

## ORIGINAL ARTICLE

# Gene amplification and protein expression of EGFR and HER2 by chromogenic in situ hybridisation and immunohistochemistry in atypical adenomatous hyperplasia and adenocarcinoma of the lung

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**Aims:** To investigate the importance of gene amplification and EGFR (epidermal growth factor receptor) and HER2 protein expression during the progression of adenocarcinoma of the lung.

**Methods:** EGFR and HER2 gene amplification was examined in atypical adenomatous hyperplasia (AAH), bronchioloalveolar carcinoma (BAC), and adenocarcinoma with mixed subtypes (MX) by chromogenic in situ hybridisation (CISH), and protein expression was examined by immunohistochemistry using paraffin wax embedded tissues.

**Results:** EGFR and HER2 gene amplification was found in four and two of 86 cases, respectively, and was detected only in the invasive components of MX. EGFR and HER2 protein expression was seen in 24 and 18 of 86 cases, respectively. EGFR and HER2 proteins were not expressed in AAH but were expressed in one BAC case each. EGFR and HER2 proteins were expressed in 23 and 17 of 55 adenocarcinomas with MX. EGFR and HER2 protein expression was seen more often in the invasive components than in the BAC components of MX, and increased significantly as lesions progressed from AAH to BAC, early MX, and overt MX. Because EGFR and HER2 protein expression was frequently seen without gene amplification, other mechanisms apart from gene amplification may be associated with protein expression.

**Conclusions:** EGFR and HER2 gene amplification may be a late event and EGFR and HER2 protein expression may be associated with the development of adenocarcinoma of the lung.

Adenocarcinoma is the most frequent histological type of carcinoma of the lung. According to the World Health Organisation classification,<sup>1</sup> atypical adenomatous hyperplasia (AAH) is a preneoplastic lesion of adenocarcinoma, and bronchioloalveolar carcinoma (BAC) is a non-invasive type of carcinoma. Adenocarcinoma with mixed subtypes (MX), which is the most common type of adenocarcinoma, usually includes BAC-type carcinoma in the periphery and invasive carcinoma in the central portion. Based on these morphological findings, it has been suggested that at least some adenocarcinomas occur as a result of progression from AAH through BAC to invasive adenocarcinoma.<sup>2–4</sup>

“Chromogenic in situ hybridisation allows comparison with histological findings obtained by microscopy”

Epidermal growth factor receptor EGFR/erbB1 and HER2/erbB2 are members of the EGFR family of type I receptor tyrosine kinases.<sup>5</sup> Homodimerisation or heterodimerisation of these receptors appears to promote signal transduction pathways, including angiogenesis, inhibition of apoptosis, and cell growth. The EGFR gene is located on chromosome 7p12. The frequency of EGFR expression is high in non-small cell lung cancer (NSCLC). In previous studies, EGFR expression was detected in 38–75% of adenocarcinomas, 30–82% of squamous cell carcinomas, and 33% of large cell carcinomas.<sup>6–8</sup> In contrast, the frequency of EGFR gene amplification, as determined by fluorescent in situ hybridisation (FISH), has been reported to be 9% in NSCLC.<sup>6</sup> HER2, which is located on chromosome 17q21, is considered a heterodimerisation partner for other members of the HER

family. Among NSCLC, HER2 expression is seen frequently in adenocarcinoma and large cell carcinoma and is associated with poor prognosis.<sup>9–10</sup> In addition, HER2 expression was reported in 29–35% of adenocarcinomas, 1–18% of squamous cell carcinomas, and 0–20% of large cell carcinomas.<sup>10–13</sup> The frequency of HER2 gene amplification, as determined by FISH, was reported to be 2–4% in NSCLC.<sup>11–13</sup>

Chromogenic in situ hybridisation (CISH) was introduced in 2000 and has been used to examine several types of carcinoma, such as breast carcinoma, prostate carcinoma, and extramammary Paget's disease.<sup>14–18</sup> However, there have been no previous reports of analysis of gene amplification in carcinoma of the lung using CISH. Although FISH is a standard technique for gene amplification, CISH has been reported to show a good correlation with gene amplification by FISH. Moreover, CISH allows comparison with histological findings obtained by microscopy.

In our present study, gene amplification and protein expression of EGFR and HER2 were examined by CISH in AAH, BAC, and MX, and the significance of the findings was analysed on the assumption of progression from AAH through BAC to invasive adenocarcinoma.

## MATERIALS AND METHODS

### Tissue samples

Table 1 shows the case profiles of the patients studied. Tissue samples were obtained from 86 patients, comprising 34 men

**Abbreviations:** AAH, atypical adenomatous hyperplasia; BAC, bronchioloalveolar carcinoma; CISH, chromogenic in situ hybridisation; EGFR, epidermal growth factor receptor; FISH, fluorescent in situ hybridisation; MX, adenocarcinoma with mixed subtypes; NSCLC, non-small cell lung cancer

**Table 1** Clinicopathological profiles of patients studied

Feature	BAC (n=31)	Early MX (n=22)	Overt MX (n=33)	p Value
Sex				
Male	11	9	14	
Female	20	13	19	
Mean (range) age (years)	41–83 (67)	40–83 (63)	41–85 (63)	
Mean (range) tumour size (cm)	0.2–2.5 (1.3)	0.8–3.0 (2.0)	1.1–7.0 (2.7)	<0.0001
Lymph node metastasis positive cases (%)	0	1/22 (4.5%)	9/30 (30%)	0.0008

The Kruskal Wallis test was used to assess the p values among BAC, early MX, and overt MX. BAC, bronchioloalveolar carcinoma; MX, adenocarcinoma with mixed subtypes.

and 52 women aged 40–85 years (median, 64.6), who underwent surgery during the period between 1988 and 2001. Some of the patients with BAC or MX had one or more lesion defined as AAH. Therefore, we examined 14 AAH lesions, 31 BAC lesions, and 55 MX lesions, defined according to the World Health Organisation classification.<sup>1</sup> Among MX, cases showing BAC in < 50% and > 50% of the area of the lesion were defined as early MX and overt MX, respectively.

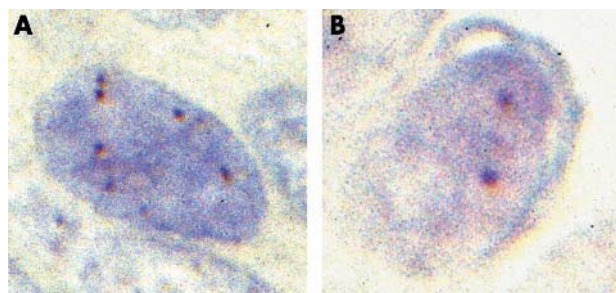
**Chromogenic in situ hybridisation**

Sections (4 µm thick) were cut from paraffin wax blocks and dewaxed. Antigen retrieval and enzyme digestion were performed using a formalin fixed, paraffin wax embedded tissue pretreatment kit (Zymed, San Francisco, California, USA). A digoxigenin labelled EGFR or HER2 DNA probe (Zymed) was applied, and the sections were covered with coverslips and denatured on a hot plate at 94°C. Hybridisation was performed overnight at 37°C. After removing the coverslips, HER2 signals were detected using a CISH detection kit (Zymed). A peroxidase quenching solution was applied to the sections, followed by non-specific blocking solution for 10 minutes at room temperature, fluorescein isothiocyanate–sheep antidigoxigenin for 30 minutes at room temperature, horseradish peroxidase–goat anti-fluorescein isothiocyanate for 30 minutes at room temperature, and DAB chromogen for 30 minutes at room temperature. Finally, the slides were counterstained with haematoxylin.

Signals were seen as dark brown dots. According to previous reports,<sup>14–18</sup> a gene copy number of six or more in the nucleus in more than 50% of tumour cells was taken to indicate amplification of the EGFR and/or HER2 genes (fig 1). Cases with three to five gene copies were considered aneuploid. Immunohistochemistry and CISH were evaluated independently.

**Immunohistochemistry for EGFR and HER2**

Immunohistochemistry was performed as reported previously.<sup>4</sup> Briefly, 2 µm thick sections were cut from paraffin wax blocks containing representative areas of the tumours.



**Figure 1** Evaluation of EGFR (epidermal growth factor receptor) gene amplification by chromogenic in situ hybridisation. (A) Six gene copies were seen. This case was considered positive for gene amplification. (B) Two gene copies were seen. This case was considered negative.

Antigen retrieval was performed by autoclaving. Anti-EGFR antibody (EGFR.113; Novocastra, Newcastle upon Tyne, UK) and anti-c-erbB-2 antibody (CB11; Novocastra) at dilutions of 1/50–1/100 were used according to the streptavidin–biotin–peroxidase method. As a negative control, the primary antibody was omitted, and a tumour specimen of invasive lobular carcinoma of the breast was used as a positive control.

**Evaluation of immunohistochemistry for EGFR and HER2**

Immunohistochemistry for EGFR and HER2 was evaluated according to the Herceptest (Dako Cytomation, Carpinteria, California, USA) protocol. If more than 10% of the tumour cells showed EGFR or HER2 expression over the entire cell membrane, the case was considered positive. Diffuse and granular cytoplasmic expression and partial membranous expression were considered negative for both EGFR and HER2.

**Statistical analysis**

Differences in the expression of EGFR and HER2 between AAH, BAC, early MX, and overt MX were analysed by the Kruskal-Wallis test and one way analysis of variance. Differences were considered significant at p < 0.05.

**RESULTS**

**EGFR and HER2 gene amplification in AAH and adenocarcinoma of the lung (table 2)**

EGFR and HER2 gene amplification was seen in four and two of 86 cases, respectively. In all of these cases, gene amplification was detected only in the invasive components of MX. EGFR aneuploidy was seen in none of the 14 cases of AAH, three of the 34 cases of BAC, and two of the 55 cases of MX. In MX, aneuploidy was seen only in the invasive components of overt MX.

**EGFR and HER2 protein expression in AAH and adenocarcinoma of the lung**

Table 3 shows the protein expression results. EGFR and HER2 expression increased significantly as the lesions progressed from AAH to BAC, early MX, and overt MX, and was seen more frequently in invasive components than in BAC components of MX (fig 2). Partial membrane expression—hemilateral or basolateral staining—was seen in less than 10% of the tumour cells in AAH and BAC. EGFR gene amplification was seen in four cases, three of which also showed protein overexpression (table 4). HER2 gene amplification was seen in two cases, both of which showed protein overexpression.

**DISCUSSION**

In our present study, we examined EGFR and HER2 gene amplification by CISH. The frequencies of EGFR and HER2 gene amplification were similar to those reported previously.<sup>6 11–13</sup> Therefore, CISH appears to be useful for the

**Table 2** EGFR and HER2 gene amplification in AAH and adenocarcinoma of the lung

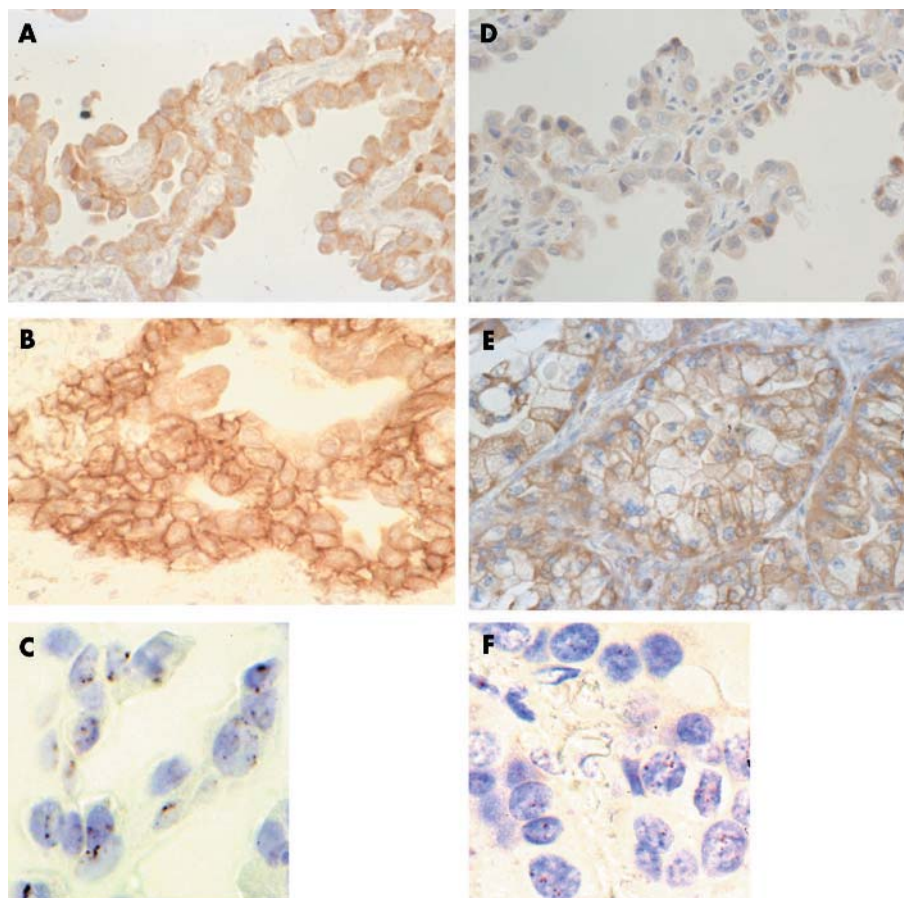
Gene	Adenocarcinoma				Total (n = 86)
	AAH (n = 14)	BAC (n = 31)	Early MX (n = 22)	Overt MX (n = 33)	
EGFR	0	0	2	2	4 (4.7%)
HER2	0	0	1	1	2 (2.3%)

AAH, atypical adenomatous hyperplasia; BAC, bronchioloalveolar carcinoma; EGFR, epidermal growth factor receptor; MX, adenocarcinoma with mixed subtypes.

detection of gene amplification in adenocarcinoma of the lung. Because gene amplification was seen only in invasive components of MX, gene amplification may be a late event in the development of adenocarcinoma of the lung. However, EGFR aneuploidy may not play an important role in the progression of adenocarcinoma of the lung, because aneuploidy was not associated with histological change.

In our present study, EGFR and HER2 protein expression increased in parallel with histological change. These results suggest that EGFR and HER2 expression may be associated with development of adenocarcinoma of the lung. Synchronous expression of both EGFR and HER2 may indicate heterodimerisation. Synchronous expression was seen in ten of the lesions, similar to previous reports,<sup>19</sup> and increased significantly as histological changes became greater. These results suggest that synchronous expression may also be associated with the development of adenocarci-

noma of the lung. The frequencies of EGFR and HER2 expression in our present study were somewhat lower than those reported previously.<sup>6-8 11-13</sup> This might be the result of case selection bias—a large number of BAC cases were included in our present study. In addition, differences in the criteria used for the evaluation of protein expression and in antibodies used may have influenced the results. Saad *et al* reported HER2 expression in 19 of 50 MX samples and three of 50 BAC samples using the Herceptest (Dako Cytomation).<sup>20</sup> The anti-c-erbB2 antibody clone CB11 used in our study has been reported to show less false positives than the Herceptest.<sup>21</sup> However, various frequencies of EGFR expression have been reported previously. Hirsch *et al* reported EGFR expression in 28 of 69 MX cases and eight of 10 BAC cases using a semiquantitative method for evaluation and anti-EGFR antibody (catalogue number 28-0005; Zymed Laboratories).<sup>6</sup> Mukohara *et al* also used an anti-EGFR



**Figure 2** The results of protein expression and gene amplification of EGFR (epidermal growth factor receptor) and HER2 in adenocarcinoma. (A) Partial EGFR expression over part of the cell membrane in bronchioloalveolar carcinoma (BAC). (B) EGFR expression was seen over the entire cell membrane in the invasive component of adenocarcinoma with mixed subtypes (MX). (C) An EGFR gene cluster (more than six gene copies) seen in the case shown in (B). (D) Absence of HER2 expression in the membrane of a BAC lesion. (E) HER2 expression seen over the entire cell membrane in the invasive component of MX. (F) More than six gene copies of HER2 seen in the case shown in (E).

**Table 3** EGFR and HER2 protein expression in AAH and adenocarcinoma of the lung

Parameter	Adenocarcinoma				p Value	Total (n = 86)
	AAH (n = 14)	BAC (n = 31)	Early MX (n = 22)	Overt MX (n = 33)		
EGFR	0	1 (3.2%)	8 (36.4%)	15 (45.6%)	<0.0001	24 (27.9%)
HER2	0	1 (3.2%)	6 (27.3%)	11 (33.3%)	0.0034	18 (20.9%)
Synchronous expression pattern	–	1 (10%)	3 (30%)	6 (60%)	<0.0001	10 (11.6%)

The Kruskal Wallis test was used to assess the p values among BAC, early MX, and overt MX. AAH, atypical adenomatous hyperplasia; BAC, bronchioloalveolar carcinoma; EGFR, epidermal growth factor receptor; MX, adenocarcinoma with mixed subtypes.

antibody (Zymed) and reported EGFR expression in 75% of adenocarcinomas using their own criteria.<sup>7</sup> Various frequencies of EGFR expression have been reported previously because there is as yet no generally accepted standard method for the evaluation of EGFR expression in carcinoma of the lung.<sup>6–8</sup> Moreover, evaluation by semi-quantitative methods may be difficult, because the staining intensity is affected by fixation conditions.<sup>22</sup> Partial membranous expression, which was considered to be positive in the study of Hirsch *et al*, was seen in a few BAC samples in our study, but we only considered entire membranous expression to be positive.

“Our results suggest that synchronous expression of EGFR and HER2 may also be associated with the development of adenocarcinoma of the lung”

EGFR and HER2 protein overexpression without gene amplification were seen frequently. Similarly, discrepancies between HER2 gene amplification detected by FISH and protein expression have been reported in NSCLC.<sup>12–23</sup> Such discrepancies have also been detected using CISH in other carcinomas.<sup>14–15</sup> These observations may be the result of the quality of fixation and tissue processing, or disturbances in transcriptional or post-transcriptional mechanisms. EGFR and HER2 are growth factors, and enhanced transcription in the absence of gene amplification is a well recognised mechanism for cellular function through enhanced transcription of mRNA by phosphorylation of tyrosine kinase acting on growth factors and regulators of cell growth and proliferation.<sup>13</sup>

In conclusion, EGFR and HER2 gene amplification may be associated with the late stages of adenocarcinoma of the lung and the expression of the protein encoded by these genes may also be associated with the development of this disease. Protein expression was not associated with gene amplification, and other mechanisms should be considered in most cases. Further studies, including analyses of the mechanism involved in upregulation of these proteins, are necessary.

**Table 4** Correlation between protein expression and gene amplification for EGFR and HER2 in adenocarcinoma of the lung

Parameter		Gene amplification	
		Present	None
Protein expression	Positive	3	21
	Negative	1	61
HER2	Positive	2	16
	Negative	0	68

EGFR, epidermal growth factor receptor.

**Take home messages**

- EGFR and HER2 gene amplification was seen only in the invasive components of adenocarcinoma with mixed subtypes (MX) and therefore appears to be a late event in the development of adenocarcinoma of the lung
- EGFR and HER2 protein expression increased significantly as lesions progressed from atypical adenomatous hyperplasia to bronchioloalveolar carcinoma, early MX, and overt MX, and may be associated with the development of adenocarcinoma of the lung
- EGFR and HER2 protein expression was frequently seen without gene amplification, so that other mechanisms apart from gene amplification may be associated with protein expression

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