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Investigation of HER2 Overexpression in Non-small Cell Lung Cancer

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Abstract. *Lung cancer is the leading cause of mortality worldwide. The median survival of advanced disease is in the range of 8 to 10 months. Intrinsic or acquired drug resistance pose major challenges to the success of chemotherapy. The HER2 gene, also known as c-erbB-2 or neu, is a proto-oncogene that encodes a membrane-bound receptor tyrosine kinase of the epithelial growth factor receptor (EGFR) family. It has a possible role in tumor cell proliferation, tumor invasion, tumor metastasis and drug resistance. We retrospectively investigated 88 samples of non-small cell lung cancer (NSCLC) and assessed the correlation between HER2 expression and tumor histology. The expression of HER2 protein was analyzed by immunohistochemical staining (IHC) and HER2 DNA amplification was detected by using fluorescence in situ hybridization (FISH). HER2 overexpression (2+, 3+) was detected in 5 (5.7%) out of 88 specimens. All of the HER2-overexpressing tumors histologically proved to be squamous cell carcinoma (SCC). Cases with 2+ HER2 immunoreactivity showed either no amplification (3.875 and 2.525), or borderline amplification (4.75). Cases with 3+ HER2 immunoreactivity showed moderate amplification (7.35) and strong amplification (15-20 – cluster), respectively. The HER2 expression in NSCLC was relatively low in the selected Hungarian population; consequently, there is no indication for introduction of trastuzumab for the treatment of lung cancer.*

Lung cancer is the leading cause of cancer death in the world. The vast majority of patients, including stage IIIA-B and IV, will progress on chemotherapy or show only moderate partial regression. The prognosis of non-small cell lung cancer (NSCLC) can be assessed using clinical, morphological and biological variables. Recently, molecular markers have been evaluated for their prognostic value, especially in early stages. Identification of specific gene abnormalities or protein expressions that may be targeted by novel therapies appear to be potentially rewarding approaches. Several proteins, such as HER2, have a possible role in tumor cell proliferation, tumor invasion, tumor metastasis and drug resistance (1). Amplification and/or overexpression of HER2 has been identified in a variety of human tumors (2-4).

The HER2 gene is overexpressed in 25% to 30% of human breast cancers (5) and, in 95% of these cases, overexpression is a direct result of gene amplification (6). This alteration correlates with poor clinical prognosis, meaning that malignancies with HER2 amplification/ overexpression are associated with shorter disease-free survival as well as overall survival. The detection of HER2 gene amplification and/or protein overexpression may be predictive of response to certain types of chemotherapy and, in addition, the HER2 protein represents a target for specific therapy with trastuzumab (a monoclonal antibody synthesized against the extracellular domain of HER2 protein). Trastuzumab, combined with chemotherapy, has already shown major clinical benefit in women with HER2-positive breast cancer, including a significant increase in survival (7).

The increased expression of HER2 has been demonstrated in several human tumors including cancers of the gastrointestinal tract (8), liver (9), testis (10), ovary (11), uterus (12), bladder (13) and prostate (14).

HER2-neu protein overexpression has been demonstrated in NSCLC, including squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma (LC) (15). The significant association of gene amplification and HER2 expression with poorly-differentiated lung carcinomas compared to the well-differentiated carcinomas suggests that HER2 may be involved in NSCLC tumor evolution. Patients with HER2 gene amplification and a strong expression of HER2 protein showed a tendency towards shorter survival (16). Studies using protein assays reported an association with

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Table I. *Correlation between tumor size and age.*

Tumor size	Age						*p* Chi-squared	*p* Fisher's
	42-49	50-56	57-64	66-71	72-79	Total		
T1	9	8	7	2	1	27	0.024	-
T2	6	4	20	13	3	46		
T3	3	3	3	5	1	15		
Total	18	15	30	20	5	88		

HER2-neu protein overexpression and inferior survival in ACs (17), whereas others did not (18).

In HER2-positive NSCLC cell lines, trastuzumab showed an antiproliferative effect *in vitro* and an even greater synergy with gemcitabine and cisplatin than it had in breast cancer cells (19). However, trastuzumab has given disappointing results in clinical studies (20). The main reason for failure was that the majority of patients had low levels (1+ and 2+) of HER2 overexpression. Nevertheless, there could be a small group of NSCLC patients with gene amplification and/or strong overexpression of HER2 who may benefit from trastuzumab (21).

The aim of our study was to investigate the prevalence and prognostic significance of the HER2 status in a clinically well-characterized group of NSCLCs and to estimate the relationship of this marker with tumor stage, grade, histological type and survival.

Materials and Methods

Between September 1999 and September 2003, 88 consecutive patients (33 females, 55 males), with previously untreated NSCLC, were enrolled into this study. The median age of the patients was 58.9 years (range; 42-79). All patients were surgically treated at the University of Szeged, Surgical Department, Hungary. All tumors were completely resected by at least a lobectomy. Fifty-seven patients were treated by surgical procedures alone; 31 patients received surgical treatment followed by radiation and/or chemotherapeutic treatment. The disease status of all patients was determined at the time of surgery according to the UICC classification (22). Fifty patients were staged pathologically as Stage I (56.8%), 24 patients as Stage II (27.3%) and 14 patients as Stage IIIA (15.9%).

The histological type and degree of differentiation were assigned according to the WHO criteria (23). Histopathological subtypes were SCC in 42 patients (47.7%), AC in 41 patients (46.6%) and LC in 5 patients (5.7%). The primary tumors were graded histopathologically as well-differentiated in 22 patients (25.3%), moderately-differentiated in 48 patients (55.2%) and poorly-differentiated in 18 patients (19.5%).

IHC detection of HER2 protein. Specimens were fixed in 4% buffered formalin. After paraffin embedding, 4-μm sections were cut. Heat-induced epitope retrieval was performed in a water-bath (95˚C, 50 minutes) and automated IC staining with the DAKO HercepTest™ performed on an automated immunostaining system (DAKO Autostainer), in strict accordance with the manufacturer's instructions. Negative and positive controls consisted of control cell lines included in the DAKO Kit (cell lines MDA-231, MDA-175 and SK-BR-3). To prove the reproducibility of IHC staining, four slides of each specimen were examined.

Each IHC case was analyzed by L.M. and L.T. The IHC-stained slides were interpreted and scored on the scale of 0 to 3+, according to the FDA-approved guidelines for HercepTest™ (24); 2+ and 3+ reactions were assessed as positive.

Determination of HER2 gene amplification by FISH. FISH was performed according to the Ventana HER2 FISH protocol as an automated procedure for use on the Ventana BenchMark™ system. In brief, tumor blocks were cut in 1-μm sections. The sections were dewaxed by adding xylene (100%, 3x2 min) and dehydrated in ethanol (100%, 2x2 min). After air-drying, the sections were digested with protease (37˚C, 40 min). Following rinsing (SCC, 3x2 min), the slides were dehydrated in a graded series of ethanol (95% and 100%, 1 min each) and were denatured (90˚C, 10 min). Hybridization was performed with fluorescent (FITC)-labelled probes for the HER2 gene (Ventana INFORM 780-2840 Probe), according to the manufacturer's recommendation. The slides were counterstained by 4,6-diamidino-2-phenylindole dihydrochloride hydrate and visualized using a fluorescent microscope with FITC/DAPI dual band filter.

At least 40 well-defined, representative, intact nuclei from each of two separate tumor areas were scored. FISH evaluation was performed only on tissue sections with uniform hybridization; overlapping nuclei were not evaluated.

The HER2 status was scored as follows: a) no amplification (up to 4 specific signals/nucleus); b) borderline amplification (4.1-5 specific signals/nucleus); c) definite amplification (more than 5 signals/nucleus).

Statistical analysis. Correlation between clinical and histological parameters was assessed by χ^2 test and Fisher's exact test. Survival probabilities were calculated with the product limit method according to Kaplan-Meier. Surviving patients were censored at the time of the last follow-up. Differences between survival curves were analyzed by means of the log-rank test.

Results

Among the variables describing patients, statistically significant correlations were found between tumor size and age (Table I),

Table II. *Correlation between tumor size and histological type.*

Tumor size	Histological type				*p* Chi-squared	*p* Fisher's
	SCC	AC	LC	Total	0.018	0.008
T1	6	18	3	27		
T2	26	19	1	46		
T3	10	4	1	15		
Total	42	41	5	88		

Table III. *Correlation between histological type and therapy.*

Therapy	Histological type				*p* Chi-squared	*p* Fisher's
	SC	AC	LC	Total		
Surgical	31	24	2	57	0.012	0.005
Surgical+Chemotherapy	0	8	0	8		
Surgical+Radiotherapy	7	2	1	10		
Chemotherapy+ Radiotherapy	4	7	2	13		
Total	42	41	5	88		

Table IV. *HER2 status by immunohistochemistry.*

HER2 overexpression	No. of cases
0	66 (75%)
+	17 (19.3%)
++	3 (3.4%)
+++	2 (2.3%)
Total	88

between tumor size and histological type (Table II) and between histological type and therapy (Table III).

Only disease stage correlated significantly with patient survival. The resulting adjusted *p* value was 0.005 (log-rank test) (Figure 1). There was no correlation between histotype and survival (p_χ=0.6205) or between tumor grade and survival (p_χ=0.6006).

IHC. HER2 overexpression (2+, 3+) was detected in 5 (5.7%) out of 88 specimens (Table IV). All of the HER2-overexpressing tumors histologically proved to be SCC (Figure 2). No statistically significant correlation was calculated for HER2 immunoreactivity and other clinicopathological parameters, such as tumor grade, stage and survival, because of the low number of positive cases.

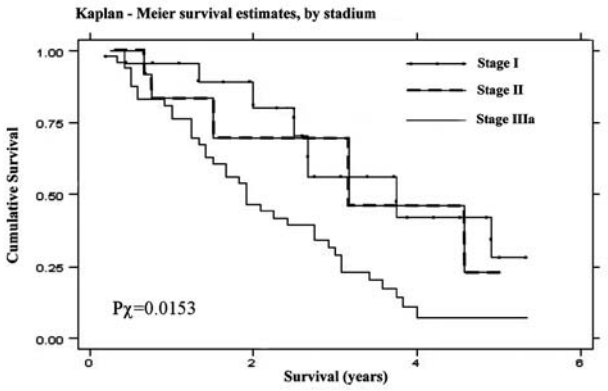

Figure 1. *Overall survival according to the stage of disease; log-rank test.*

FISH. Materials from the immunohistochemically HER2-overexpressing tumors were investigated. Cases with 2+ HER2 immunoreactivity showed either no amplification (3.875 and 2.525), or borderline amplification (4.75). Cases with 3+ HER2 immunoreactivity showed moderate amplification (7.35), and strong amplification (15-20 – cluster), respectively (Figure 3).

Discussion

The *c-erbB-2* oncogene, found on chromosome 17, also known as HER2, is one of the family of related transmembrane growth factors. HER2 protein is expressed at low levels by normal cells of breast, ovaries, skin, lung, liver, intestines and prostate. It has been shown, in a number of studies, that defective signal regulation by HER2 plays a role in the genesis and progression of many types of cancer (25). Amplification of the HER2 gene results in an increased amount of HER2 mRNA and increased HER2 protein level, which are associated with more rapid growth of tumor cells and worse prognosis. Tumor cells with HER2 activation tend to become resistant to chemotherapeutic agents.

Recently, some monoclonal antibodies that block EGFR at the cell surface have been developed (26). The best known of these is trastuzumab, which is currently undergoing different clinical trials in combination with the cytotoxic drug cisplatin in HER2-overexpressing breast and lung tumors (27). Immunhistochemical staining (IHC) is the most frequently used test to assess tumor HER2 status. FISH is sensitive and specific and is the most commonly used test to detect HER2 DNA amplification.

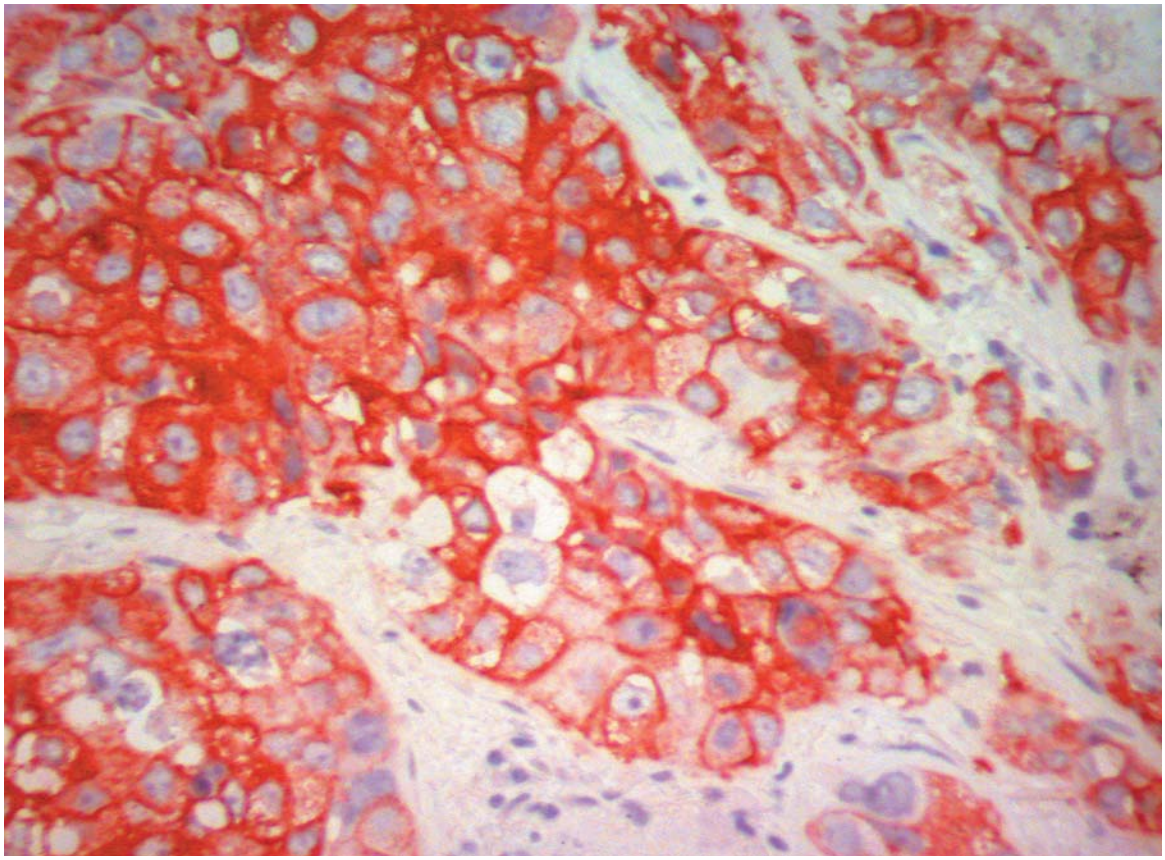

Figure 2. *Squamous cell carcinoma. An intense staining of the entire membrane is observed in about 50% of the epithelial cells (3+ HER2 positivity) (HercepTest™ x100).*

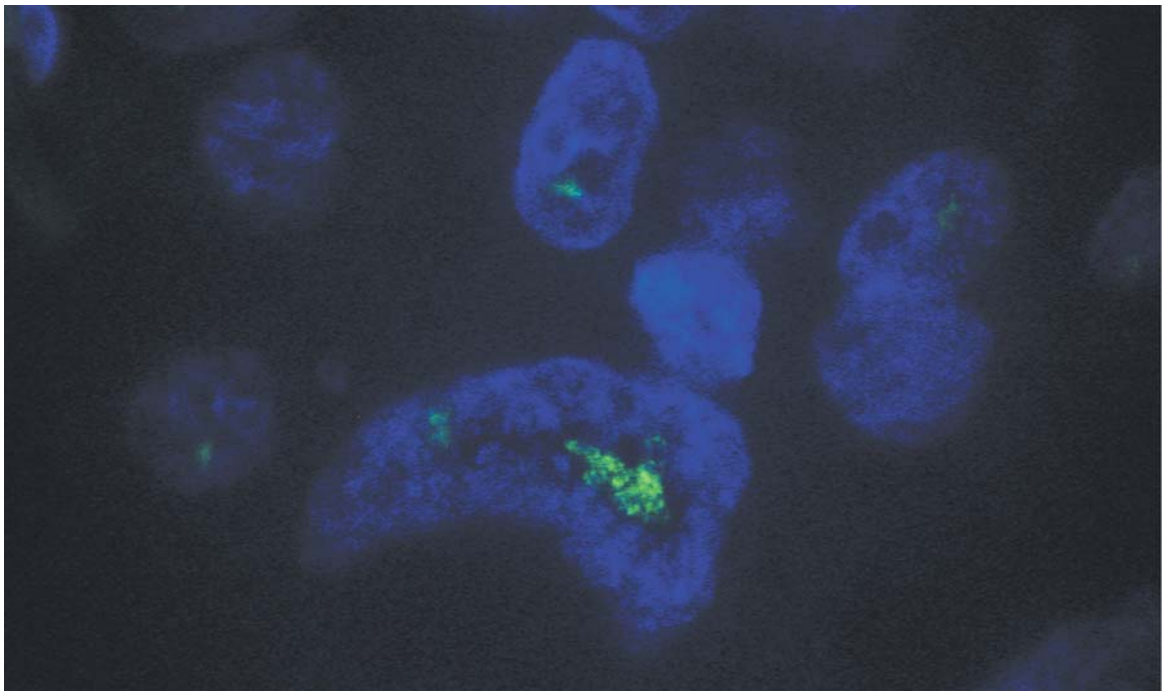

Figure 3. *Squamous cell carcinoma. Strong HER2 gene amplification (cluster) with fluorescence in situ hybridization (x1000).*

In the current study, we retrospectively analyzed the clinical data of 88 NSCLC patients and investigated the expression of HER2 overexpression by IHC and the gene amplification by FISH to estimate the prevalence and the prognostic value of HER2 positivity in NSCLC. Among the clinicopathological variables describing patients, only the disease stage correlated significantly with patient survival. HER2 overexpression (2+, 3+) was detected only in 5 out of 88 patients (5.7%), which was lower than in the majority of trials.

Regarding the HER2 expression (gene or protein level) in lung cancer, several studies with inconsistent results have been reported. The proportion of HER2-positive tumors varies widely among studies (range; 5-80%) (28, 29).

The different proportion of HER2 positivity partly depends on the applied method. We believe that, in this study, the cause of the relatively low number of positive cases was the application of the standard diagnostic kit (HercepTest™), approved by the U.S. Food and Drug Administration (FDA) for assessment of HER2 overexpression, based on an automated staining method and cell controls with a known receptor density to avoid false conclusions.

According to the literature, the impact of HER2 overexpression on survival was maintained in the long-term follow-up of radically resected patients (30). Because of the low number of positive cases, we did not look for a statistically significant correlation between HER2 immunopositivity and other clinicopathological parameters such as tumor grade, stage and survival.

The HER2 receptor may be overexpressed in the absence of gene amplification (31, 32). The discrepancy between the FISH and IHC results is well-documented in several other tumor types as well. In our study, cases with 2+ HER2 immunoreactivity (3.4%) showed either no amplification or borderline amplification. Cases with 3+ (2.3%) HER2 immunoreactivity showed moderate and strong amplification. According to Pellegrini *et al.*, other mechanism could be responsible for HER2 mRNA gene amplification and protein overexpression, but a firm association exists between strongly immunoreactive (3+) cancers and FISH-documented gene amplification (33). In the case of 2+ overexpressing tumors, perhaps mechanisms other than gene amplification (autocrine loop, mutation) are the cause of the immunopositive results. This theory could explain the fact that, in these cases, the correlation of protein overexpression with gene amplification was reduced.

In the literature, the majority of HER2-positive cases are lung adenocarcinomas (range; 28-80%) (34). It was surprising that, contrary to previous literature findings, in this study all of the HER2-overexpressing tumors histologically proved to be SCC.

The use of monoclonal antibody combined with chemotherapy could increase the overall survival for patients with advanced, metastatic breast cancers that are HER2 3+ by IHC or show HER2 gene amplification by FISH (35).

The results of clinical trials evaluating trastuzumab in lung cancer overexpressing HER2 indicated that trastuzumab therapy might be less effective in lung cancer than in breast cancer (36). The results of the ECOG trial could help to identify the benefit of trastuzumab combined with chemotherapy for patients with advanced NSCLC (37). The data suggest that only the few HER2 3+ or FISH-positive patients (<5%) had a higher response rate compared to other patients. According to Gatzeimer *et al.*, only a small percentage of patients (<2%) had HER2 3+ by IHC and HER2 amplification measured by FISH, so it is difficult to assess the benefit of trastuzumab therapy (38).

HER2 expression in NSCLC was relatively low in the selected Hungarian population; consequently, there is no indication for introduction of trastuzumab for the treatment of lung cancer.

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