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Epidermal growth factor receptor localized to exosome membranes as a possible biomarker for lung cancer diagnosis

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Detection of drug-target proteins and biomarkers that are expressed in cancer tissue has significant potential for both diagnosis and treatment of cancer. However, current immuno-histochemical and cytogenetic analyses of biopsy specimens for pre-operational diagnosis are highly invasive and often difficult to apply to lung cancer patients. The purpose of this study was to evaluate the possible utility of determining epidermal growth factor receptor (EGFR) expression on exosomal membranes using a targeted ELISA with an anti-CD81 antibody as a capture antibody for lung cancer diagnosis. While soluble EGFR (sEGFR) levels in plasma were not remarkably different between lung cancer patients and normal controls, significantly higher exosomal EGFR expression levels were observed in 5/9 cancer cases compared to normal controls. These results suggest that measurement of exosomal protein levels could be useful for *in vitro* diagnosis, and that exosomal EGFR is a possible biomarker for characterization of lung cancer.

1. Introduction

Lung cancer has been recognized as a heterogeneous disease, since its development is unique in terms of clinical characterizations, prognosis, response and tolerance to treatment for every patient. Epidermal growth factor receptor (EGFR) expression is significantly elevated in some of the tumors and has been associated with tumor growth, invasion, and metastasis in non-small cell lung cancer. The function of EGFR is upregulated in lung cancers and thus its blockade by EGFR tyrosine kinase inhibitors (TKI) improves outcomes for lung cancer patients. Therefore expression of EGFR could possibly serve as a marker to predict which lung cancer patients would be most likely benefit from such a treatment (Philip et al. 2011; Gusterson et al. 1984; Modjtahedi et al. 1993). Although tumor biopsy is currently recommended as the standard method to detect EGFR expression in patients, it has several disadvantages such as its high invasiveness, potential sampling error and risk of trauma. Considering these limitations, there is a need to develop novel and less invasive methods to detect lung cancer.

Exosomes are endogenous nano-vesicles (40–100 nm in diameter) secreted by various cells (Deng et al. 2012; Simons and Raposo 2009; Théry et al. 2009) and they are known to contain many kinds of RNAs and proteins derived from their parent cells. Micro RNAs (miRNAs) included in blood exosomes could potentially be used for cancer diagnosis (Kosaka et al. 2010). The membranes of exosomes secreted from cancer cells contain proteins related to characteristics of specific cancers such as melanoma and colon cancer (Mathivanan et al. 2010; Simpson et al. 2008). These observations support the idea that detection of exosomal EGFR as a blood biomarker could prove useful for cancer diagnosis and characterization. In this study, we established a simple ELISA method to detect and quantify exosomal EGFR. We also examined the possibility of utilizing exosomal EGFR as a blood biomarker for lung cancer diagnosis. Our demonstration of exosomal EGFR in the plasma from a lung cancer patient provides a rationale for further studies to investigate the utility of exosomal EGFR as an easily measurable biomarker for lung cancer diagnosis.

2. Investigations and results

2.1. Characteristics of exosomes derived from HPAEpiC and HARA-B cell lines

Exosomes derived from HPAEpiC and HARA-B cells were compared using transmission electron microscopy (TEM) and dynamic light scattering. Electron microscopy analysis revealed that the exosomes collected from the culture medium of various cancer cell lines consisted primarily of small membrane vesicles having an average diameter of 90–120 nm (Fig. 1A, 1B), similar to previously described exosome preparations (Valadi et al. 2007).

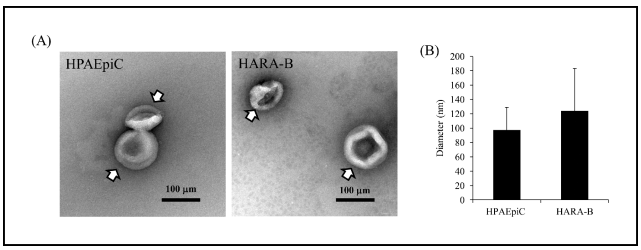

Fig. 1: Characteristics of purified exosomes. A) TEM images of exosomes derived from HPAEpiC and HARA-B cells negatively stained with uranyl acetate. B) The average diameters of exosomes. The average particle diameters obtained by dynamic light scattering. Data are shown as means and standard deviations of 6 measurements.

2.2. Comparison of EGFR expression between cancer cell lines

In order to select lung cancer cells that expressed EGFR at high levels, western blots were performed for cancer cell lines and HPAEpiC cells (Fig. 2A). Quantitative analysis revealed that lung cancer cell lines expressed EGFR while EGFR was seldom detected on HPAEpiC cells. EGFR expression was particularly high on HARA-B cells (Fig. 2B). Furthermore, EGFR expression levels were parallel on exosomes and the cells from which they were derived (Fig. 2B and 2C).

2.3. Expression analysis of exosomal EGFR derived from conditioned media and plasma of tumor-bearing mice

Detection, quantification and characterization of target molecules by a blood test is ideal for performing a cancer diagnosis. To determine whether EGFR was expressed on the exosomes derived from cancer cells and whether this could be a useful biomarker indicating the presence or the expression level of the cancer-related molecules of cancer, exosomal EGFR levels in conditioned media and plasma from tumor-bearing mice were measured by an ELISA assay. At first, purified exosomes derived from cell culture supernatants were captured using an anti-human CD81 antibody. Furthermore, Figs. 3A and 3B show that EGFR in cell supernatants and purified exosomes derived from HARA-B cells, which showed the highest levels of EGFR among the examined cell lines (Fig. 2C), were detected in a dose-dependent manner. In contrast, EGFR expression on exosomes derived from HPAEpiC cells was almost undetectable. Thus, exosomal EGFR expression in cell supernatants could be assessed by this ELISA system. Next, the exosomal EGFR levels in plasma of HARA-B tumor-bearing mice were investigated. Expression of EGFR in HARA-B tumor tissue was confirmed by immunostaining using an anti-human EGFR antibody (Fig. 3C). The plasma samples of the tumor-bearing mice were measured using an established ELISA, and then exosomal EGFR levels were found to increase in parallel with the tumor size (Fig. 3D). These results indicate that detection of exosomal EGFR levels in plasma could be used to estimate the sizes of tumors.

2.4. Comparison of soluble and exosomal EGFR levels in human plasma

A previous study has indicated that sEGFR is not a useful blood biomarker for lung cancer diagnosis because it is detected in blood from both healthy individuals and cancer patients (Jacot et al. 2004). Therefore, in order to evaluate the possible utility of exosomal EGFR as a lung cancer biomarker, we compared the expression levels of soluble and exosomal EGFR in plasma from nine lung cancer patients and from normal controls. The sEGFR levels did not differ remarkably between normal and lung cancer

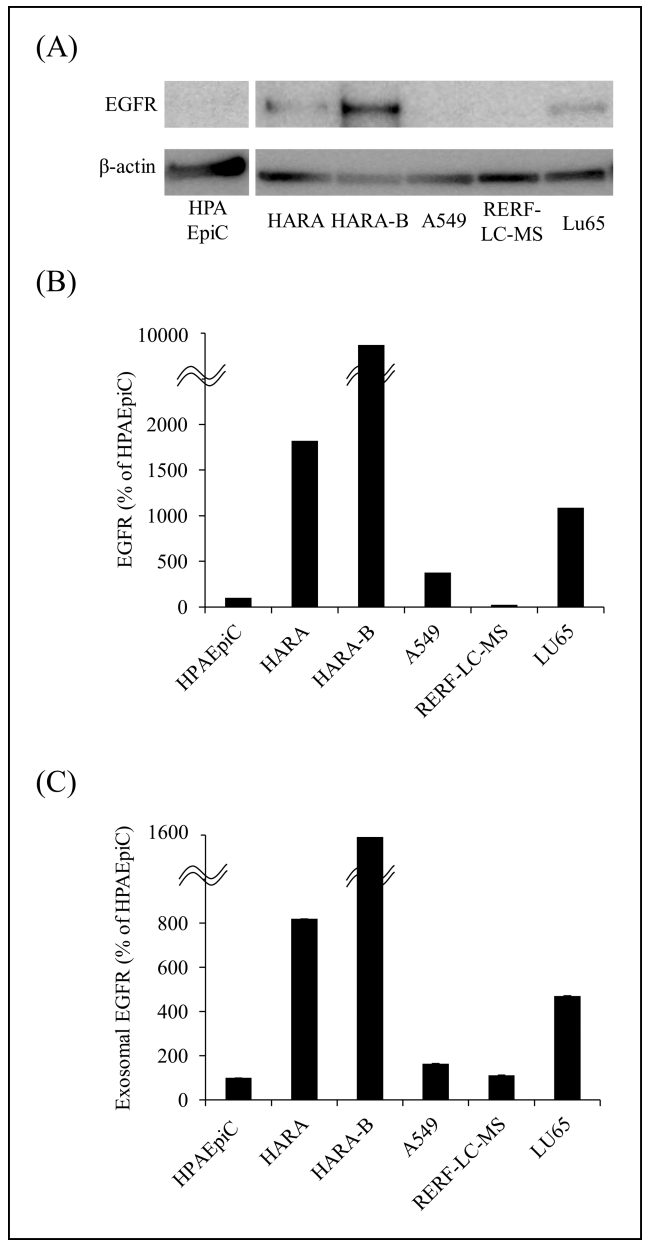

Fig. 2: Expression of EGFR on cancer cell-derived exosomes. A) Western blotting analysis of EGFR expression in lysates from lung cancer cell lines. B) Quantitative analysis of the Western blotting results of A. Signal quantification was performed by densitometry with normalization by b-actin. C) Expression of EGFR on exosomes in conditioned media. Ultracentrifuge-purified exosomes derived from the indicated cells were captured by anti-CD81 and detected by anti-EGFR. Data are shown as means and standard deviations (n = 3).

patient plasma. On the other hand, exosomal EGFR was significantly higher in five lung cancer patients plasma compared to healthy controls.

3. Discussion

Several blood biomarkers are commonly used for clinical cancer detection, e.g., carcinoembryonic antigen (CEA) and cytokeratin 19 fragment (CYFRA 21–1) for lung cancer (MacSween et al. 1972; Pujol et al. 1993). However, these biomarkers show low specificity and poor capability to detect tumors, although they continue to be used for cancer diagnosis. There is a clear need for improved biomarkers with higher specificity for cancer diagnosis and therapy. EGFR is known to be a good biomarker for lung cancer because it is highly expressed in tumor tissue

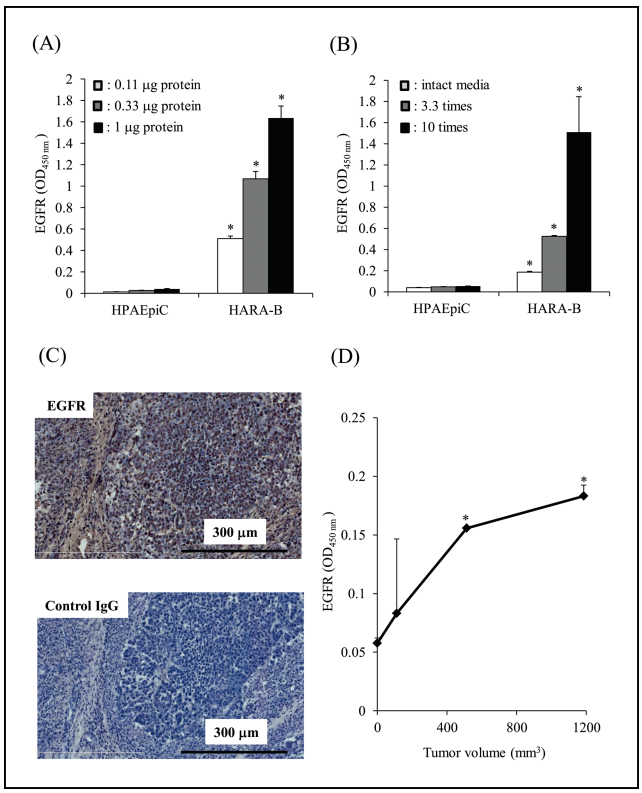

Fig. 3: Exosomal EGFR derived from conditioned medium and plasma from tumor-bearing mice. A) Concentration dependence analysis of EGFR expression on ultracentrifuge-purified exosomes (0.11, 0.33 and 1.0 μg exosomal protein) derived from HARA-B cells. Exosomes were captured and detected using an anti-CD81 antibody B) EGFR expression in exosomes derived from conditioned media of HARA-B cells (intact and concentrated 3 and 10 times by ultrafiltration). Exosomes were captured by an anti-CD81 antibody and then detected using an anti-EGFR antibody. C) Expression of EGFR on HARA-B xenograft tumors. The HARA-B tumor sections were stained using an anti-EGFR antibody. Isotype control staining was also performed. Representative 200x photomicrographs are shown. D) Relationship between plasma levels of CD81 + /EGFR + exosomes and tumor volume. Plasma samples were collected from mice sacrificed 10, 17 and 21 days after inoculation with HARA-B cells. Data are shown as means and standard deviations (n = 3).

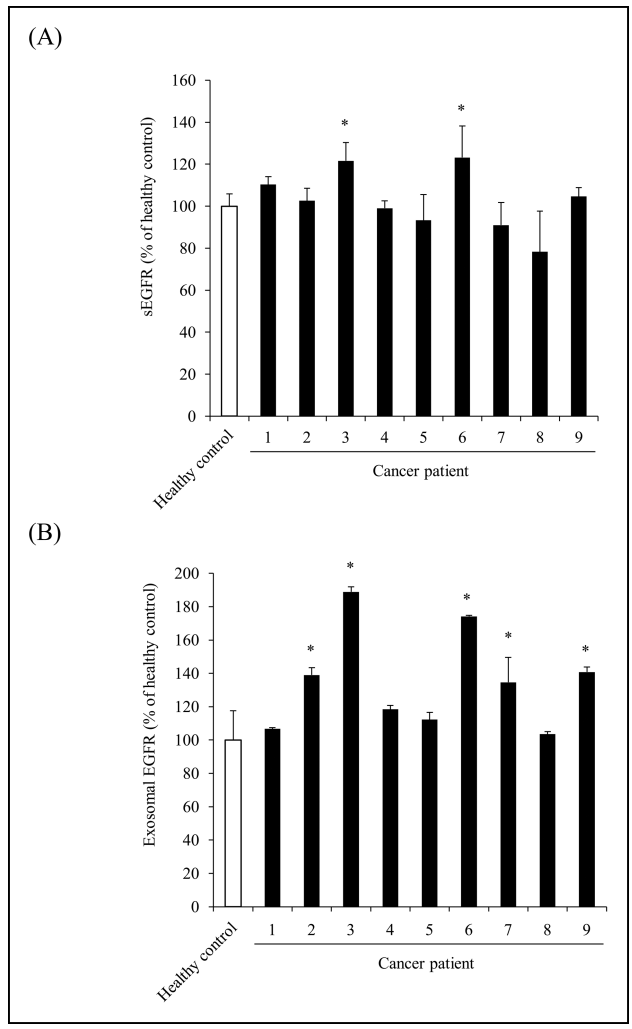

Fig. 4: Expression of sEGFR and exosomal EGFR in human plasma from lung cancer patients. A) Expression analysis of sEGFR using an ELISA, which consisted of two different antibodies recognizing other epitopes of EGFR. B) Expression analysis of exosomal EGFR using an ELISA, which utilized anti-EGFR and anti-CD81 antibodies. Exosomes were captured by an anti-CD81 antibody and then detected using an anti-EGFR antibody. Data are shown as means and standard deviations (n = 3). *$P < 0.05$ (vs. normal).

and also is a target molecule of EGFR TKIs, which are used to treat lung cancer patients. However, EGFR expression is evaluated by performing a biopsy that is highly invasive, and it would be quite desirable to develop a less invasive method to quantify it. The present study demonstrates that exosomal EGFR is a possible biomarker for lung cancer detection and characterization, and its expression may be evaluated by an EGFR-targeted ELISA using an anti-CD81 antibody as a capture antibody. This ELISA system should be capable of detecting EGFR on membranes of exosomes. This diagnostic method could be applied to lung cancer detection and pharmacometrics analysis, etc.

A previous report showed that the levels of exosomes in blood derived from cancer patients are higher than in healthy persons (Taylor and Gercel-Taylor 2008). Therefore, an ELISA system could be used for lung cancer diagnostics if EGFR was expressed at higher levels on exosomes from lung cancer patients. However, it has been reported that sEGFR levels are similar in blood from lung cancer patients and normal controls. Furthermore, exosomes are detected in plasma from healthy individuals as well as from cancer patients because they are secreted from various cells in normal and disease situations (Fevrier et al. 2004; Witek et al. 2009). Therefore, the tumor-derived exosomes must be distinguished from other exosomes for diagnostic purposes. The results of our animal study indicate that exosomes in plasma that express high levels of EGFR are clearly derived from tumor tissue, because this mouse model was inoculated with a human lung cancer cell line and the ELISA system can only detect human CD81 and human EGFR. Furthermore, our results indicate that exosomal EGFR detection could potentially be used in blood tests to diagnose lung cancer because the exosomal EGFR level was higher in lung cancer patients than in normal individuals (Fig. 4).

It is thought that secretion of exosomal EGFR derived from tumor tissue is increased in lung cancer patients. However, the present study did not reveal any relationship between EGFR expression levels in exosomes *versus* tumor tissues. Further studies are necessary to investigate the relationships among size, stage, EGFR expression of tumor tissues and plasma exosomal EGFR levels. Future large-scale validation studies are also anticipated. Tetraspanins such as CD9, CD63 and CD81 are a family of small proteins expressed on membranes. They are highly enriched in late endosomes, lysosomes, and multi-vesicular bodies, where they associate to form complexes (Andre et al. 2002; Abache et al. 2007; Pols and Klumperman 2009). Secretion of exosomes from dendritic cells of CD9 knockout mice was found to be reduced (Chairoungdua et al. 2010), suggesting that tetraspanins are essential molecules for generation of exosomes. Another study utilized an anti-CD63 antibody and caveolin-1 antibodies to detect endosomes in plasma from melanoma

patients (Logozzi et al. 2009). CD63-positive exosomes were found to be highly expressed in *in vitro* culture supernatants and were also detected in plasma from melanoma patients. In contrast, we carried out a proteomics study to determine what kinds of tetraspanins were expressed on exosomes derived from lung cancer cell lines. The results indicated that exosomal CD63 was seldom detected in some lung cancer cells but CD81 was widely expressed (in the lines HARA, HARA-B, A549, and LU65). Chiba et al. also reported that CD81 was widely expressed on exosomes derived from colon cancer cell lines (HCT-15, SW480, and WiDr), as indicated by western blotting experiments (Chiba et al. 2012). Furthermore, it was reported that CD81 promotes cell proliferation by ERK/MAP Kinase activation and tyrosine phosphorylation in hepatocellular carcinoma cells (Carloni et al. 2004). Thus, CD81 may be useful as an appropriate marker for exosomes derived from cancer cells.

This study demonstrates for the first time that exosomal EGFR may be detected and quantified using CD81 and EGFR targeted ELISA assays. The alteration of plasma EGFR concentration in cancer patients could be detected by a targeted ELISA capturing exosomes because soluble EGFR is usually observed in the healthy state. Although this ELISA system could be useful for cancer detection, optimization of this method (e.g. exploitation of radio-isotopes or fluorescence, affinity maturation of antibodies, etc.) will be needed to estimate EGFR expression levels in cancer tissues.

It has been reported that tumor-specific gene mutations of EGFR could be detected by reverse transcription-PCR analysis in serum vesicles from glioblastoma patients (Skog et al. 2008). The exosomes include not only tumor derived membrane or cytosolic proteins but also mRNA expressed in tumor cells. In the future, exosome analysis, which allows simultaneous measurement of EGFR gene mutations and EGFR protein expression, will likely provide more valuable information for diagnosis and to inform therapeutic decisions for cancer patients, compared to previous methods.

4. Experimental

4.1. Cell lines

HARA, HARA-B, A549, RERF-LC-MS and LU65 cells were purchased from the JCRB cell bank. HARA, HARA-B and LU65 cells were maintained in RPMI1640 medium containing 10% fetal calf serum (FCS). A549 and RERF-LC-MS cells were maintained in Eagle's minimal essential medium with non-essential amino acids containing 10% FCS under a 5% CO_2 atmosphere at 37 °C. Primary human pulmonary alveolar epithelial cells (HPAEpiC) were purchased from Science Cell Research Laboratory. These cells were maintained in Alveolar Epithelial Cell Medium (Science Cell Research Laboratory, California, USA) under a 5% CO_2 atmosphere at 37 °C.

4.2. Electron microscopy

Electron microscope images of exosomes were taken by floating a carbon-coated 400-mesh Formvar EM grid on top of 5 µl of freshly prepared exosomes in deionized water for 20 min. The grid was then briefly washed with deionized water and floated on a drop of 2% uranyl acetate. Samples were examined using an H-7650 Transmission Electron Microscope (Hitachi High-Technologies).

4.3. Preparation of conditioned media and exosomes

Prior to exosomes collection, confluent cells were incubated for 72 h without FCS. Exosomes were prepared from the supernatant of lung cancer cells and HPAEpiC by centrifugation. Six hundred ml volumes of supernatants containing 1 x 10^8 HPAEpiC or 6 x 10^8 lung cancer cells were centrifuged at 300 g for 5 min to eliminate cells and at 16,000 x g for 20 min, followed by filtration through a 0.22 µm filter to clear cell debris. Exosomes were precipitated by ultracentrifugation at 140,000 x g for 70 min. The pellets of exosomes were washed once in PBS and their protein contents were measured using a Micro BCA protein assay kit (Thermo Fisher Scientific). Particle sizes were measured by dynamic light scattering using a Zetasizer nano (Malvern).

4.4. Western blots

All cells were lysed in RIPA buffer (Thermo Fisher Scientific) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics). Determination of protein concentrations was performed using a micro BCA protein assay kit (Thermo Fisher Scientific). Cell lysates were electrophoresed in 10% SDS-polyacrylamide gels (10 µg/ lanes) and transferred to PVDF membranes (Millipore). After blocking with 4% Block Ace (DS Pharma Biomedical) for 60 min at room temperature, the blots were reacted with anti-EGFR polyclonal antibody (clone AF231, R&D Systems) in a buffer containing 0.4% block ace, and then with an HRP conjugated anti-goat IgG secondary antibody (Jackson Immuno Research) in the same buffer and detected using the ECL-plus system (GE Healthcare). Equal amounts of protein loading were confirmed by parallel β-actin immunoblotting, and signal quantification was performed by densitometric scanning.

4.5. Detection of exosomes in plasma and conditioned media using targeted ELISA with anti CD81 and anti EGFR antibody

Ninety-six well Maxisorp plates (Nunc) were coated with 20 µg/ml mouse anti human CD81 monoclonal antibody (clone 1D6, Gene Tex) in volumes of 100 µl/well of carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After incubation, 100 µl/well of 4% BLOCK ACE (DS-pharma) were added and incubated overnight at 4 °C. After 3 washes with PBS, several concentrations of purified exosomes, conditioned media and mouse or human plasma were added to a final volume of 100 µl and incubated for 60 min at 37 °C. Sample concentrations are given in the figure legends. After 3 washes with PBS, 0.8 µg/ml goat anti-human EGFR polyclonal antibody was added and incubated for 60 min at 37 °C. After 3 washes with PBS, the plates were incubated with 100 µl of HRP-conjugated anti-goat IgG antibody per well diluted 1:5,000 in 0.4% block ace for 60 min at room temperature. After the final 3 washes with PBS, the reaction was developed with tetramethyl benzidine reagents, blocked with H_2SO_4 and optical densities were recorded at 450 nm. Human plasma samples were purchased from Kohjin Bio (Saitama, Japan: healthy control) and Tissue Solutions Ltd (Glasgow, UK: lung cancer samples). Soluble EGFR (sEGFR) was detected by EGFR Human ELISA kit (R & D Systems).

4.6. Human tumor xenograft mice model

Six-week-old male BALB/c Slc-nu/nu mice (Japan SLC) were maintained at the animal care facility of National Institute of Biomedical Innovation under a regulated protocol. A suspension of HARA-B cells (1 x 10^6 cells/mouse) was inoculated s.c. into the backs of the mice. The two perpendicular diameters of the tumors were obtained using a slide caliper 10, 17 and 21 days after inoculation and then tumor volumes were calculated using the formula 0.5 x (A x B^2), where A and B are the longest and shortest dimensions of the tumor, respectively (Andre et al. 2002). Six-hundred microliters of plasma were collected from tumor-bearing mice sacrificed at 10, 17 and 21 days after inoculation.

4.7. Immunohistochemistry staining

Frozen HARA-B tumor tissues in 5 µm thick sections were fixed in 4% paraformaldehyde for 15 min at 4 °C. Heat-induced epitope retrieval was performed in the Target Retrieval Solution pH 9 (Dako) according to the manufacturer's instructions. After the epitope retrieval, endogenous peroxidase was blocked with the Peroxidase Blocking Reagent (Dako). Following peroxidase blocking, the slides were incubated with 10% BSA solution for 30 min at room temperature. After BSA blocking, the slides were incubated for 60 min with goat anti human EGFR polyclonal antibody, 5 µg/ml in 3% BSA at room temperature. After 3 washes with Wash Buffer (Dako), each slide was incubated for 60 min with HRP-conjugated anti-goat IgG antibody diluted 1:1,000 in 3% BSA at room temperature. After the final 3 washes with wash buffer, the slides were stained with 3,3'-diaminobenzidine. After development, the slides were lightly counterstained with Mayer's hematoxylin. All procedures were performed using AutoStainer (Dako).

4.8. Statistical analysis

Statistical significance in ELISA signals between the control and target groups was analyzed using the one-way ANOVA followed by Tukey post-hoc test.

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