p=0.81$) or clinical stage ($p=0.39$).

By recursive partitioning with complete response set as the outcome, the most predictive markers were ranked as NF-kB, TGF-B, COX-2, Her-2/neu, p53, B-catenin, E-cadherin, and MMP-1, with no single combination reaching statistical significance (Fig. 4).

Table 1 Demographics and clinical characteristics of all enrolled patients

	Non-responders ($n=34$)		Complete responders ($n=19$)		p value
Gender					0.349[a]
Female	21 %	(7)	11 %	(2)	
Male	79 %	(27)	89 %	(17)	
Age (range)	66	(61.5–79.0)	71	(62.5–73.0)	0.715[b]
TNM stage					
Tumor					0.920[a]
cT0	3 %	(1)	0 %	(0)	
cT1	3 %	(1)	5 %	(1)	
cT2	18 %	(6)	16 %	(3)	
cT3	74 %	(25)	74 %	(14)	
cT4	3 %	(1)	5 %	(1)	
Nodes					0.843[a]
cN0	3 %	(1)	0 %	(0)	
cN1	24 %	(8)	26 %	(5)	
cN2	9 %	(3)	5 %	(1)	
Clinical stage					0.478[a]
IB	3 %	(1)	5 %	(1)	
IIA	21 %	(7)	26 %	(5)	
IIB	12 %	(4)	5 %	(1)	
III	53 %	(18)	63 %	(12)	
Pathologic tumor status					<0.001[a]
ypTis	3 %	(1)	0 %	(0)	
ypT0	3 %	(1)	100 %	(19)	
ypT1	12 %	(4)	0 %	(0)	
ypT2	32 %	(11)	0 %	(0)	
ypT3	44 %	(15)	0 %	(0)	
ypT4	3 %	(1)	0 %	(0)	
Pathologic nodal status					0.002[a]
ypN0	32 %	(11)	95 %	(18)	
ypN1	53 %	(18)	5 %	(1)	
ypN2	6 %	(2)	0 %	(0)	
ypN3	6 %	(2)	0 %	(0)	
ypNx	3 %	(1)	0 %	(0)	
Pathologic stage					
0	3 %	(1)	100 %	(0)	<0.001[a]
I	3 %	(1)	0 %	(0)	
IIA	29 %	(10)	0 %	(0)	
IIB	26 %	(9)	0 %	(0)	
III	29 %	(10)	0 %	(0)	
IVb (M1b)	3 %	(1)	0 %	(0)	

[a] Pearson test

[b] Wilcoxon test

Discussion

If neoadjuvant therapy can affect survival by inducing a complete pathologic response, then the molecular mechanism through which it exerts this effect should be identifiable prior to the initiation of therapy. However, to date, no such marker has been established as predicting response to conventional chemotherapy and radiation. A number of markers have been identified by prior authors as playing a significant role in the development of esophageal adenocarcinoma or in effecting response to therapy or outcome after treatment.[10] In this study, we have attempted to link these known markers of esophageal cancer with response to neoadjuvant therapy.

All of the markers in our study have been previously shown to have significant prognostic value in esophageal cancer. Her-2/neu is a proto-oncogene encoding a tyrosine kinase involved in growth factor signaling and when expression is upregulated, has been associated with the development of multiple cancers, including gastric and esophageal cancer.[11] Overexpression has been shown in dysplastic Barrett's metaplasia and esophageal adenocarcinoma. In those patients with adenocarcinoma of the distal esophagus, neoadjuvant therapy has been shown to alter the expression status of Her-2/neu 26 % of the time.[12] Assessment of Her-2 status can be easily and reliably assessed using immunohistochemistry,[13] which when

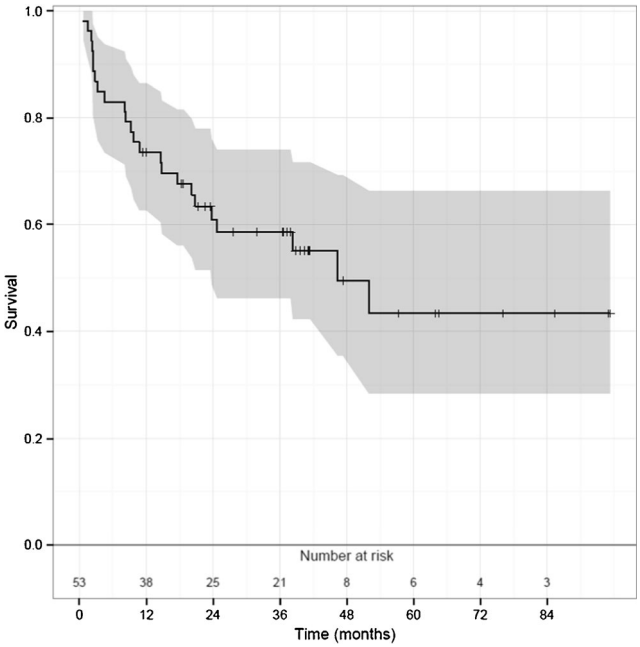

Fig. 1 Overall survival of all 53 enrolled patients in this study

combined with its potential predictive power, make it an ideal candidate for potentially predicting response to neoadjuvant therapy in esophageal adenocarcinoma. Likewise, p53 is a nuclear tumor suppressor involved in the maintenance of genomic integrity, and when mutated, is over-expressed and detectable by immunohistochemistry. While it is thought to contribute to the development of many cancers, it has been shown to directly correlate with the degree of inflammation in the distal esophagus,[14] as well as the development of dysplastic

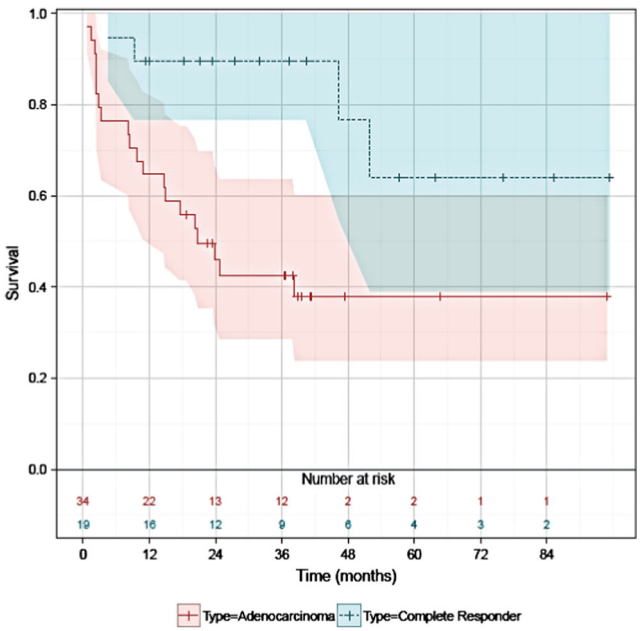

Fig. 2 Survival for pCRs (*blue*) and NRs (*red*)

Barrett's and adenocarcinoma.[15] E-cadherin and Beta-catenin together play pivotal roles in cellular structure and intercellular signaling. E-cadherin forms a calcium dependent cellular adhesion complex which allows cells to bind one another, while Beta-catenin links E-cadherin to the cytoskeleton and plays a pivotal role in intracellular signaling to trigger mitosis. It is thought that loss of expression of either molecule may affect cell adhesion, facilitating tumor infiltration and promote metastasis. Loss of expression has recently been shown to directly impact survival in patients with esophageal cancer.[16] MMP-1 is a metalloprotease thought to play a role in the degradation of the extracellular matrix, and when expression is elevated may also facilitate the infiltration and spread of esophageal cancer, contributing to known worsening of prognosis in those who have elevated expression.[17] NF-kB is a transcription factor which upregulates a variety of genes involved in the inflammatory and immune response. It has been shown to have no expression in normal esophageal tissue but is upregulated in 40 % of Barrett's specimens and 61 % of esophageal adenocarcinomas.[18] Moreover, expression of NF-kB is inversely correlated with pathologic complete response to neoadjuvant therapy, potentially making it a good marker for non-responders.[18] Cox-2 is a housekeeping gene, which is rapidly induced in response by a variety of tumor promoters, oncogenes, and carcinogens and has been found to be constitutively expressed in many tissues, but expression is substantially over-expressed in both esophageal squamous cell cancers and adenocarcinomas.[19–21] Both Cox-2 and NF-kB showed a distinct trend toward significance when expression was compared between complete responders and non-responders. This may be related to the relatively small sample size of this study, and with a greater sample size these differences might have reached significance.

Because each of these proteins is thought to contribute to the dysplastic progression to cancer in the esophagus, we hypothesized that increased dysregulation, as indicated by the total number of driver mutations, might correlate with worse prognosis or increased susceptibility to neoadjuvant therapy. This did not appear to be the case and we found no correlation between the overall number of disregulated proteins and either survival, clinical stage at diagnosis, or response to neoadjuvant therapy.

With 38 different combinations of marker expression in a sample of 53 patients, there is clearly a great deal of heterogeneity in expression of these markers between patients. This may reflect differences in the timing of when the tissue sample was taken from the tumor during tumor cell clonal evolution, or it may reflect the intratumoral heterogeneity of expression which may result in a sampling error when taking a limited sample of the tumor for analysis. It is also clear that none of these markers alone is either necessary or sufficient to cause

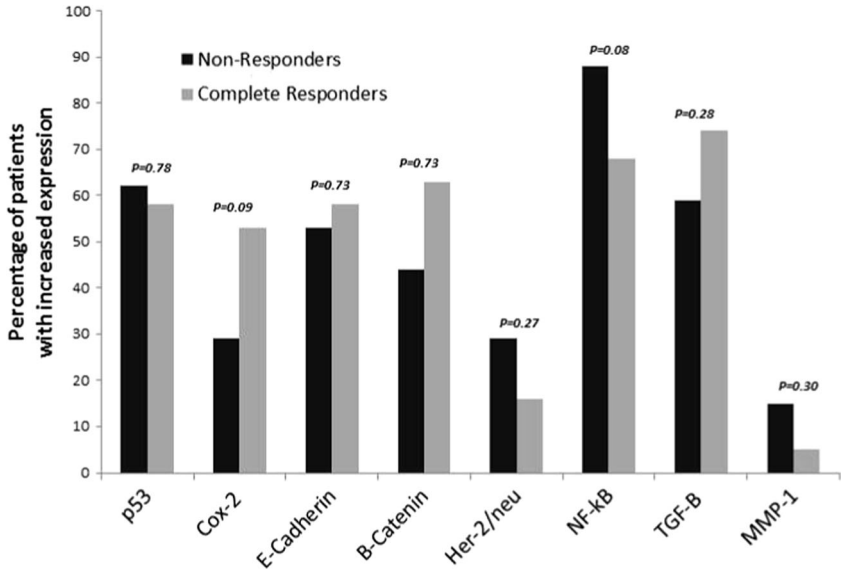

Fig. 3 Specific tumor marker expression between pCR and NR

cancer in these patients and none of these markers imparts a clear susceptibility to neoadjuvant therapy.

This study is limited by a small sample size with only 53 patients and an endpoint which occurs in only about 30 % of treated patients. The relative rarity of this disease and the consequent scarcity of pre-treatment biopsy tissue makes this limitation unavoidable. We believe it is a strength of this study that pre-treatment tissue was used for analysis. While other authors have used surgical specimens for similar studies,[21] the confounding effect of neoadjuvant therapy on the expression of these markers could be profound, and by utilizing tissue taken at the time of diagnosis, we have avoided this potential confounder. Because this is the tissue that would be used for a clinical prediction tool, these results are more generalizable to the newly diagnosed esophageal cancer patient than results obtained from surgical specimens that have already been exposed to neoadjuvant therapy.

This study demonstrates that the known tumor markers of esophageal cancer are expressed in a highly heterogeneous fashion and are not predictive of response to neoadjuvant therapy in esophageal cancer. Any tools that may be developed in the future to predict response to neoadjuvant therapy by esophageal cancer will need to take into account this heterogeneity and will likely need to utilize a much broader array of markers to better tailor therapy to patient-specific tumor biology.

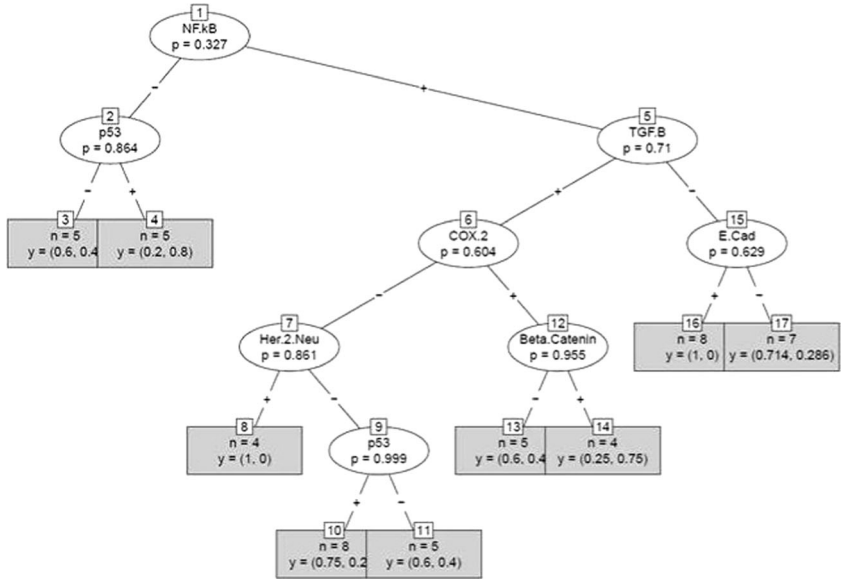

Fig. 4 Optimal combination and regression tree analysis predictive of pCR in patients who have undergone neoadjuvant therapy. MMP-1 was omitted by the algorithm due to low predictive power and small group sizes

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References

1. Cooper JS, Guo MD, Herskovic A, et al. Chemoradiotherapy of locally advanced esophageal cancer: Long-term follow-up of a prospective randomized trial (RTOG 85-01). radiation therapy oncology group. *JAMA*. 1999;281(17):1623-1627.
2. Perry KA, Enestvedt CK, Diggs BS, Jobe BA, Hunter JG. Perioperative outcomes of laparoscopic transhiatal inversion esophagectomy compare favorably with those of combined thoracoscopic-laparoscopic esophagectomy. *Surg Endosc*. 2009;23(9):2147-2154.
3. Holscher AH, Drebber U, Schmidt H, Bollschweiler E. Prognostic classification of histopathologic response to neoadjuvant therapy in esophageal adenocarcinoma. *Ann Surg*. 2014;260(5):779-84; **discussion 784-5**.
4. Meredith KL, Weber JM, Turaga KK, et al. Pathologic response after neoadjuvant therapy is the major determinant of survival in patients with esophageal cancer. *Ann Surg Oncol*. 2010;17(4):1159-1167.
5. Slotta-Huspenina J, Wolff C, Drecoll E, et al. A specific expression profile of heat-shock proteins and glucose-regulated proteins is associated with response to neoadjuvant chemotherapy in oesophageal adenocarcinomas. *Br J Cancer*. 2013;109(2):370-378.
6. Wang E, Zaman N, Mcgee S, Milanese J, Masoudi-Nejad A, O'Connor-McCourt M. Predictive genomics: A cancer hallmark network framework for predicting tumor clinical phenotypes using genome sequencing data. *Semin Cancer Biol*. 2015;30C:4-12.
7. Noble F, Hopkins J, Curtis N, et al. The role of systemic inflammatory and nutritional blood-borne markers in predicting response to neoadjuvant chemotherapy and survival in oesophagogastric cancer. *Med Oncol*. 2013;30(3):596-**013-0596-6**.
8. Smit JK, Faber H, Niemantsverdriet M, et al. Prediction of response to radiotherapy in the treatment of esophageal cancer using stem cell markers. *Radiother Oncol*. 2013;107(3):434-441.
9. Zhang SS, Huang QY, Yang H, et al. Correlation of p53 status with the response to chemotherapy-based treatment in esophageal cancer: A meta-analysis. *Ann Surg Oncol*. 2013;20(7):2419-2427.
10. Lagarde SM, ten Kate FJ, Richel DJ, Offerhaus GJ, van Lanschot JJ. Molecular prognostic factors in adenocarcinoma of the esophagus and gastroesophageal junction. *Ann Surg Oncol*. 2007;14(2):977-991.
11. Thompson SK, Sullivan TR, Davies R, Ruszkiewicz AR. Her-2/neu gene amplification in esophageal adenocarcinoma and its influence on survival. *Ann Surg Oncol*. 2011;18(7):2010-2017.
12. Schoppmann SF, Jesch B, Friedrich J, et al. Expression of her-2 in carcinomas of the esophagus. *Am J Surg Pathol*. 2010;34(12):1868-1873.
13. Hofmann M, Stoss O, Shi D, et al. Assessment of a HER2 scoring system for gastric cancer: Results from a validation study. *Histopathology*. 2008;52(7):797-805.
14. Binato M, Fagundes R, Gurski R, Meurer L, Edelweiss MI. Immunohistochemical overexpression of the p53 protein and ki-67 (MIB-1) antigen in patients with GERD and chronic esophagitis. *Appl Immunohistochem Mol Morphol*. 2010;18(3):236-243.
15. Hritz I, Gyorffy H, Molnar B, et al. Increased p53 expression in the malignant transformation of Barrett's esophagus is accompanied by an upward shift of the proliferative compartment. *Pathol Oncol Res*. 2009;15(2):183-192.
16. Zhao XJ, Li H, Chen H, et al. Expression of e-cadherin and beta-catenin in human esophageal squamous cell carcinoma: Relationships with prognosis. *World J Gastroenterol*. 2003;9(2):225-232.
17. Murray GI, Duncan ME, O'Neil P, McKay JA, Melvin WT, Fothergill JE. Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer. *J Pathol*. 1998;185(3):256-261.
18. Abdel-Latif MM, O'Riordan J, Windle HJ, et al. NF-kappaB activation in esophageal adenocarcinoma: Relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. *Ann Surg*. 2004;239(4):491-500.
19. Zhang W, Wang L, Chang A, Jin Y, Rao J. Immunohistochemical analysis of cyclooxygenase-2 expression in premalignant and malignant esophageal glandular and squamous lesions in Cixian, China. *Cancer Detect Prev*. 2003;27(4):243-249.
20. Shamma A, Yamamoto H, Doki Y, et al. Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus. *Clin Cancer Res*. 2000;6(4):1229-1238.
21. Garcia-Aguilar J, Chen Z, Smith DD, et al. Identification of a biomarker profile associated with resistance to neoadjuvant chemoradiation therapy in rectal cancer. *Ann Surg*. 2011;254(3):486-92; **discussion 492-3**.