Discovery and Validation of a Serologic Autoantibody Panel for Early Diagnosis of Esophageal Squamous Cell Carcinoma

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Abstract

Background: Esophageal squamous cell carcinoma (ESCC) accounts for the highest incidence rate worldwide and is responsible for the fourth leading cause of cancer-related death. Currently, serologic biomarkers for early ESCC diagnosis are needed for timely treatment.

Methods: The performance of a four-autoantibody panel (i.e., anti-TP53, HRAS, CTAG1A, and NSG1) was evaluated by ELISA for the early diagnosis of ESCC with 569 retrospective serum samples. A training set comprising 129 patients with early-stage ESCC, 130 patients with esophageal benign lesion (EBL), and 150 healthy controls (HC) was used to develop an early ESCC predictive model. Data obtained from an independent validation set were used to evaluate and validate the predictive model to distinguish the early ESCC from the controls (EBL+HC). Finally, a multiplexed assay based on the Luminex xMAP technology platform was developed to enable simultaneous detection of the four-autoantibody panel using the validation set.

Results: The four-autoantibody panel significantly discriminated early ESCC cases from the controls with 62.8% sensitivity at 88.9% specificity in the training set and with 58.0% sensitivity at 90.0% specificity in the independent validation set. The results of the multiplexed assay using xMAP technology for early ESCC showed a significant correlation with that of the ELISA assays with 66.0% sensitivity at 90.9% specificity.

Conclusions: A four-autoantibody panel showed good performance for early ESCC diagnosis with ELISA and could be further developed into a multiplex assay using the Luminex xMAP technology.

Impact: The four-autoantibody panel could be used for serologic screening for early ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the common malignant diseases of the upper digestive tract worldwide, especially in China, where ESCC accounts for the highest incidence rate worldwide and is responsible for the fourth leading cause of cancer-related death (1, 2). The five-year overall survival rate for ESCC remains a dismal 15%–25% during the last several decades, largely because most cases are still diagnosed in the late stages mainly due to the lack of specific symptoms in patients at the early stages (3). Traditionally, endoscopy examination and mucosa biopsy are widely used in center for early ESCC detection, yet invasive examinations limit their application in the screening of asymptomatic populations. Therefore, it is essential to develop new noninvasive biomarkers as screening tests for the early detection of ESCC to improve the patients’ survival rate.

Recent advances in the biomarker field have demonstrated that circulating autoantibodies against tumor-associated antigens (TAA) present in sera may be detectable several years before the development of symptomatic cancer and may serve as new screening markers for patients with cancer at the early stages (4–6). In addition, the development of a panel comprising several autoantibodies is recommended because the cause of most cancers is high heterogeneity, and because the performance of a biomarker panel usually trumps that of individual biomarkers (4). Although some studies investigated the serologic autoantibody biomarker for early ESCC detection, the performance is still unsatisfactory (7, 8).

Recently, using an approach based on HuProt array (i.e., v3.0; 20,240 individual human proteins), we successfully discovered and validated eight IgG autoantibody biomarkers, including anti-TP53, -ETHE1, -CTAG1A, -C1QTNF1, -TEX264, -CLDN2, -NSG1, and -HRAs, for lung cancer diagnosis at the early stages (9). In a
small cohort study, we observed that of the eight LC autoantibodies, anti-TP53, -HRAS, -CTAG1A (P < 0.01), and -NSG1 (P < 0.05), exhibited significantly higher immunity and positive rates in an ESCC group of early stages (n = 50) than those in EBL (n = 50) or HC group (n = 50; Supplementary Fig S1). Considering the obvious advantages of serologic biomarkers in early diagnosis of cancer, the performance of the four autoantibodies for early diagnosis of ESCC in a large cohort is worth exploring.

In this study, we evaluated the performance of the four-autoantibody panel for early diagnosis of ESCC using ELISA in a large cohort of 569 retrospective sera samples, including the following: a training set (n = 409) consisting of 129 patients with early-stage ESCC, 130 patients with esophageal benign lesion (EBL), 150 healthy controls (HC); and an independent matched validation set (n = 160). The four-autoantibody panel could significantly distinguish early ESCC cases from the controls (EBL+HC). Moreover, we successfully developed a multiplex assay based on the Luminex xMAP technology platform to assay the four autoantibodies simultaneously. The development of this biomarker panel will pave the road for improving early diagnosis of ESCC.

Materials and Methods
Study participants
This prospective cohort comprised 569 serum samples collected from 210 healthy persons, 179 patients with early ESCC, and 180 patients with EBL from February 2016 to June 2018 at Fujian Provincial Hospital, in Fujian Province, China. Among the participants, the healthy persons selected during annual health examinations showed no evidence of disease; the early ESCC patients with stages 0, I, and II based on the TNM classifications showed no evidence of disease; the early ESCC patients recruited after histopathologic confirmation of ESCC tumors; the patients with EBL were diagnosed with benign esophageal tumor, benign esophageal ulcer, esophageal erosion, and reflux esophagitis after accurate clinical assessment. Characteristics including gender, age, tumor stage, and smoking and drinking history of all participants are listed in Supplementary Table S1. This study was approved by the Institutional Review Board of Fujian Provincial Hospital, and all participants provided written informed consent.

Identification of four serum autoantibodies by Western blotting
GST-tagged recombinant proteins, TP53 (71 kDa), HRAS (46 kDa), CTAG1A (45 kDa), and NSG1 (48 kDa) at 200 ng (expressed by yeast and provided by CDI Laboratories, Inc.) and GST-tagged recombinant proteins, TP53, HRAS, CTAG1A, and NSG1 were coated onto 96-well plates at 50 ng/well at 4°C overnight, followed by three washes with PBST. The nonspecific binding was blocked by 3% BSA/well at 37°C for 1 hour and then washed one time with PBST. The wells were incubated with serum samples (1:500) at 37°C for 1 hour and then washed four times with PBST. Subsequently, 100 μL HRP-labeled goat anti-human IgG antibody (Jackson Immuno; 1:40,000) was added to each well at 37°C for 30 minutes. After 4 washes with PBST, 50 μL tetramethyl benzidine (TMB) and 3% hydrogen peroxide (H2O2; Xiamen Yingke XinChuang) were added successively and incubated at 37°C for 10 minutes. The reaction was terminated by adding 50 μL of 2 mol/L H2SO4, and immunoreactivity (OD) was measured by reading the A450. All assays were performed in duplicate, and the averaged OD value was calculated.

Luminex bead coupling and multiplex assay procedure
Bead coupling were performed according to the manufacturer’s instructions (Luminex Corporation coupling protocol). Briefly, Sulfo-NHS and EDC were added to the stock beads and then incubated for 20 minutes at room temperature. Subsequently, 10 μg of recombinant protein was added and incubated for 2 hours by rotation at room temperature. Then, the coupled beads were washed twice with PBS and stored at 2°C–8°C in the dark.

Multiplex immunoassay for autoantibody markers was performed using the xMAP technology platform (Luminex Corp.). Serum sample (1:20) at 10 μL was incubated with recombinant protein coupled beads in 96-well microtiter plate (Millipore) at 37°C for 15 minutes on a plate shaker (1,000 rpm). The antigen–antibody complex was incubated with the PE-labeled goat anti-human IgG antibody (Millipore) at 37°C for 15 minutes on a plate shaker (1,000 rpm). The washed and resuspended beads were then analyzed on the Luminex-200. For each analyte, median fluorescence intensity (MFI) values were calculated using Luminex xPONENT software. All assays were performed in duplicate, and the averaged intensity value was calculated.

Discovery and validation of biomarker panels
The performance of each candidate biomarker was assessed after obtaining signals of the ELISA assays and normalizing using serum samples of the discovery cohort. Combinatorial biomarker panels with better performance were identified based on the sensitivity and specificity of the four autoantibodies in the validation cohort, after the performances of all possible combinations between two and four autoantibodies were evaluated. The proteins of the best combination were further validated in an independent cohort. The detailed methods were described in ref. 9.

Results
Study design
The objective of this study was to evaluate the performance of a panel of four autoantibodies (i.e., anti-TP53, -HRAS, -CTAG1A, and -NSG1) and develop a multiplex assay for ESCC early diagnosis. Two independent studies were performed to evaluate the performance of a four-autoantibody panel for ESCC early diagnosis in 569 retrospective serum samples by ELISA assay (Fig. 1). First, a training set (n = 409) comprising 129 patients diagnosed with early-stage (0/I/II) ESCC, 130 patients diagnosed with EBL,
and 150 HCs was used to develop the early ESCC predictive model. Second, an independent and matched validation set ($n = 160$), comprising 50 patients diagnosed with early-stage (0/I/II) ESCC, 50 patients diagnosed with EBL, and 60 HCs was used to evaluate the developed model and to determine the ability of the panel to distinguish early ESCC cases from controls (EBL + HC).

Finally, a multiplex assay based on the Luminex xMAP technology platform was developed to simultaneously test serum immunity with the four autoantigens.

**Conformation of serum immunity of the four autoantigens using Western blot**

To confirm the serum reactivity observed in our previous ELISA assay, we used the Western blot analysis by employing recombinant proteins, TP53, HRAS, CTAG1A, and NSG1 expressed in yeast. We also included GST protein as a negative control, because all the recombinant proteins were N-terminal GST fusions. As expected, when four early ESCC serum samples previously tested positive in ELISA for anti-TP53, HRAS, CTAG1A, and NSG1 were incubated individually in the Western blot analysis, all four recombinant proteins showed stronger and substantially higher serum reactivity than the GST control. None of the four proteins showed any detectable signals with the negative control serum samples (i.e., EBL and HC; Supplementary Fig. S2). Therefore, our Western blot analysis confirmed the previous ELISA results.

**Testing of biomarkers for early-stage ESCC diagnosis with ELISA**

To validate the usefulness of the four autoantibodies as biomarkers for early diagnosis of ESCC, we assembled a much larger cohort, comprising 129 serum samples collected from 129 patients diagnosed with early stage ESCC, as well as 150 and 130 serum samples from healthy subjects and EBL patients, respectively, as negative controls. Statistical analysis did not show any significant differences in age, gender, or smoking history between the ESCC groups and negative controls (Table 1; Supplementary Table S1). We next developed an ELISA-based assay with the four candidate biomarkers targeting autoantigens, purified recombinant TP53, HRAS, CTAG1A, and NSG1 proteins. All four candidates showed significantly higher ELISA signals using the ESCC samples than the negative control group (Fig. 2A–D). For example, the AUC values for anti-TP53, -HRAS, -CTAG1A, and -NSG1 were determined to be 0.574, 0.701, 0.809, and 0.613, respectively. Similarly, the sensitivity values determined for anti-TP53, -HRAS, -CTAG1A, and -NSG1 were 25.6%, 29.5%, 30.2%, and 18.6%, respectively, at a specificity of higher than 95%.

To further improve the performance, we exhaustively analyzed the performance of all possible combinations among the four biomarkers (Fig. 2E). The sensitivity of a given biomarker panel was substantially improved without dramatically sacrificing the specificity values. Indeed, the best combination consisted of all four biomarkers. It produced the highest discriminative ability with 62.8% sensitivity at 88.9% specificity (Fig. 2E; Supplementary Fig. S3).

**Validation of biomarker panel for early-stage ESCC diagnosis with ELISA**

To further evaluate the diagnostic performance of the four-biomarker panel, we assembled an independent cohort with

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**Table 1. Characteristics of the samples in phase I**

| Variable     | Early ESCC ($n = 129$) | EBL ($n = 130$) | Healthy ($n = 150$) | $P$  |
|--------------|------------------------|----------------|---------------------|------|
| Age (years)  | $n$ Mean %             | $n$ Mean %     | $n$ Mean %          |      |
| Sex          | Male 92 71.3 86 66.2 101 67.3 | Female 37 28.7 44 33.8 49 32.7 | | 0.114 |
| Smoker       | 48 37.2 44 33.8 47 31.3 | | | 0.087 |
| Drinker      | 19 14.7 20 15.4 21 14.0 | | | 0.077 |
| Type         | 0 40 31.0               | 1 36 27.9      | 2 53 41.1           | 0.551 |

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serum samples collected from 50 patients with ESCC at early stages and from 60 healthy subjects and 50 patients with EBL. Statistical analysis did not show any significant differences in age, gender, or smoking history between the ESCC groups and negative controls (Table 2; Supplementary Table S1). All four candidates showed significantly higher intensity in ESCC than the negative control group (Fig. 3A). The sensitivity of the panel was 58.0% at 90.0% specificity (Fig. 3B).

Figure 2. Performance of the biomarkers in phase I. Boxplot and ROC curve for TPS3 (A), HRAS (B), CTAG1A (C), and NSG1 (D). E, The performance of the combined panel. The performance of individual candidates and combined biomarkers to distinguish between early ESCC and controls using ELISA assay in phase I is shown.

Table 2. Characteristics of the samples in phase II

| Variable       | Early ESCC (n = 50) | EBL (n = 50) | Healthy (n = 60) | p       |
|----------------|---------------------|--------------|------------------|---------|
| Age (years)    |                     |              |                  |         |
| Mean           | 56.1                | 55.8         | 56.3             | 0.969   |
| SD             | 9.5                 | 9.6          | 10.9             |         |
| Sex            |                     |              |                  | 0.379   |
| Male           | 34                  | 32           | 40               |         |
| Female         | 16                  | 18           | 20               |         |
| Smoker         |                     |              |                  | 0.198   |
| Male           | 18                  | 16           | 18               |         |
| Female         | 7                   | 6            | 7                |         |
| Drinker        |                     |              |                  | 0.361   |
| Type           |                     |              |                  |         |
| Type 0         | 19                  | 14           | 17               |         |
| Type I         | 14                  | 14           | 14               |         |
| Type II        | 17                  | 14           | 17               |         |

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Development of a multiplexed assay with the four-biomarker panel for the Luminex platform

To enable a multiplexed assay for the four-biomarker panel, we coupled the four recombinant proteins to the beads separately and were able to accurately detect the conjugated proteins on the Luminex-200 (Supplementary Fig. S4). The developed multiplex assay based on the Luminex xMAP technology platform made it possible to simultaneously profile the serum autoimmunity against the four autoantigens, which is crucial for future clinical application of early ESCC diagnosis. As illustrated in Supplementary Fig. S5, the intensity measured on the Luminex platform correlated well with that obtained with the ELISA assays for all four autoantibodies. Using a similar bioinformatics approach as described above, all four candidates showed significantly higher intensity in ESCC than the negative control group (Fig. 4A), and the sensitivity of the panel was calculated to be 66.0% at 90.9% specificity on the Luminex platform (Fig. 4B). The agreement of positive hits for the combination was 76.1% (Fig. 4C).

Discussion

Mounting lines of evidence demonstrated that humoral immune responses to autologous TAAAs can be exerted, and they trigger the release of large amounts of autoantibodies into sera during early tumorigenesis (4–7). Compared with the direct detection of TAAAs in body fluids, detection of serum autoantibodies targeting TAAAs can be a more sensitive approach to early diagnosis of cancer in asymptomatic patients (5, 6, 9). TAA-based autoantibodies, particularly the IgG and IgA isotypes, are highly stable and persistent in sera (4). Therefore, these unique characteristics make it possible for TAA-based autoantibodies to serve as valuable serologic markers for the ESCC early diagnosis (8).

On the basis of our previous study, in which we used a relatively small cohort, we aimed to determine the diagnostic value of a four-autoantibody panel comprising anti-TP53, -HRAS, -CTAG1A, and -NSG1 for early ESCC using a much larger cohort, that is, 569 retrospective sera samples, including a training set (n = 409) and an independent matched validation set (n = 160), using ELISA assay. Interestingly, the panel of proteins selected for investigation comprised a number of TAAAs related to tumorigenesis. In brief, TP53, a well-recognized tumor suppressor gene, is reportedly involved in the regulation of cell processes, such as cell cycle, apoptosis, senescence, and DNA repair (10, 11). It is often mutated and overexpressed, in various cancers, including ESCC (12–15). For instance, high frequency of TP53 mutation closely correlated with loss of heterozygosity (LOH) at 17p13 site, and high positive rate of TP53 protein expression, have been reported in a comprehensive study for 94 surgically resected Japanese cases of ESCC (14), and loss of TP53 function is thought to be very important event at early stage of ESCC (15). HRas, a small GTPase, is a member of the Ras oncogene family, whose mutations are responsible for the development of bladder cancer, thyroid cancer, salivary ductal carcinoma, epithelial–myoepithelial carcinoma, and renal cancer (16, 17). Interestingly, HRAS mutations in cancer is thought to be very important event at early stage of ESCC (15). HRAs, a small GTPase, is a member of the Ras oncogene family, whose mutations are responsible for the development of bladder cancer, thyroid cancer, salivary ductal carcinoma, epithelial–myoepithelial carcinoma, and renal cancer (16, 17). Interestingly, HRAS mutations in cancer is found to be responsible for the activation of the RAS and mTOR pathways, which might serve as a novel target for MEK and mTOR inhibitors (18). Moreover, CTAG1A is considered a good candidate targets for immunotherapy as it is the most immunogenic among the CTA family members, with a restricted expression in normal somatic tissues concomitant with a reexpression in solid epithelial cancers (19). CTAG1A, also known as New York Esophageal Squamous Cell Carcinoma Antigen 1 (NY-ESO-1), is a member of the cancer testis (CTA) subfamily that induces a strong immune response against tumor cells. The aberrant expression of CTAG1A has been described in a variety of cancer tissues (20). NSG1 is an endosome protein expressed in neuronal cells, and its transcription may be directly regulated by the tumor suppressor gene TP53, as evidenced by the observations that many non-neuronogenous cancer cells can express NSG1 in a TP53-dependent manner under the...
effect of doxorubicin, hydrogen peroxide, UV, and γ-rays. Moreover, NSG1 overexpression significantly inhibits cell proliferation and induces cell apoptosis (21). In addition, as a TP53-responsive gene, NSG1 is demonstrated to be accumulated in endoplasmic reticulum (ER) and play an important role for the apoptosis induction in response to DNA damage (22). Of the four autoantigens, autoantibodies targeting TP53 and CTAG1A are detectable in ESCC patient sera even before the cancer is diagnosed (7, 23). However, the other two autoantibodies targeting HRas and NSG1 have not yet been described in the context of ESCC diagnosis.

In this study, we first confirmed the presence of the autoantibodies targeting the four autoantigens in sera of patients with early ESCC by using Western blot analysis. Next, similar to the results observed in our pilot study, the ELISA assays with the 569 retrospective sera samples confirmed that each of the four autoantigens could significantly differentiate early ESCC from the EBL and HC controls in terms of their sensitivity and specificity values. At above 95% specificity, HRAS and CTAG1A could reach about 30% sensitivity individually. To further improve the sensitivity for early ESCC diagnosis, we evaluated the diagnostic performance of 11 panels including different combinations of the four candidates. The sensitivity of the panel including HRAS and CTAG1A could reach 49%, while the sensitivity of the panel including all four candidates could reach 60% at 90% specificity. These results were based on two independent cohorts that highlighted the robustness and great potential of this panel for early ESCC diagnosis.

ELISA is the conventional method used for detecting the presence of autoantibodies. However, this assay cannot be easily multiplexed to simultaneously profile autoantibodies targeting multiple autoantigens, thereby limiting its application to one autoantibody detection at a time. Luminex xMAP is FDA-approved for diagnostic use, and is also a preferred method for measuring serologic autoantibody panels (24, 25), because it offers a highly multiplexed and high-throughput platform with relatively superior sensitivity (26, 27). Therefore, in this study, we successfully converted a panel of four individual ELISA tests into a multiplexed assay on the basis of the Luminex xMAP technology to enable simultaneous detection of the four autoantibodies in a single reaction. We demonstrated that the performance of our biomarker panel on the Luminex platform showed significant correlation with that obtained with the ELISA tests but with a slightly better sensitivity of 66% than the ELISA tests (i.e., 58%) at the same specificity. As a result, considering the obvious advantages of reducing labor costs and assay time, and relatively superior sensitivity, there is no doubt that the Luminex-based multiplex assay will benefit future application of four-autoantibody panel to clinical settings.

In conclusion, to the best of our knowledge, this is the first study to define the good diagnostic value of a multi-autoantibody panel for early ESCC diagnosis in a large cohort. The development of a multiplex assay based on the Luminex xMAP technology for simultaneous serologic profiling with the four autoantibodies would facilitate the improvement of serologic screening for the

Figure 4.
Performance of the biomarkers in phase III. A, Performance of four biomarkers. B, Performance of the biomarker panel. C, Agreement of positive hits between ELISA and Luminex. The performance of individual candidates and combined biomarkers to distinguish between early ESCC and controls using the Luminex xMAP technology in phase III and the detection agreement with phase II are shown.
early ESCC diagnosis, thereby possibly reducing the mortality of the patients. Nevertheless, a prospective study in a larger cohort comprised of other serum samples from patients with different cancer types and autoimmune diseases for comparison, will still be required to further validate the utility of four-autoantibody panel for clinical use prior to its future implementation. We anticipate the current panel will be refined as follow-up data accrue.

Disclosure of Potential Conflicts of Interest
J. Qian is a consultant/advisory board member for CDI. H. Zhu is a consultant/advisory board member for CDI Labs. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: Q.-Z. Zheng, Y. Li, J. Qian, H. Zhu, Y. Huang Development of methodology: J. Pan, Q.-Z. Zheng, Y. Li, J.-Y. Zheng Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Q.-Z. Zheng, Y. Li, J.-Y. Zheng, B.-S. Xie Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Pan, Q.-Z. Zheng, Y. Li, L.-L. Yu, J.-Y. Zheng, X.-J. Pan, Y.-A. Wu, J. Qian

References
1. Felty J, Soerjomataram I, Dikhvit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:1359–86.
2. Arnold M, Soerjomataram I, Felty J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. Gut 2015;64:381–7.
3. Pennathur A, Gibson MK, Jobe BA, Luketic JD. Oesophageal carcinoma. Lancet 2013;381:400–12.
4. Finn OJ. Immune response as a biomarker for cancer detection and a lot more. N Engl J Med 2001;353:1288–90.
5. Tan HT, Low J, Lim SG, Chung MC. Serum autoantibodies as biomarkers for early cancer detection. FEBS J 2009;276:6880–904.
6. Macdonald IK, Parys-Kowalska CB, Chapman CJ. Autoantibodies: opportunities for early cancer detection. Trends Cancer 2017;3:198–213.
7. Xu YW, Peng YH, Chen B, Wu ZY, Wu JY, Shen JH, et al. Autoantibodies as potential biomarkers for the early detection of esophageal squamous cell carcinoma. Am J Gastroenterol 2014;109:36–45.
8. Zhang H, Xia J, Wang K, Zhang J. Serum autoantibodies in the early detection of esophageal cancer: a systematic review. Tumour Biol 2015;36:95–109.
9. Pan J, Song C, Chen D, Li Y, Liu S, Hu S, et al. Identification of serological biomarkers for early diagnosis of lung cancer using a protein array-based approach. Mol Cell Proteomics 2017;16:2069–78.
10. Shaw PH. The role of p53 in cell cycle regulation. Pathol Res Pract 1996;192:669–75.
11. Higashitsuji H, Higashitsuji H, Masuda T, Liu Y, Iuss K, Fujita J. Enhanced deacetylation of p53 by the anti-apoptotic protein HSCO in association with histone deacetylase 1. J Biol Chem 2007;282:13716–25.
12. Ozaki T, Nakagawa A. Role of p53 in cell death and human cancers. Cancers 2011;3:994–1013.
13. Mogi A, Kuroano H. TP53 mutations in nonsmall cell lung cancer. J Biomed Biotechnol 2011;2011:583929.
14. Egashira A, Morita M, Yoshiida R, Sakei H, Oki E, Sadanaga N, et al. Loss of p53 in esophageal squamous cell carcinoma and the correlation with survival: analyses of gene mutations, protein expression, and loss of heterozygosity in Japanese patients. J Surg Oncol 2011;104:169–75.
15. Kim SG, Hong SJ, Kwon KW, Jung SW, Kim WY, Jung IS, et al. The expression of p53, p16, cyclin D1 in esophageal squamous cell carcinoma and esophageal dysplasia. Korean J Gastroenterol 2006;48:269–76.
16. Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. Cancer Res 2012;72:2457–67.
17. Ouerhani S, Elgaied AB. The mutational spectrum of HRAS, KRAS, NRAS and FGFR3 genes in bladder cancer. Cancer Biomark 2011;10:259–66.
18. Kissling MK, Curioni-Fontecedro A, Samaras P, Atrott K, Cosin-Roger J, Lang S, et al. Mutant HRAS as novel target for MEK and mTOR inhibitors. Oncotarget 2015;6:42183–96.
19. Thomas R, Al-Khadir G, Roelands J, Hendrickx W, Derminie S, Bedognetti D, et al. NY-ESO-1 based immunotherapy of cancer: current perspectives. Front Immunol 2018;9:947.
20. Jungbluth AA, Chen YJ, Stockert E, Busam KJ, Kolb D, Iversen K, et al. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer 2001;92:856–60.
21. Ohashi S, Futamura M, Kamino H, Nakamura Y, Kitamura N, Miyamoto Y, et al. Identification of NEEP21, encoding neuron-enriched endosomal protein of 21 kDa, as a transcriptional target of tumor suppressor p53. Int J Oncol 2010;37:1133–41.
22. Kudosh T, Kimura J, Izu GC, Miki Y, Yoshida K. DAS234E, a novel p53-responsive gene, induces apoptosis in response to DNA damage. Exp Cell Res 2010;316:2849–58.
23. Chai Y, Peng B, Dai L, Qian W, Zhang JY. Autoantibodies response to MDM2 and p53 in the immunodiagnosis of esophageal squamous cell carcinoma. Scand J Immunol 2014;80:109–16.
24. Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. Cancer Res 2012;72:2457–67.
25. Casiano CA, Medralla-Varela M, Tan EM. Tumor-associated antigen arrays for the serological diagnosis of cancer. Mol Cell Proteomics 2006;5:1745–59.
26. Farlow EC, Patel K, Baru S, Lee BS, Kim AW, Coon JS, et al. Development of a multiplexed tumor-associated autoantibody-based blood test for the detection of non-small cell lung cancer. Clin Cancer Res 2010;16:3452–62.
27. Elshafy MF, Mcgory JP. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. Methods 2006;38:317–23.