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Immunohistochemical staining with EGFR mutation-specific antibodies: high specificity as a diagnostic marker for lung adenocarcinoma

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We previously demonstrated a high specificity of immunohistochemistry using epidermal growth factor receptor (EGFR) mutation-specific antibodies in lung adenocarcinoma and correlation with EGFR mutation analysis. In this study, we assessed EGFR mutation status by immunohistochemistry in a variety of extrapulmonary malignancies, especially those that frequently show EGFR overexpression. Tissue microarrays containing triplicate cores of breast carcinomas ($n=300$), colorectal carcinomas ($n=65$), pancreatic adenocarcinoma ($n=145$), and uterine carcinosarcoma or malignant mixed müllerian tumors ($n=25$) were included in the study. Tissue microarray of lung adenocarcinoma with known EGFR mutation status was used as reference. Immunohistochemistry was performed using antibodies specific for the E746-A750del and L858R mutations. In pulmonary adenocarcinoma, a staining intensity of 2+ or 3+ correlates with mutation status and is therefore considered as positive. Out of 300 breast carcinomas, 293 (98%) scored 0, 5 (2%) had 1+ staining, 2 (1%) were 2+ for the L858R antibody. All breast carcinomas scored 0 with the E746-A750 antibody. All the colorectal, pancreatic carcinomas and malignant mixed müllerian tumors were negative (0) for both antibodies. Molecular analysis of the breast carcinomas that scored 2+ for L858R showed no mutation. Our results show that EGFR mutation-specific antibodies could be an additional tool distinguishing primary *versus* metastatic carcinomas in the lung. False-positivity can be seen in breast carcinoma but is extremely rare (1%).
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Epidermal growth factor receptor (EGFR) is a member of the ERBB receptor tyrosine kinase family. It is the first receptor tyrosine kinase to be linked directly to human tumors.[1] Binding to its specific ligands induces the receptor dimerization and auto phosphorylation of the intracellular tyrosine kinase domain, activating downstream signal pathways that regulate crucial cellular processes, such as proliferation, differentiation, motility, angiogenesis, and survival. Overexpression and structural alterations of EGFR are frequent in a number of human malignancies (lung, colorectal, head and neck, pancreatic, renal, bladder, breast, ovarian, esophageal, gastric, prostate carcinomas, and glioblastomas, uterine malignant mixed müllerian tumor)[2–4] and the overexpression of EGFR is associated with adverse disease characteristics and poor clinical outcome.[5]

The identification of EGFR as an oncogene has led to the development of EGFR-targeted cancer therapies. EGFR inhibitors can be mainly categorized into two classes: monoclonal antibodies against the extracellular domain of EGFR, such as cetuximab; and small-molecule tyrosine kinase inhibitors that target the kinase domain, such as erlotinib and gefitinib.[6–12] A major breakthrough in the field of EGFR-targeted therapy was the discovery of somatic mutations in the tyrosine kinase domain of *EGFR* gene in lung adenocarcinomas that are associated with good clinical response to EGFR–tyrosine kinase inhibitors.[13–15] The incidence of EGFR mutation in

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lung adenocarcinomas from patients in the United States is about 15–20%.[16] Approximately 90% of the mutations occur in two hot spots: the in-frame deletions in exon 19 centered around codons 746 to 750 make up 45–50% of mutations, and the point mutation at codon 858 (L858R) in exon 21 consists of 35–45% of the mutations.[17–21]

Molecular testing for EGFR mutation in non-small cell lung cancer has been implemented into routine clinical practice.[22–24]

Recently, two mutation-specific antibodies to the two most common mutations in the *EGFR* gene have been developed: rabbit monoclonal antibody clone 6B6 and clone 43B2 (Cell Signalling Technology), are specific for EGFR with E746-A750del and L858R mutation, respectively.[25] These antibodies detect specific EGFR mutations in lung adenocarcinoma by immunohistochemistry, a simple, rapid, and cost-effective method. We have previously demonstrated that both antibodies show high sensitivity and positive predictive value for detecting these two specific EGFR mutations in lung adenocarcinoma.[26,27] It is worth mentioning that negative results for immunohistochemistry using these specific antibodies do not eliminate the need for further testing, as the antibodies will not detect other types of EGFR mutations and the antibody to the E746-A750del deletion may not reliably detect other less common types of deletions in exon 19. The goal of this study is to assess whether the two major forms of mutant EGFR are present in other types of human malignancies, especially those in which EGFR overexpression has been reported, and, more generally, whether these antibodies could be potentially useful in assigning lung origin to adenocarcinoma, given that these mutations are highly specific for lung adenocarcinoma.

Materials and methods

Tissue Microarrays

Tissue microarrays containing carcinomas of the breast ($n=300$), colon and rectum ($n=65$), pancreas ($n=145$), and uterine malignant mixed müllerian tumor ($n=25$) were used in this study. Among the 65 colorectal adenocarcinomas, 39 were primary tumor, 20 were lung metastases, and 6 were liver metastases. All other tissue microarrays contain only the primary tumors. Triplicate 0.6-mm diameter cores were taken from each formalin-fixed, paraffin-embedded block for the construction of the tissue microarrays. Tissue microarrays of lung adenocarcinomas with known EGFR mutation status ($n=194$) were included for comparison.

Immunohistochemistry

Immunohistochemical stains were performed on 4-μm-thick sections cut from the tissue microarrays, using rabbit monoclonal antibodies that are specifically against EGFR with L858R point mutation in exon 21 (clone 43B2, Cell Signalling Technology) or E746_A750 deletion mutation in exon 19 (clone 6B6, Cell Signalling Technology). The EGFR mutation-specific staining was scored based on membrane staining intensity as previously described: 0= no staining; 1+ =faint cytoplasmic staining in >10% of tumor cells; 2+ =moderate membranous staining; 3+ =strong membranous staining.[26,27] Although the scoring system used is subjective, the classification as negative (0 and 1+) and positive (2+ and 3+) has been shown to correlate with the presence of mutations detected by standard molecular techniques.[26,27] A similar system of immunohistochemistry interpretation has been validated for reporting of HER2/neu in breast carcinoma.[28] Lung adenocarcinomas with mutation status confirmed by molecular testing were used as positive and negative controls. The intensity of the staining was assessed by two pathologists (YHW and ALM).

Molecular Analysis

We evaluated EGFR mutation with molecular analysis in non-pulmonary tumors showing positive staining (2+ to 3+) by immunohistochemistry. DNA was extracted from the archived formalin-fixed paraffin-embedded tissue samples using the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) as described by manufacturer. Samples were analyzed using the Sequenom platform (Sequenom, San Diego, CA), which uses matrix-assisted laser desorption/ionization-time of flight mass spectrometry to distinguish the products of primer extension reactions (performed on polymerase chain reaction products) in a sequence-specific manner based on mass and charge. Briefly, mutant and wild-type alleles for a given point mutation produce single-allele base extension reaction products of different mass that can then be resolved by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. This high throughput assay detects 93 mutations in 9 genes (*EGFR, ERBB2, KRAS, BRAF, NRAS, PIK3CA, MEK1, MAP2K1* and *AKT*). All mutations detected by Sequenom are confirmed by conventional sequencing.[29] Other mutations detected apart from EGFR mutations were not relevant to this study.

Results

Breast Cancer

A total of 300 invasive breast carcinomas, including 220 triple-negative breast cancers and 80 non-triple-negative breast cancers were included in this study. We chose to include more triple-negative breast cancers because these tumors are known to be associated with high expression of EGFR.[30] The

clinicopathological features of the patients with triple-negative breast cancers and non-triple-negative breast cancers are summarized in Table 1. All patients were female, treated at our institution between 2002 and 2006. The mean age at diagnosis of patients with triple-negative breast cancers was 55 years (range, 29–85). The mean age of patients with non-triple-negative breast cancers was 53 years (range, 30–87). The average tumor size of triple-negative breast cancers and non-triple-negative breast cancers was 3.0 cm (range, 0.7–28.0 cm) and 2.2 cm (range, 0.9–9.5 cm). Among the 220 triple-negative breast cancers, 201 (91%) were invasive ductal carcinoma not otherwise specified; 9 (4%) were invasive apocrine carcinoma; 6 (3%) were metaplastic carcinoma; the remaining were invasive mammary carcinoma with mixed ductal and lobular features ($n=2$), invasive lobular carcinoma pleomorphic type ($n=1$), and micropapillary carcinoma ($n=1$). Among the 80 non-triple-negative breast cancers, 60 (75%) were invasive ductal carcinoma not otherwise specified; 8 (10%) were invasive lobular carcinoma (7 classical type and 1 pleomorphic type); 4 (5%) were invasive mammary carcinoma with mixed ductal and lobular features; 4 (5%) were micropapillary carcinoma; the remaining were apocrine carcinoma ($n=2$), mucinous carcinoma ($n=1$), and papillary carcinoma ($n=1$). The ER, PR, and HER2 status in the 80 non-triple-negative breast cancers was ER/PR+, HER2− ($n=63$), and ER/PR+, HER2+ ($n=17$).

Results of the immunohistochemical staining for the EGFR mutation-specific antibodies are summarized in Table 2. Of the 220 triple-negative breast cancers, 219 scored 0 and 1 was scored 1+ for the EGFR L858R antibody. Of the 80 non-triple-negative breast cancers, 74 (93%) were scored 0, 4 (5%) were 1+, 2 (3%) were 2+ (Figure 1), and none was 3+ for the EGFR L858R antibody. Both breast carcinomas that are 2+ for the EGFR L858R antibody were invasive ductal carcinoma, NOS, ER/PR positive, HER2 positive (3+). All TNBC and non-triple negative breast cancer cases scored 0 for the EGFR E746_A750 deletion-specific antibody.

We performed molecular analysis on two breast carcinomas that were scored 2+ by immunohistochemistry for EGFR L858R, and no mutation was detected. Overall, there was false positive (2+) staining for EGFR L858R in 2 (1%) of 300 breast cancer cases screened.

Table 1 Clinicopathological characteristics of the patients with triple-negative breast carcinoma and non-triple-negative breast carcinoma

	TNBC (n = *220*)	*Non-TNBC* (n = *80*)
Age, mean (range), years	55 (29–85)	53 (30–87)
Size, mean (range), cm	3.0 (0.7–28.0)	2.2 (0.9–9.5)
Histological subtypes		
IDC, NOS	201 (91%)	60 (75%)
ILC, classical	0	7 (9%)
ILC, pleomorphic	1 (0.5%)	1 (1%)
Mixed ductal and lobular	2 (1%)	4 (5%)
Apocrine	9 (4%)	2 (3%)
Metaplastic	6 (3%)	0
Micropapillary	1 (0.5%)	4 (5%)
Mucinous	0	1 (1%)
Papillary	0	1 (1%)
Lymph node		
Positive	126 (57%)	46 (58%)
Negative	93 (42%)	33 (41%)
Unknown	1 (0.5%)	1 (1%)
ER, PR, HER2 status		
ER/PR+, HER2−	0	63 (79%)
ER/PR+, HER2+	0	17 (21%)
ER/PR−, HER2+	0	0
ER/PR−, HER2−	220	0

Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; NOS, not otherwise specified; TNBC, triple-negative breast carcinoma.

Table 2 Results of immunohistochemistry using EGFR mutation-specific antibodies in triple-negative breast carcinoma and non-triple-negative breast carcinoma

	TNBC (n = *220*)	*Non-TNBC* (n = *80*)
Anti-L858R antibody		
0	219 (99.5%)	74 (93%)
1+	1 (0.5%)	4 (5%)
2+	0	2 (3%)
3+	0	0
Anti-del E746_A750 antibody		
0	220	0
1+	0	0
2+	0	0
3+	0	0

Abbreviation: TNBC, triple-negative breast carcinoma.

Colorectal Cancer

Primary and metastatic colorectal carcinoma form 65 patients treated at our institution between 1992 and 2003 were included in this study. Thirty-nine were primary tumors, 26 were metastatic tumors (20 lung metastases and 6 liver metastases). The average age of the patients was 63 years (range, 32–85). Thirty-seven (57%) patients were male, 28 (43%) were female. The clinicopathological features were summarized in Table 3. All tumors were negative (score 0) for both EGFR mutation-specific antibodies.

Pancreatic Cancer

The pancreatic cancer tissue microarrays contained pancreatic adenocarcinomas from 145 patients treated at our institution between 2000 and 2009. Patient characteristics are summarized in Table 4. There were 62 (43%) male and 83 (57%) female patients. Mean patient age was 67 years

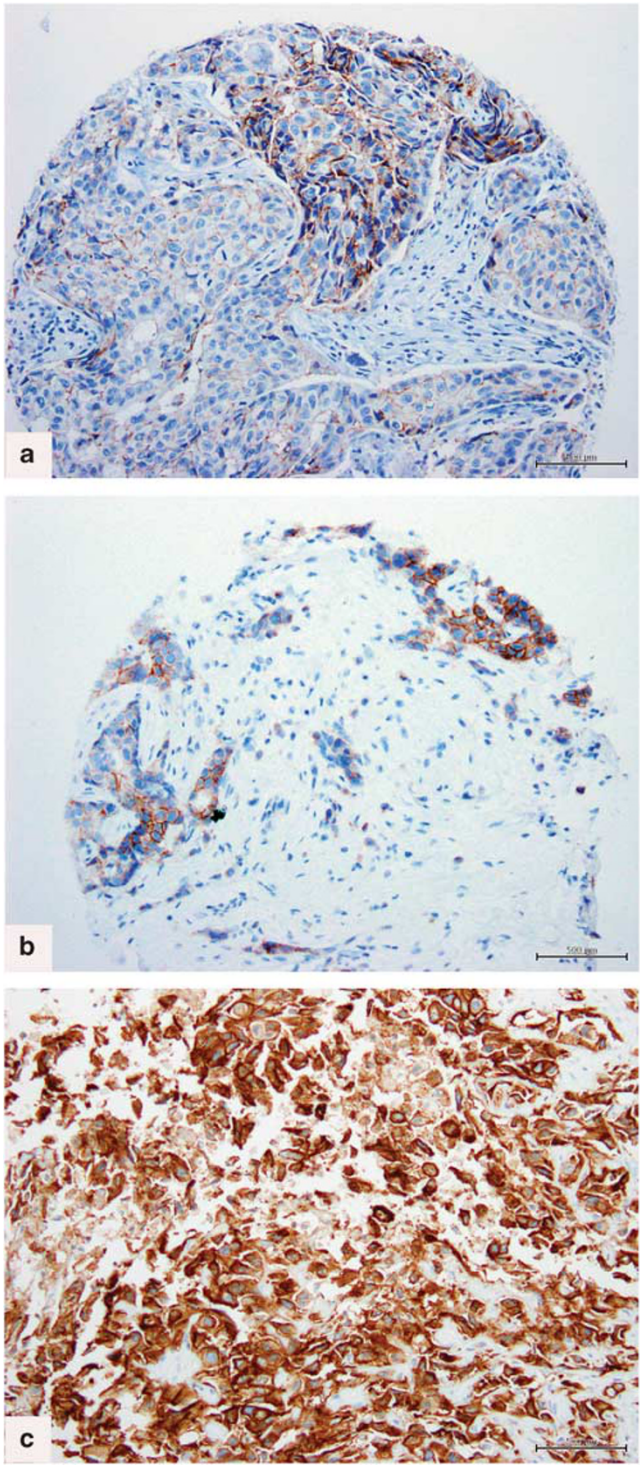

Figure 1 Immunohistochemical stain for epidermal growth factor receptor (EGFR) L858R mutation-specific antibody in breast cancer. (**a** and **b**) Two breast cancer cases with 2+ staining. (**c**) The positive control, using lung adenocarcinoma with known EGFR L858R mutation. Magnification, ×200.

(range, 41–86). The mean tumor size was 3.4 cm, (range, 1.2–10.2). Six (4%) tumors were well-differentiated, 91 (63%) were moderately differentiated, and 48 (33%) were poorly differentiated. Metastases to lymph nodes were found in 106 (73%) patients. All tumors were negative (score 0) for both EGFR mutation-specific antibodies.

Uterine Malignant Mixed Müllerian Tumor

Twenty-five primary tumor samples from patients diagnosed with uterine malignant mixed müllerian tumor between 1995 and 2005 were included in this study. The average age of the patients was 64 years (range, 54–76 years). Twenty-two (88%) patients had stage I disease (IA: $n=3$; IB: $n=17$; IC: $n=2$) at the time of diagnosis, 1 (4%) had stage III disease and 2 (8%) had stage IV disease. Patient clinical information is presented in Table 5. All cases were negative (scored 0) for both EGFR mutation-specific antibodies.

Discussion

Molecular testing of patients with adenocarcinoma of the lung for selection of specific therapy is the standard of care in clinical practice. Determination of mutational status in pulmonary adenocarcinoma has entered our daily routine and it is now an integral part of the pathological evaluation. However, there are barriers to the widespread implementation of molecular testing due to cost limitations, tissue availability, and the need for specialized skills to perform and evaluate molecular tests results.

Tissue utilization and speed of tests results availability are the main limiting factors for molecular tests. Most patients that may benefit from molecular testing, to determine the choice of drugs for target therapy, present with an advanced stage of disease, where only small biopsies or cytological material are available. We have previously demonstrated that EGFR mutation-specific antibodies are a reliable method for mutational status determination in small biopsy specimens including decalcified material,[27] such as bone biopsies, which are very often not suitable for molecular evaluation.[31,32] In this article, we compare the use of EGFR mutation-specific antibodies in a set of different tumors that express high levels of wild-type EGFR to determine the specificity of mutation-specific antibodies. We have shown that positivity for EGFR mutation-specific antibodies is only seen in adenocarcinomas of the lung that harbor the mutation and are negative in other tumors, such as adenocarcinoma of the pancreas, colon, breast, and poorly differentiated endometrial tumors. We observed that in 1% of estrogen receptor, progesterone receptor, and Her2/neu-positive breast cancer, a moderate staining pattern (2+) with the L858R-specific mutation antibody is present. All cases that were positive for the antibody did not harbor the EGFR mutation when tested with molecular techniques, therefore representing false positive results. It is possible that the positive reaction with the EGFR

Table 3 Clinicopathological features of the 65 patients with colorectal carcinoma

	Primary tumor (n = *39*)	*Lung metastasis* (n = *20*)	*Liver metastasis* (n = *6*)	*Total* (n = *65*)
Age, mean (range), years	64 (32–85)	62 (32–78)	60 (43–73)	63 (32–85)
Sex				
Male	28 (72%)	5 (25%)	4 (67%)	37 (57%)
Female	11 (28%)	15 (75%)	2 (33%)	28 (43%)
Tumor size, mean (range), cm	4.5 (2–9)			
Mucinous component				
None	28 (72%)			
<50%;	5 (13%)			
>50%;	6 (15%)			
T stage				
pT1	1 (3%)			
pT2	11 (28%)			
pT3	27 (69%)			
Lymph node				
Negative	21 (54%)			
Positive	18 (46%)			

Table 4 Characteristics of the 145 patients with pancreatic adenocarcinoma

	N *(%)*
Age, mean (range), years	67 (41–86)
Sex	
Male	62 (43%)
Female	83 (57%)
Tumor size, mean (range), cm	3.4 (1.2–10.2)
Tumor histology	
Well differentiated	6 (4%)
Moderately differentiated	91 (63%)
Poorly differentiated	48 (33%)
Lymph node	
Positive	106 (73%)
Negative	39 (27%)
Stage	
pT2	2 (1%)
pT3	139 (96%)
pT4	4 (3%)

Table 5 Characteristics of the 25 patients with uterine carcinosarcoma/malignant mixed müllerian tumor

	N *(%)*
Age, mean (range), years	64 (54–76)
Stage at initial diagnosis	
I	22 (88%)
II	0
III	1 (4%)
IV	2 (8%)

L858R mutation-specific antibody may represent cross-reactivity with an epitope on another, as yet undetermined protein expressed by these tumor cells.

In a setting of disseminated metastatic disease of unknown primary, it is not uncommon to find tumors that are positive for cytokeratin 7 only without expression of tissue-specific markers, which makes it very difficult to pin-point the site of origin for selection of appropriate therapy. The only exception in this group is colon cancer that expresses cytokeratin 20. Therefore, EGFR mutation-specific antibodies could be included in the diagnostic work-up of patients with disseminated metastatic diseases, where pulmonary carcinoma is in the differential diagnosis, a positive result will likely indicate that these tumors are indeed from the lung. These will not only offer diagnostic confirmation, but also a predictive indicator for therapy with EGFR inhibitors. Although the diagnostic sensitivity of these two antibodies for lung adenocarcinoma used together would be low, approaching that of the prevalence of EGFR mutations in this group (~15–20%), the predictive significance of a positive result would be clinically invaluable. In the case of breast cancer as discussed above, the false positive cases were only seen in tumors that are positive for Her2/neu, estrogen and progesterone receptors, not triple-negative breast cancer. Positivity for L858R-specific antibody and positivity for estrogen receptor and other breast markers should raise the possibility of breast cancer and not pulmonary carcinoma.

In conclusion, we showed that immunohistochemical stains for mutation-specific EGFR antibodies are a reliable source for the diagnosis of mutation, confirming previous reports, and also suggesting that these are specific staining for pulmonary adenocarcinoma that harbor the mutation. EGFR mutation-specific antibodies are negative in other tumors that overexpress wild-type EGFR protein.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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