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Original Article

HER-2/neu alterations in non-small cell lung cancer: A comprehensive evaluation by fluorescence in situ hybridization and immunohistochemistry

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Abstract

Aim: The molecular relationships of protein overexpression and HER-2/neu gene amplification in lung cancers are not clearly defined. The aim of the study is to evaluate HER-2/neu changes in non-small cell lung cancer with in situ hybridization and immunohistochemistry, to compare the effectiveness of both methods and to examine the influence of HER-2/neu gene and protein level alterations on prognosis.

Material and Methods: HER-2/neu alterations were analyzed with fluorescence in situ hybridization and immunohistochemistry in 24 patients with non-small cell lung cancer.

Results: Of 24 patients, 13 (54%) were found immunohistochemistry (IHC) positive, on the other hand 24 (100%) were found fluorescence in situ hybridization (FISH) negative. Balanced trisomy was detected in 23 out of FISH negative cases and balanced quadrisomy was detected in one case. Our study has revealed that overexpression of HER-2/neu protein is not associated with real gene amplification. No significant correlation was detected between HER-2/neu overexpression with amplification, and prognosis of non-small cell lung cancer.

Conclusion: No correlation is present between fluorescence in situ hybridization and immunohistochemistry results. HER-2/neu alterations are present in non-small cell lung cancers. Therefore, HER-2/neu overexpression may be associated with transcriptional mechanisms rather than gene amplification.

Keywords: Non-Small Cell Lung Cancer; HER-2/neu; Fluorescence In Situ Hybridization; Immunohistochemistry; Gene Amplification.

INTRODUCTION

Lung cancer is the main cause of cancer deaths worldwide. Non-small cell lung cancer (NSCLC) account for approximately 85% of all lung cancers (1,2). Prognosis is quite poor and only 10-15% of the patients survive 5 years or longer (3).

ErbB gene family codes growth factor tyrosine kinase receptors. ErbB is composed of four receptors including the ones located on cell membrane: ErbB1, ErbB2, ErbB3 and ErbB4, also called Her-1, Her-2, Her-3 and Her-4, respectively. Four receptors of ErbB gene family are structurally similar. They contain membrane spanning region, cysteine-rich domains and intracellular tyrosine kinase domain. These receptors are generally co-expressed with various combinations and found in various tissues except hematopoietic system (4).

ErbB family has significant oncogenic potential in many human cancers. Deregulation of the family has been defined particularly in breast, stomach, lung, colorectal, head and neck cancers and glioblastomas. Overexpression develops as the result of point mutation in kinase domains of family members, deletion in their extracellular domains, and gene amplification (5).

HER-2/neu, which is located on 17q12, encodes a

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transmembrane tyrosine kinase of 185 kDalton (6). HER-2/neu gene amplification and receptor overexpression lead to an increase in cell division and acceleration in cell growth. HER-2/neu overexpression is determined in 13%-20% of NSCLC patients although HER-2/neu amplification is detected in 2%-4% (7). A meta-analysis has revealed that HER-2/neu alterations are observed more in adenocarcinomas and HER-2/neu amplification is a negative prognostic marker (8). However, conflicting results have been reported in the studies investigating HER-2/neu expression (at gene or protein level) in lung cancer (8).

Trastuzumab (Herceptin) is a recombinant DNA-derived monoclonal antibody that is binds to HER2/neu gene product p185HER2. Herceptin has been proven to provide a longer life compared to conventional therapy of breast cancer. Evidence has been obtained about the feasibility of Herceptin and chemotherapy combination in recent years (7,9). Trastuzumab emtansine (T-DM1), a HER-2-targeted antibody-drug conjugate, has been approved for clinical use in HER2-positive breast cancer patients in many countries (10). Effectiveness of T-DM1 is being investigated for HER-2 positive NSCLC patients (11). Studies are also available about the use of novel drugs for HER2-targeted therapies. HER-2 alterations should be detected with proper techniques for using HER-2-based medications in lung cancers (8).

We aimed to analyze the influence of HER-2/neu alterations on prognosis and prevalence in non-small cell lung cancers.

MATERIAL and METHODS

Patients and Tissue Samples

Paraffin blocks were obtained from the cases that underwent operation due to NSCLC at Izmir Suat Seren Pulmonology and Thoracic Surgery Research and Training Hospital between 2002-2004. The study has been approved by Medical Ethics Committee of Dokuz Eylul University. After fixation in NBF (10% neutral buffered formalin) overnight, tumor samples were taken followed by standard tissue tracking procedure. Sections of 2 micron (µ) were taken from 24 paraffin blocks onto lysine lams, stained with Hematoxylin-Eosin and tumor regions were marked. Clinical and pathologic characteristics of the patients are given in detail in Table 1.

DAKO probes and DAKO automated standard staining system were used in immuno-histochemical (IHC) staining for HER-2/neu. Each slide was independently evaluated by two pathologists blinded to clinic-pathological characteristics of the tumor. A four step (0, 1, 2, 3) system which is based on membranous staining and takes cut-off value as 10% was used for evaluation. The samples in which less than 10% cells are stained were accepted as negative, 10-50% staining as ++ (positive) and >50% staining as +++ (positive).

Table 1. Histopathologic characteristics and clinic stages and of patients (n=24)

Parameter	n (%)
Gender	
Female	2 (8)
Male	22 (92)
Age	
Range: 51-80	
Median: 63.25	
Smoking	
Smoker	23 (96)
Nonsmoker	1 (4)
Histology	
Adenocarcinoma (ADC)	8 (33)
Squamous-cell carcinoma (SCC)	13 (54)
Large-cell carcinoma (LCC)	3 (13)
Differentiation	
Poor	7 (29)
Moderate	17 (71)
Tumor status	
T1	3 (13)
T2	15 (62)
T3	6 (25)
Nodal status	
N0	20 (83)
N1	3 (13)
N2	1 (4)
Tumor stage	
1A	3 (13)
1B	11 (46)
2B	8 (33)
3A	2 (8)

IHC and Scoring Criteria

FISH Assay and Scoring Criteria

Twenty-four NSCLC cases were analyzed with fluorescence in situ hybridization (FISH) method. Sections of 2 µ were taken from 24 paraffin blocks onto lysine lams, stained with Hematoxylin-Eosin and tumor regions were marked. The 2 µ sections were kept at 55^{0}C overnight. Paraffin melting process was performed on the other day. Slides were waited at 55^{0}C for 10 min in xylene, waited in 100% ethanol for 5 min twice and dried thereafter. Slides were treated with pretreatment kit (Q Biogene) after paraffin melting, waited in 20% pretreatment solution at 45^{0}C for 15 min, washed with 2xSSC at room temperature for 5-10 sec. At protease stage, slides were waited in protease solution at 45^{0}C for 20 min, washed with 2xSSC at room temperature for 5-10 sec, dried after waited in 70%, 80%, 100% ethanol for one min. Slides were waited in formamide (70%) at 73^{0}C for 3 min. Thereafter waited in 70%, 80%, 100% (-20^{0}C) ethanol for one min. Preparations were dried at room temperature.

Probe was denaturated at 75^{0}C for 5 min. At hybridization stage, 10 µl probe cocktail (17q12 (HER-2/neu) specific DNA probe, Rhodamine, chromosome 17 Alpha-Satellite probe, directly marked with Fluorescein was dropped. It was closed with cover slip and covered with rubber band adhesive, left incubation overnight at 37^{0}C in damp environment. Post-hybridization washing was done on the other day. Slides were washed with 1x in situ wash buffer at 37^{0}C for 5-10 sec, with 1x in situ wash buffer at 65^{0}C for 3 min, with 1xPBD at room temperature for 5 min. 10 µl DAPI was dropped onto dried lams. Analyses were done using Macprobe 4.0 program under Nikon ECLIPSE E 600 fluorescein microscope (12,13).

Minimum 100 nuclei were counted for each case (except one). FISH analysis was not performed in partially or totally overlapped nuclei. Red signal was observed for HER-2/neu, green signal was observed for alpha satellite 17. Analysis results were evaluated as "gene amplification is present" if HER-2/alpha satellite 17 ratio≥ 2, "gene amplification is not present" if HER-2/alpha satellite 17 ratio<2.

Overall, HER-2/neu and alpha satellite 17 signals were calculated in each case and mean HER-2/neu and alpha satellite 17 values were obtained by dividing with total cell count (14).

Of both values, being between 1.5 and 2.5 together or alone was evaluated as "balanced disomy" (normal), 2.5-3.5 as "balanced trisomy", 3.5-4.5 as "balanced quadrisomy" (14). In each case, number of alpha satellite signals was divided with the number of HER-2/neu signals and the result was accepted as (+) for gene amplification if ≥2 and (-) if <2 (15).

Statistical Analysis

Inter-group differences were compared using chi-square or Fisher's exact test. Statistical analysis was performed using SPSS statistical software version 20. The p-value < 0.05 was considered statistically significant.

RESULTS

IHC

Of the cases, 11 (46%) were negative and 7 (29%) were +2, 6 (25%) were +3. HER-2/neu over-expression (+2, +3) was detected in 13 (%54) out of 24 cases. Figure 1, A shows an adenocarcinoma which is stained +2 and figure 1, B shows an adenocarcinoma which is stained + 3.

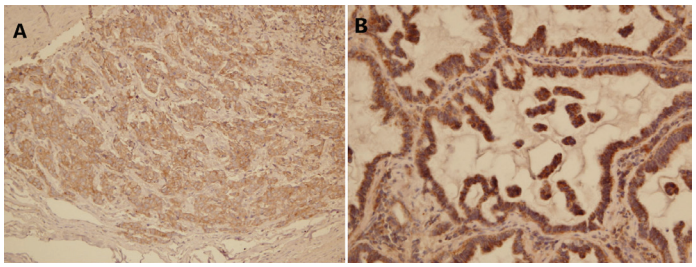

Figure 1. HER-2/neu patterns of immunoreactivity in NSCLC. Adenocarcinoma , 2+ HER-2 positivity (A). Adenocarcinoma , 3+ HER-2 positivity (B)

Dual Color FISH

HER-2/neu alterations were detected in 1 out of 24 (4.17%) cases. While FISH analysis performed in the sections obtained from paraffin blocks revealed balanced disomy in 23 cases, balanced polysomy (trisomy, quadrisomy) was detected in one +3 case, real amplification of HER-2/neu gene was observed in no case. HER-2/neu protein over-expression was shown not to be associated with real gene amplification in IHC +2, +3 positive cases (54%) (Table 2). P-values were estimated according to Fisher and Pearson for the parameters and HER-2/neu gene amplification status. A statistically significant difference was not detected between clinical data and the elevation in copy count detected in HER-2/neu gene (p>0.05) (Table 3).

Table 2. Comparison of FISH results with prognostic data and IHC results (1 case not included)

	Survival		Metastasis			Recurrence		Immunohistochemistry		
FISH	Alive	Dead	Distant	Local	Absent	Present	Absent	0 + 1	2	3
Not gene amplification	19	4	6	4	13	10	13	11	7	5
Polysomy	1	-	-	-	1	-	1	-	-	1

Table 3. Comparison of clinical data with FISH results

	Histological type			Stage			
FISH	SCC	AC	LCC	1A	1B	2B	3A
Not gene amplification	13	8	3	3	11	8	2
Polysomy	-	-	1	-	-	1	-

Abbreviations: SCC= Squamous cell carcinoma, AC= Adenocarcinoma, LCC= Large cell carcinoma

HER-2/neu gene anomaly and IHC were positive in 4.16% of the patients, normal gene and IHC were negative in 45.83%. Results were inconsistent in 50% of tumors (50% positive by IHC with a normal gene status).

Figure 2A shows interphase regions with normal copy count, figure 2B show inter-phase regions in which copy count elevation is observed.

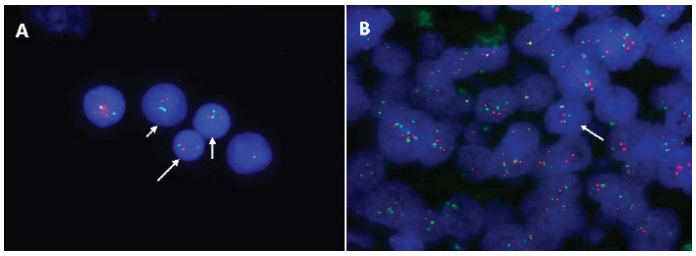

Figure 2. Dual color FISH on NSCLC nuclei with specific HER-2/neu (red fluorescent signals) and α-satellite chromosome 17 (green fluorescent signals) probes. Tumor section showing two normal copy of both probes (A). Tumor section with HER-2/neu amplification (B)

DISCUSSION

In our study, HER-2/neu amplification was evaluated with FISH, HER-2/neu over-expression was evaluated with IHC in 24 NSCLC paraffin block samples and results were compared with clinical data of the patients.

Studies investigating HER-2/neu expression (at gene or protein level) have revealed conflicting results. Ratio of HER-2/neu positive tumors widely varies among various studies (5-80%) (8).

While some studies report no association between IHC results and FISH results of HER-2/neu, some others propose the opposite. D.Tan et al. detected a significant relationship between FISH and IHC methods in lung cancer ($r=0.83$, $p<0.001$). While all of +3 tumors showed amplification, the authors detected amplification in none of Hercept test negative (0 or +1) cases (16). They indicated a significant relationship between histological types of lung and HER-2/neu anomalies. D.Tan et al. detected amplification in 72% (5/7) of adenocarcinoma, 14% (1/7) of squamous cell carcinoma, 14% (1/7) of large cell carcinoma.

G. Cox et al. detected no amplification in 19 negative (0 and +1) and four +2 cases. While amplification was detected in all of four +3 cases, polysomy was detected in only one +2 case (17). According to G.Cox et al., Hercept test is a quite reliable method for determining of HER-2/neu amplification particularly in +3 cases. A significant association is present between IHC results and FISH results.

In our study, while balanced disomy was detected in 23 cases, balanced quadrisomy was detected in one +3 case, real amplification was detected in none of the cases as the result of FISH test performed in paraffin block sections of the cases with 11 (46%) negative cases, seven (29%) +2 cases and 6 (25%) +3 cases according to IHC results. Although the consequences of our study are consistent with those of the mentioned studies with regard to negative cases, they are not consistent with the results of others with regard to +3 cases.

Pellegrini et al. have detected low amplification in 2 out of 23 negative tumors, low amplification in 3 out of 18 positive cases, high-level amplification was detected in 3, polysomy was detected in 1 and amplification was not detected in 11 in their study conducted with 41 cases (15). Nakamuro et al. found an elevation in HER-2/neu copy count in 44% of tumors and amplification in 2% of tumors. While elevated HER-2/neu copy was not encountered in large cell carcinoma, it was determined in squamous cell carcinoma and adenocarcinoma (18). Nakamuro et al. have proposed that the elevation in HER-2/neu copy count was associated with polysomy 17 and but not with p185 overexpression.

While elevated HER-2/neu copy count was not detected in squamous cell cancer and adenocarcinoma cases, we detected elevated HER-2/neu copy count in one large cell carcinoma case. HER-2/neu copy count was found as >3 in 4.17% of tumors. In our study, balanced polysomy was seen in chromosome 17 in the only +3 case which showed elevated copy count.

Polysomy 17-dependent HER-2/neu elevation is suggested to lead to overexpression of p185. While this is true for some tumors, protein overexpression is encountered despite normal HER-2/neu copy count in some tumors. Moreover, overexpression is not present in 62% of the tumors with elevated copy count. Slaman et al. reported a similar phenomenon for breast and ovarian cancer. Therefore overexpression is suggested to develop due to the alterations in regulation of transcriptional or post-transcriptional mechanisms. These mechanisms are considered to be able to common in overexpression as mRNA overexpression of HER-2/neu gene is frequently seen in NSCLC (18).

HER-2/neu overexpression was investigated with FISH, IHC and FACS methods in the studies conducted with lung cancer cell lines and a moderate expression was detected in small number of cell lines (19). Overexpression was associated with duplication and polysomy of chromosome 17. In this study, trastuzumab was found to influence cell proliferation in cell lines with real gene amplification for only HER-2/neu. However, phase II clinical trials indicate that IHC +3 cases benefited from combination therapy with trastuzumab more than conventional therapy. However use of Herceptin in treatment of NSCLC is still of debate and being investigated. The ideal method for examination of HER-2/neu is also still of debate. Considering mechanism of actions of trastuzumab, we consider that using the combination of both methods and HER-2 ECD serum levels may be better.

Her-2/neu amplification has been observed in 20-30% of breast cancer cases. 20-30% of breast cancer patients have Her-2/neu amplification. FISH technique has been

determined as gold standard for detection of HER-2/neu amplification in breast cancer. HER-2/neu amplification observed in breast cancer leads to an elevation in HER-2 RNA and thereby overexpression of HER-2/neu (20). In addition, IHC and FISH results are more consistent in breast cancer. The patients eligible for Herceptin use are detected with IHC and FISH methods (21). However, HER-2/neu overexpression is encountered less in lung cancer and results of IHC and FISH are not always consistent.

In some studies, Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR) method was used to evaluate HER-2/neu expression. The consequences of qRT-PCR analysis for HER-2 assay appear to be encouraging and could supply much more clinically relevant information.

However, the results of the studies show incompatibility between IHC, FISH and qRT-PCR (15,22). This incompatibility originate from low quality nucleic acids isolated from formalin-fixed paraffin-embedded (FFPE).

On the other hand; a dual-color FISH testing can simply distinguish chromosome 17 polysomy from HER2 gene amplification. Chromosome 17 polysomy was observed in patients with low or limited protein concentrations without gene amplification (23). Quantitative RT-PCR is a favourable method to determine HER-2 overexpression and a supplementary array to FISH. But; to the results of the present works it cannot be used as a gold standard technique (24,25).

HER-2/neu positivity is reported to be associated with poor prognosis in the literature (8). In this study an association was not detected between overexpression and amplification of HER-2 with prognosis of lung cancer.

CONCLUSION

Studies have shown that dual-ISH (DISH) method is more advantageous than FISH in case of heterogeneity of HER-2 amplified cells (26). Therefore, studies may be planned for comparison of FISH, IHC and different methods for detection of HER-2 alterations in NSCLC.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018;68:7-30.
2. Wang D, Pham NA, Tong J, et al. Molecular heterogeneity of non-small cell lung carcinoma patient-derived xenografts closely reflect their primary tumors. Int J Cancer 2017;140:662-73.
3. Sasaki H, Suzuki A, Tatematsu T, et al. Prognosis of recurrent non-small cell lung cancer following complete resection. Oncol Lett 2014;7:1300-4.
4. Iqbal N, Iqbal N, et al. Human epidermal growth factor receptor 2 (HER2) in Cancers: overexpression and therapeutic lmplications. Mol Biol Int 2014;2014:1-9.
5. Roskoski R. The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacol Res 2014;79:34-74.
6. Kobyakov DS, Avdalyan AM, Bobrov IP, et al. Correlation of HER-2/ Neu protein expression and HER2 gene amplification with clinical morphological parameters of nonsmall cell lung cancer. Bull Exp Biol Med 2014;157:789-93.
7. Rothschild SI. Targeted therapies in non-small cell lung cancer—beyond EGFR and ALK. Cancers (Basel) 2015;7:930-49.
8. Liu L, Shao X, Gao W, et al. The role of human epidermal growth factor receptor 2 as a prognostic factor in lung cancer: A meta-analysis of published data. J Thorac Oncol 2010;5:1922-32.
9. Rosell R. Toward customized trastuzumab in HER-2/neu-overexpressing non-small-cell lung cancers. J Clin Oncol 2004;22:1171-3.
10. Barok M, Joensuu H, Isola J. Trastuzumab emtansine: mechanisms of action and drug resistance. Breast Cancer Res 2014;16:R22.
11. Ohashi K, Hotta K, Hirata T, et al. Trastuzumab Emtansine in HER2+ Recurrent Metastatic Non–Small-Cell Lung Cancer: Study Protocol. Clin Lung Cancer 2017;18:92-5.
12. Ozer O, Sahin FI, Aydemir F, et al. HER-2 / neu gene amplification in paraffin- embedded tissue sections of meningioma patients meningiom olgular › na ait parafin blok doku kesitlerinde her2 /neu. Turk Neurosurg 2009;19:135-8.
13. Amplification G, Immunohistochemistry W, Ciampa A, et al. HER-2 status in breast cancer. Appl Immunohistochem 2006;14:132-7.
14. Meert AP, Martin B, Verdebout JM, et al. Is there a relationship between c-erbB-1 and c-erbB-2 amplification and protein overexpression in NSCLC? Lung Cancer 2005;47:325-36.
15. Pellegrini C, Falleni M, Marchetti A, et al. HER-2/Neu alterations in non-small cell lung cancer : a comprehensive evaluation by real time reverse transcription-pcr, Fluorescence in Situ hybridization, and immunohistochemistry. Clin Cancer Res 2003;9:3645-52.
16. Tissue T, Winston J, Wiseman S, et al. HER-2 / neu protein expression and gene alteration in stage I-IIIa non – small-cell lung cancer : a study of 140 cases using a combination. Cancer 2003;12:201-11.
17. Cox G, Vyberg M, Melgaard B, et al. HER2 expression and gene amplification in non-small cell lung cancer 2001;15:480-3.
18. Nakamura H, Saji H, Ogata A, et al. Correlation between encoded protein overexpression and copy number of the HER2 gene with survival in non-small cell lung cancer. Int J Cancer 2003;103:61-6.
19. Bunn PA, Helfrich B, Soriano AF, et al. Expression of Her-2/neu in human lung cancer cell lines by immunohistochemistryand fluorescence in situ hybridization and its relationship to in vitro cytotoxicity by trastuzumab and chemotherapeutic agents. Expression of Her-2/neu in human lung cancer cell lines by immunohistochemistryand fluorescence in situ hybridization and its relationship to in vitro cytotoxicity by trastuzumab and chemotherapeutic agents. Clin Cance 2001;7:3239-50.

20. Hicks DG, Tubbs RR. Assessment of the HER2 status in breast cancer by fluorescence in situ hybridization: a technical review with interpretive guidelines. Hum Pathol 2005;36:250-61.
21. Horton J. Trastuzumab use in breast cancer: clinical issues. Cancer Control 2002;9:499-507.
22. Cuadros M, Talavera P, López FJ, et al. Real-time RT-PCR analysis for evaluating the Her2/neu status in breast cancer. Pathobiology 2010;77:38-45.
23. Barberis M, Pellegrini C, Cannone M, Quantitative PCR and HER2 testing in breast cancer: A technical and cost-effectiveness analysis. Am J Clin Pathol 2008;129:563-70.
24. Homaei Shandiz F, Fani A, Shakeri S, et al. Fluorescent in situ hybridization and real-time quantitative polymerase chain reaction to evaluate HER-2/neu status in breast cancer. Iran J Pathol 2017;12:67-73.
25. Park S, Wang HY, Kim S, et al. Quantitative RT-PCR assay of HER2 mRNA expression in formalin-fixed and paraffin-embedded breast cancer tissues. Int J Clin Exp Pathol 2014;7:6752-9.
26. Yoshizawa A, Sumiyoshi S, Sonobe M, et al. HER2 status in lung adenocarcinoma: A comparison of immunohistochemistry, fluorescence in situ hybridization (FISH), dual-ISH, and gene mutations. Lung Cancer 2014;85:373-8.