Overexpression of PY1289-HER3 in sporadic pulmonary carcinoid from patients bearing *MEN1* gene variants

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Abstract. The present study aimed to investigate the expression of human epidermal growth factor receptors (HERs) (HER1/HER2/HER3/HER4) and their phosphorylated forms (p-HER1/p-HER2/p-HER3/p-HER4) in pulmonary carcinoids (PCs). HER and p-HER protein expression was assessed by immunohistochemistry on tissue microarrays in 37 specimens of sporadic PCs, 29 typical carcinoids (TCs) and 8 atypical carcinoids (ACs). When compared with the ACs, the TCs did not exhibit any differences in terms of HER/p-HER expression. The tumors of this study have previously been characterized for the expression of menin and the mutational status of menin 1 (*MEN1*), a gene strongly implicated in the pathogenesis of PCs. In the present study, it was found that the cytoplasmic ('disarrayed'), but not nuclear ('arrayed') expression of menin was positively correlated with HER3 (P=0.004), HER4 (P=0.015), p-HER1 (P=0.005), p-HER3 (P<0.001), and p-HER4 (P=0.001) expression. Moreover, HER3 and p-HER3 were found to be significantly more expressed in PCs with *MEN1* variants, than in tumors with *MEN1* wild-type (P=0.000 and P=0.025, respectively). These findings suggest the potential clinical use of HER inhibitors in the treatment of patients with PCs, particularly for individuals with p-HER3-positive PCs harboring *MEN1* gene variants.

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Introduction

Pulmonary carcinoids (PCs), which account for 2-5% of all lung primary tumors (1) and are comprised of the typical carcinoid (TC) and atypical carcinoid (AC) types, are components of neuroendocrine neoplasms, a heterogenous group of tumors whose prevalence is increasing (2). TCs, which are up to four times more frequent than ACs, are low-grade tumors characterized by scarce mitotic figures, an absence of necrosis and a lower average size in comparison with ACs. ACs are considered to be intermediate-grade tumors with more mitotic figures than TCs, plus areas of focal necrosis and a greater size than TCs (3). The mean 5-year survival rate for patients with TCs is 88% and ranges from 25-56% for ACs (4-6). Surgical resection of the tumor in the affected lung is the standard treatment, but complete surgical excision can be difficult or unattainable depending upon the location of the tumor in the chest and whether it has metastasized. The outcome for patients with widely metastatic disease is poor, and chemotherapy and radiation therapy have limited success in treating PCs (7-9). Several studies designed to identify molecular alterations associated with the development and unpredictable progression of this type of tumor have only been partially successful (10-12). A better understanding of PC biology could lead to the development of novel therapeutic strategies.

The human epidermal growth factor receptor (HER) family, comprising the epidermal growth factor receptor (EGFR; also known as HER1), HER2, HER3 and HER4, is not only essential for the development and maintenance of normal tissue, but is also strongly involved in the development of numerous tumor types (13,14). HER dimerization is required for signal transduction to occur, through formation of homodimeric or heterodimeric complexes. Dimer formation leads to activation of the intrinsic tyrosine kinase domain, followed by phosphorylation on specific tyrosine residues, which serve as docking sites for downstream signaling proteins (15). An increasing number of small molecules and monoclonal antibodies are being recognized as targeting the HER family (15-17).

In the present study, the expression pattern of unphosphorylated (HER) and phosphorylated (p-HER) forms of HER receptors were defined in archival tumor specimens of 37 patients

with PC tumors (29 with TC and 8 with AC), in order to assess the expression of possible molecular targets of anti-HER therapy.

Materials and methods

Tissue samples. Archived formalin-fixed paraffin-embedded blocks derived from resection material of 37 apparently sporadic PCs consecutively diagnosed between January 2001 and December 2008 at the Institute of Pathology, 'S.S. Annunziata' Hospital (Chieti, Italy) were retrieved. All tumors were reviewed for diagnosis. Cases were classified as TC (29 cases) or AC (8 cases) according to the World Health Organization classification (18). For each sample, tumor and normal tissues were available. The study was approved by the Ethical Committee of the 'S.S. Annunziata' Hospital.

Tissue microarray (TMA) construction and immunohistochemistry (IHC). Duplicate TMAs were constructed by extracting cores (2-mm in diameter) of histologically confirmed neoplastic areas, as previously described (19). TMA sections were stained using antibodies to HER1 (EGFR PharmDx kit; Dako, Milano, Italy), pY1197-HER1 (rabbit polyclonal, 1:400 dilution; 30-min incubation; Thermo Fisher Scientific Inc., Waltham, MA, USA), HER2 (HercepTest kit; Dako), pY1248-HER2 (clone PN2A; 1:25 dilution; overnight incubation; Dako), HER3 (clone RTJ1; 1:20 dilution; overnight incubation; Novocastra; Leica Microsystems, Milano, Italy), pY1289-HER3 (clone 21D3; 1:50 dilution; overnight incubation; Cell Signaling Technology Inc., Danvers, MA, USA), HER4 (rabbit polyclonal; 1:50 dilution; overnight incubation; Santa Cruz Technology Inc., Dallas, TX, USA) and pY1162-HER4 (rabbit monoclonal; 1:100 dilution; overnight incubation; Epitomics/Abcam, Cambridge, UK). Epitope retrieval was performed in a heated citrate buffer (pH 6.0) for p-HER2, HER3, HER4 and p-HER4, and in 1 mmol/l EDTA (pH 8.0) for p-HER3. Antigen-antibody reactions were visualized using a polymer-based detection system (EnVision kit; Dako), using diaminobenzidine as a chromogen. The expression of HER1 and HER2 was evaluated according to the manufacturer's protocols (EGFR PharmDx and HercepTest Dako kits, respectively). Appropriate positive controls (HER1, EGFR PharmDx control slide; HER2, HER2 HercepTest control slide; HER3, HER4, p-HER1, p-HER2 and p-HER4, breast cancer tissue; and p-HER3, lung cancer tissue) of human tumor tissues known to express the marker of interest were used. For negative control sections, the specific primary antibody was omitted or replaced with non-immune serum or isotype-matched immunoglobulins. The expression of markers was quantified as a percentage of the immunoreactive tumor cells. Cases were considered HER-positive when ≥1% of tumor cells were positively stained (PharmDx kit; Dako, Milano, Italy).

Statistical analysis. Comparisons between molecular markers were performed using Spearman's Rho correlation. Student's t-test was used to evaluate the statistical differences between markers (HER and p-HER) in PC according to the *MEN1* gene status and the tumor histotype (TC and AC). SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was employed throughout, and $P<0.05$ was considered to indicate a statistically significant difference.
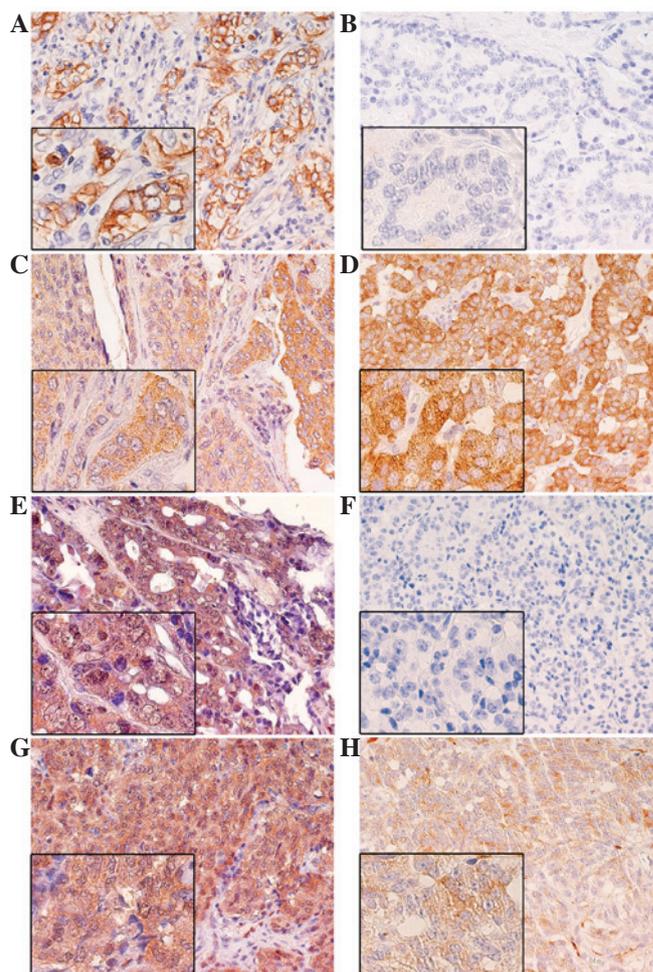

Figure 1. HER and p-HER immunostaining patterns in pulmonary carcinoids. (A) Membrane staining for HER1; (B) absence of HER2 reactivity; (C) HER3 and (D) HER4 cytoplasmic expression; (E) p-HER1 cytoplasmic expression with tumor cells showing concomitant nuclear staining; (F) lack of expression for p-HER2; (G) p-HER3 cytoplasmic expression with tumor cells showing concomitant faint nuclear staining; and (H) p-HER4 cytoplasmic expression. Inset images (magnification, x640) show enlargements of reported images (magnification, x400). HER, human epidermal growth factor receptor; p-HER, phosphorylated HER.

Results

Immunoreactivity for HER1 was present mainly in the tumor cell membranes. Reactivity for HER2 and p-HER2 receptors was absent in all samples. Specific positive staining for HER3 and HER4 was exclusively found in the cytoplasm of the tumor cells (Fig. 1). p-HER1 and p-HER3 were mainly detected in the cytoplasm. However, in association with cytoplasmic expression, nuclear staining for p-HER1 and p-HER3 was also observed in a small proportion of tumor cells [mean ± standard error (SE), 6.3±4.1 and 2.3±1.0%, respectively]. For analytical purposes, only cytoplasmic expression for p-HER1 and p-HER3 was considered.

The analysis showed that 65.5% of TC and 50.0% of AC tumors expressed HER1. HER3-positive tumors occurred in 79.3 and 100% of TC and AC cases, respectively, while HER4-positive tumors occurred in 82.8 and 87.5%, respectively. In the cases of TC, the following proportions of positive tumors were recorded; 51.7% for p-HER1, 75.9% for p-HER3

Table I. Expression of HER markers and their activated forms in pulmonary carcinoids.

Marker[a]	All cases (n=37)	Typical carcinoids (n=29)	Atypical carcinoids (n=8)	P-value[b]
HER1	22.2±5.6	19.0±5.8	33.0±14.6	0.296
HER2	0.0±0.0	0.0±0.0	0.0±0.0	
HER3	58.0±6.2	56.9±7.5	57.0±11.0	0.690
HER4	62.5±5.9	62.6±7.0	62.0±11.1	0.966
p-HER1	36.8±8.1	36.0±9.6	38.7±16.5	0.884
p-HER2	0.0±0.0	0.0±0.0	0.0±0.0	
p-HER3	35.0±6.4	38.7±7.8	25.5±11.2	0.370
p-HER4	19.4±5.4	20.1±6.2	17.7±11.5	0.849

[a]Results are expressed as the mean percentage of positive tumor cells ± standard error; [b]TCs vs. ACs, P-values from independent-samples t-test. HER, human epidermal growth factor receptor; p-HER, phosphorylated HER; TC, typical carcinoid; AC, atypical carcinoid.

Table II. Correlations[a] among HER and p-HER expression (percentage values) in pulmonary carcinoids.

Marker	HER1	HER3	HER4	p-HER1	p-HER3	p-HER4
HER1						
rho	1.000	0.003	-0.088	0.367	0.067	0.292
P-value		0.987	0.615	0.078	0.734	0.177
HER3						
rho	0.003	1.000	0.493[b]	0.392	0.687[b]	0.495[b]
P-value	0.987		0.002[b]	0.058	0.000[b]	0.014[b]
HER4						
rho	-0.088	0.493[b]	1.000	0.482[b]	0.508[b]	0.550[b]
P-value	0.615	0.002[b]		0.017[b]	0.005[b]	0.005[b]
p-HER1						
rho	0.367	0.392	0.482[b]	1.000	0.535[b]	0.882[b]
P-value	0.078	0.058	0.017[b]		0.009[b]	0.000[b]
p-HER3						
rho	0.067	0.687[b]	0.508[b]	0.535[b]	1.000	0.728[b]
P-value	0.734	0.000[b]	0.005[b]	0.009[b]		0.000[b]
p-HER4						
rho	0.292	0.495[b]	0.550[b]	0.882[b]	0.728[b]	1.000
P-value	0.177	0.014[b]	0.005[b]	0.000[b]	0.000[b]	

[a]Spearman's correlation: Correlation coefficient (rho) and P-values. [b]Significant positive correlations (P<0.05). HER, human epidermal growth factor receptor; p-HER, phosphorylated HER.

and 48.3% for p-HER4. In the cases of AC, the proportions of positive tumors were as follows: 62.5% for p-HER1, 62.5% for p-HER3 and 37.5% for p-HER4. The distribution of HER and p-HER expression is reported as a box-and-whisker plot in Fig. 2.

The expression (mean % ± SE) of the HERs and their activated forms, in all cases and in TC vs. AC tumor types, are reported in Table I. As assessed by independent-samples t-test, the TCs and ACs did not significantly differ in terms of HER/p-HER expression.

By Spearman's correlation analysis of the expression of HERs and p-HERs in PCs (Table II), HER3 expression was found to be positively correlated with that of HER4 (rho=0.493; P=0.002), p-HER3 (rho=0.687; P<0.001) and p-HER4 (rho=0.495; P=0.014). The expression of HER4 was also directly correlated with that of p-HER1 (rho=0.482; P=0.017), p-HER3 (rho=0.508; P=0.005) and p-HER4 (rho=0.550; P=0.005). In addition, significant direct correlations were found between p-HER3 and p-HER1 (rho=0.535; P=0.009), p-HER3 and p-HER4 (rho=0.728; P<0.001), and p-HER4 and p-HER1 (rho=0.882; P<0.001) (Table II).

PC samples utilized in this study have previously been characterized for the expression of menin and the mutational status of the *MEN1* gene (20). Therefore, in the present study,

Table III. Correlations[a] among menin and HER/p-HER expression (percentage values) in pulmonary carcinoids.

Localization	n-menin	c-menin	HER1	HER3	HER4	p-HER1	p-HER3	p-HER4
n-menin[b]								
rho	1.000	-0.059	-0.046	0.073	0.167	-0.066	-0.233	-0.073
P-value		0.723	0.792	0.668	0.323	0.759	0.224	0.736
c-menin[b]								
rho	-0.059	1.000	-0.094	0.457[b]	0.398[b]	0.551[b]	0.641[b]	0.635[b]
P-value	0.723		0.592	0.004[b]	0.015[b]	0.005[b]	0.000[b]	0.001[b]

[a]Spearman's correlation: Correlation coefficient (rho) and P-values. [b]Significant positive correlations (P<0.05). n-menin, nuclear menin; c-menin, cytoplasmic menin. HER, human epidermal growth factor receptor; p-HER, phosphorylated HER.

Table IV. Expression of HERs and their activated forms in pulmonary carcinoids according to *MEN1* gene status.

	All cases		Typical carcinoids		Atypical carcinoids	
Markers	Mean ± SE	P-value	Mean ± SE	P-value	Mean ± SE	P-value
HER1						
MEN1 variants	18.6±9.1	0.648	18.4±10.9	0.949	19.0±19.0	0.379
MEN1 wild-type	24.0±7.1		19.2±7.0		47.0±22.6	
HER3						
MEN1 variants	84.8±5.4	0.000[a]	85.1±7.0	0.001[a]	84.0±9.3	0.012[a]
MEN1 wild-type	43.5±7.7		44.2±9.1		40.0±8.1	
HER4						
MEN1 variants	79.5±4.8	0.009[a]	79.6±6.5	0.039[a]	79.3±6.8	0.156
MEN1 wild-type	53.3±8.2		55.0±9.3		44.8±18.3	
p-HER1						
MEN1 variants	45.9±15.7	0.443	57.5±23.1	0.226	34.3±22.9	0.785
MEN1 wild-type	32.3±9.5		29.4±10.2		44.7±28.6	
p-HER3						
MEN1 variants	54.5±8.5	0.025[a]	59.7±9.5	0.045[a]	46.8±16.7	0.046[a]
MEN1 wild-type	24.8±7.9		30.3±9.5		4.3±2.5	
p-HER4						
MEN1 variants	26.6±9.9	0.354	25.5±9.0	0.643	27.8±19.3	0.358
MEN1 wild-type	15.8±6.5		18.4±7.8		4.3±4.3	

Data are presented as the mean percentage of positive tumor cells ± standard error, with P-values from independent-samples t-test. [a]Significant positive correlations (P<0.05). HER, human epidermal growth factor receptor; p-HER, phosphorylated HER; *MEN1*, menin 1.

possible correlations were searched for among the expression results of the HERs/p-HERs and that of menin, by Spearman's rho test. As reported in Table III, the cytoplasmic but not the nuclear expression of menin was positively correlated with HER3 (rho=0.457; P=0.004), HER4 (rho=0.398; P=0.015), p-HER1 (rho=0.551; P=0.005), p-HER3 (rho=0.641; P<0.001), and p-HER4 (rho=0.635; P=0.001) expression.

Table IV shows the expression of HERs and their activated forms according to *MEN1* gene status. By independent-sample t-test, HER3 and p-HER3 were found to be significantly more expressed in PCs with *MEN1* variants than in tumors with the *MEN1* wild-type (P=0.000 and P=0.025, respectively). This was true for the TCs and the ACs. HER4, but not its activated

form, was also found to be more expressed in PC tumors (P=0.009) with *MEN1* variants. No statistical significances were found between *MEN1* status and the expression levels of HER1 and p-HER1 (Table IV).

Discussion

HER1 is the first member of the HER family to be identified in PCs, and the existence of an autocrine growth-promoting circuit based on HER1 and transforming growth factor-α has previously been documented (21,22). Lack of HER2 expression in PCs was first observed by Wilkinson *et al* (23). More recently, Rickman *et al* (12) investigated a series of PCs by IHC analysis
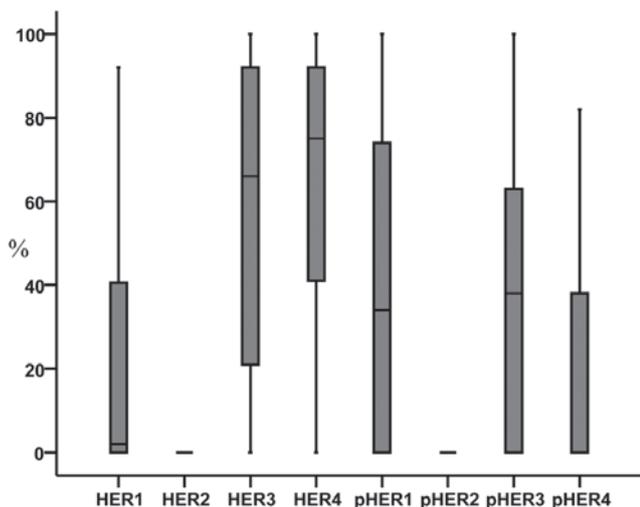
Figure 2. Box-and-whisker diagram of the percentage of pulmonary carcinoid cells stained with anti-HER and anti-p-HER antibodies. Upper and lower ends of boxes represent the 75th and 25th percentiles, respectively. The median value is shown as a solid line. HER, human epidermal growth factor receptor; p-HER, phosphorylated HER.

performed in TMAs to detect the expression of the HER family, and found that 46% of TCs and 28% of ACs expressed HER1, while none expressed HER2 and 100% showed intense to moderate staining for HER3 and HER4. The present study is the first report including data on the expression of p-HER forms. Notably PCs were found to express HER1, HER3 and HER4 receptors, as well as their phosphorylated forms.

Several malignancies, including lung, breast, stomach, colorectal, head and neck, and pancreatic carcinomas, and glioblastoma, melanoma and ovarian cancers, are associated with the mutation or increased expression of members of the HER family. HER3 is the only member of this receptor tyrosine kinase family that does not have intrinsic kinase activity, but is unexpectedly the most robust signaling complex of the HER family (17). Different degrees of HER3 expression have been observed in lung, breast, ovarian, prostate, gastric and colorectal cancer, and high expression usually correlates with a poor prognosis (24). HER2 is unlikely to be the catalytic partner of HER3, as HER2 expression is low or absent in the majority of melanomas. HER3 may function as an allosteric activator of HER1 or HER4 in melanomas (25) and PCs, as suggested by the observation that HER2 is absent in these tumors. In addition, the present study found that the expression of activated HER3 was directly correlated with that of activated HER1 and HER4. The role of HER4 in tumorigenesis is complex and not completely understood. In a breast cancer study, HER4 expression was found to be correlated with a favorable prognosis (26). By contrast, non-small cell lung cancer patients with tumors that expressed HER4 experienced decreased survival times compared with patients with tumors that did not express HER4, and a positive correlation was also found with lymph node metastasis and HER4 expression (27).

The MEN1 gene is implicated in the pathogenesis of hereditary and sporadic PCs, and MEN1 gene mutations are associated with a poor prognosis in these tumors (28). This association with poor prognosis appears to be specific to PCs, as it is not observed in pancreatic neuroendocrine tumors (29).

Menin, the protein encoded by the MEN1 gene, is a component of histone methyltransferase complexes and is ubiquitously expressed (18,30). We previously observed that PCs displaying MEN1 nucleotide variants were characterized by higher menin accumulation in the cytoplasm (disarrayed distribution), but not in the nucleus, compared with those without MEN1 variants (20). In the pancreas, IHC staining for menin showed that normal islet cells exhibited intense nuclear and extremely faint cytoplasmic staining, while 40% of pancreatic endocrine tumors lacked nuclear immunostaining and expressed high levels of cytoplasmic menin (31). The present study results showed that the presence of the activated HER1, HER3 and HER4 forms was directly correlated with the disarrayed cytoplasmic menin expression, and more importantly, that higher proportions of p-HER3-positive cells are present in PCs harboring MEN1 variants when compared with the wild-type counterpart. The association between HERs and the aberrant cytoplasmic expression of menin in tumors with MEN1 variants is noteworthy, and future studies may address this observation. At present, several HER3-targeting drugs are being tested in clinical trials (16)

Thus, the inhibition of HER signaling in these tumors by monoclonal antibodies or small tyrosine kinase inhibitors may represent a potential therapeutic strategy in the treatment of patients with p-HER-positive tumors, particularly for those patients whose cancer cannot be surgically resected, or in patients with advanced disease. In particular, HER3 could constitute a relevant therapeutic target in patients with MEN1 gene variants. The present study findings may have clinical implications for the treatment of patients with PCs.

Acknowledgements

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