



HER2 CODON 655 POLYMORPHISM IN HER2 NEGATIVE LUNG CANCER PATIENTS – NEW PROPOSAL FOR ONE OLD DIAGNOSIS

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ABSTRACT

The significance of HER2 and Ile/Val single nucleotide polymorphism (SNP) of HER2 in lung cancer can be useful for predicting progression of this neoplasm.

Materials and Methods. We investigate immunohistochemically the expression of HER2 and single nucleotide polymorphism (SNP) in HER2 codon 655 we used RFLP – PCR analysis in 20 cases. We evaluated the correlation between the collected data and clinical and pathological parameters of investigated patients. The results were compared with clinical and pathological parameters of investigated patients.

Results. After analysis of the results we found that 100% of the cases were negative for HER2. HER2 RFLP - PCR analysis showed genotype AG in five patients (25%) and the rest 15 cases (75%) had AA (Ile/Ile) genotype. The analysis of SNP and the presence of lymph node metastasis showed tendency. Among the 20 patients with metastasis 80% had genotype AA (Ile/Ile) and 20% genotype AG (Ile/Val) ($\chi^2=2.857$; $p=0.091$).

Conclusion. Our results indicate that SNP in HER2 655 codon and investigation of HER2 expression could be helpful to outline the prognosis for lung adenocarcinomas.

Key words: lung adenocarcinoma, HER2/neu, HER2 codon 655,

INTRODUCTION

Lung cancer is most common cause of death from the neoplastic diseases [4]. Non-small cell lung cancer is the cause in up to 85% of cases and adenocarcinoma is the leading subtype among smokers in many countries [3]. The fact that the prognosis in these patients still remains poor with 5-year-survival rate in 15% of cases is particularly disturbing [6, 8].

Polymorphism of HER2 gene (HER2, located at 17q21.1) that results in the substitution of valine for isoleucine at codon 655 of the HER2 protein (Ile655Val, GTC > ATC; refSNP number, rs1136201) has been extensively investigated as risk

factor for different types of cancer [9]. This extensive body of evidence has been driven by an initial positive study that reported a strong association of the valine-encoding allele with breast cancer in an East Asian population from Shanghai, China but there is no data for the lung cancer [13].

We investigated immunohistochemically the expression of HER2 and single nucleotide polymorphism (SNP) in HER2 codon 655 we used RFLP – PCR analysis in 20 cases and evaluated the correlation between the collected data and clinical and pathological parameters of investigated lung cancer patients.

MATERIALS AND METHODS

Patients and materials

We investigated biopsy specimens collected from 20 patients who underwent lung resection in Clinical centre of Serbia, Belgrade, Serbia between 2000 and 2009. Eight of the patients are

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male and 12 female, with age from 46 to 68 years (58.8 years mean). Retrospective data of some clinical and histological findings was performed.

DNA extraction and genotype analysis

Genomic DNA was extracted from paraffin-embedded tissues of patients' tumor biopsies using a genomic DNA purification kit (GeneJet Genomic DNA Purification Kit, Thermo Scientific). Extracted DNA was stored at -20°C for further use. The concentration of resulting DNA was spectrophotometrically measured at 260 nm by NanoVue™ Spectrophotometer (GE Healthcare, Buckinghamshire, UK). Absorptions ratio at 260 vs. 280 nm was calculated to assess the purity of DNA samples.

Genotyping for the single nucleotide polymorphism at codon 655 in the HER2 gene was performed by restriction fragment length polymorphisms RFLP-PCR assay as previously described [19]. The PCR primers for amplification were as follows: 5'-AGA GCG CCA GCC CTC TGA CGT CCA T-3' (forward) and 5'-TCC GTT TCC TGC AGC AGT TCT CGC A-3' (reverse). Sample DNA (approx. 100ng) was amplified in 30µl of a reaction mixture, containing 1xPCR buffer, 0.4 µmol/L of each primer, 0.2 mmol/L dNTPs, 1.5 mmol/L MgCl_2 , and 1 unit of *Taq* polymerase (Thermo Scientific). The PCR cycle conditions consisted of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute. Final elongation was performed for 7 minutes at 72°C .

The gene specific primers amplify a 148 bp region of the gene. Amplification was checked electrophoretically on a 2% agarose gel. The PCR product was digested with 10 U of the restriction enzyme *Alw26I* in a total volume of 20 µl with 1xTango buffer (Thermo Scientific) at 37°C overnight. The fragments were separated on a 3% agarose gel and visualized with ethidium bromide. This restriction enzyme recognizes the sequence GTC, and therefore the G allele is cleaved by the enzyme giving rise to two fragments (116 and 32 bp), whereas the A allele is not cleaved by the enzyme. For all genotype analysis, laboratory personnel were unaware to subject status; agarose restriction fragment gels were interpreted by at least two

independent readers. In each PCR run, heterozygous control template was used to ensure accuracy.

Immunohistochemistry

Lung tissues from all 20 cases were immunohistochemically analysed. Tumor specimens were fixed in 10% buffered formalin, embedded in paraffin and cut to 4 µm thickness. Specimens were dewaxed and endogenous peroxidase was blocked for 5 minutes with blocking reagent according to the protocol. Then the slides were washed 3 times with PBS and incubated with primary antibody for 1 hour. After washing 3 times the slides were incubated with marked polymer and then washed again. In the last phase they were incubated with DAB substrate-chromogen and were washed again. At the end they were contrastained with Mayer's hematoxylin.

Following antibodies were used for the immunohistochemistry: antibodies c-erbB-2 oncoprotein (A0485, DAKO) as well as detection system EnVision™ FLEX+, Mouse, High pH, (Link) (K8002, DAKO). The analysis was performed according to the manufacturer's protocols.

Statistical analysis

The data was statistically analyzed by SPSS for Windows, Version 16 (SPSS, Inc., Chicago, IL, USA). Non-parametrical Mann-Whitney U test was used. Chi-squared test was used to compare the sampling distribution of investigated parameters. The accepted level of significance was set at $p < 0.05$.

RESULTS

We performed immunohistochemical analysis on 20 cases with lung adenocarcinoma for HER2 and analyse of HER2 expression showed that all 20 (100%) cases were negative and they were scored 0 (**Figure 1**).

HER2 RFLP - PCR analysis of 20 cases of lung adenocarcinoma showed presence of SNP in *HER2* gene codon 655 (**Figure 2**). Five (25%) of the cases had genotype AG (Ile/Val) and the rest 15 cases (75%) had AA (Ile/Ile) genotype.

The analysis of SNP and the presence of lymph node metastasis showed tendency. Among the 20 patients with metastasis 80% had genotype AA (Ile/Ile) and 20% genotype AG (Ile/Val)

($\chi^2=2.857$; $p=0.091$) (**Table 1**). No statistically significant correlation between it parameter and gender, age, smoking habits, tumor size, distant

metastasis, stage, the presence of necrosis and the desmoplastic reaction were noted.

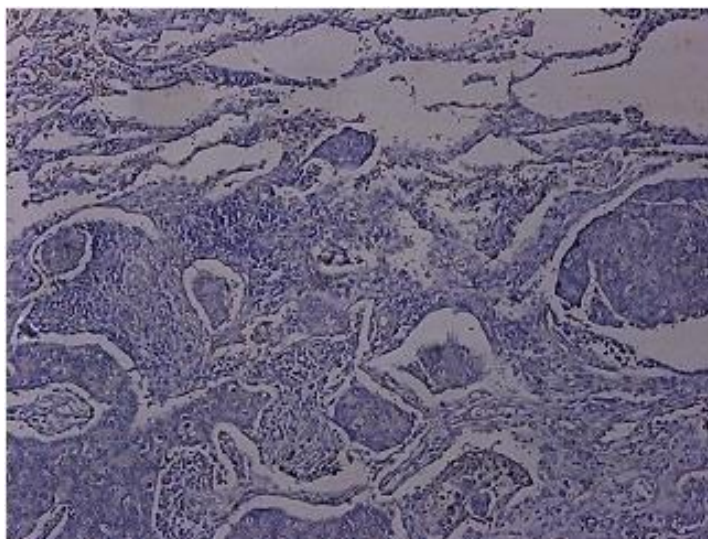


Figure 1. Representative image of HER2 negative Case of lung adenocarcinoma (score 0, x100).

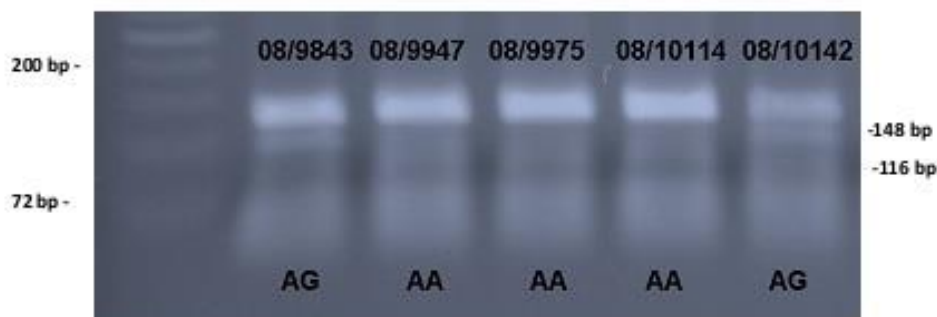


Figure 2. Representative image of SNP analysis in codon 655 of HER2 in lung adenocarcinomas.

Table 1. Correlation between genotype and lymph node metastases.

| HER2 SNP codon 655 | pN | |
|-----------------------|-----------|------|
| | N0 | N1-3 |
| AA | 3 | 12 |
| AG | 3 | 2 |
| | $p=0,091$ | |

DISCUSSION

We examined IHC expression of HER2 in 20 cases of lung adenocarcinoma, also in these cases RFLP - PCR analysis in order to find a single nucleotide polymorphism (SNP) in HER2 codon 655 was performed.

HER2/neu expression is investigated in different type of tumors. In breast cancer it is expressed in about 20 % of cases and usually is connected with poor outcome. The data for other tumor types such as lung cancer is performed on cell lines and/or are limited [1].

Hirsch et al. reported that in a series of non-small cell lung cancers 25% have moderate expression of HER2 and only between 6% and 8% have strong expression scored 3+ [5]. Later Takenaka described limited positive cases of lung adenocarcinomas and relation between stronger expression and unfavorable prognosis in patients [11]. The impact of expression could be analysed in process of determination of type of cancer treatment. It is known that in some cases, patients overexpressing HER2 are resistant to certain chemotherapeutic regimens, while the same patients have been proposed for therapy with monoclonal antibody – trastuzumab [2, 7]. Due to small number of cases we investigated, none of them showed HER2 expression which did not allowed to correlate it with the other clinicopathological parameters.

The biological significance of HER2 polymorphism is still not clear. The reports are rare and they are predominantly about codon 655 polymorphism in breast cancer. It is thought that alteration of codon 655 nucleotide A→G leads to substitution Ile→Val and this is the cause for structural and functional changes in HER2 protein [10]. Despite the lack of statistical significance our results show that the patients with lymph node metastasis more often have AA genotype. Wang researched the same type polymorphism among patients with bladder carcinoma and reported that patients with AA genotype are in more advanced stage in comparison to the size of the tumor but his results showed no connection with the tumor grade [12].

In resume - the lack of genotyping quality control to have influenced the results published to date and need to be addressed in the design of future studies.

REFERENCES

1. Bunn PA Jr, Helfrich B, Soriano AF, Franklin WA, Varella-Garcia M, Hirsch FR, et al (2001) Expression of Her-2/neu in human lung cancer cell lines by immunohistochemistry and fluorescence in situ hybridization and its relationship to in vitro cytotoxicity by trastuzumab and chemotherapeutic agents. *Clin Cancer Res.* 7(10):3239-50.
2. Calikusu Z, Yildirim Y, Akcali Z, Sakalli H, Bal N, Unal I, et al (2009) The effect of HER2 expression on cisplatin-based chemotherapy in advanced non-small cell lung cancer patients. *J Exp Clin Cancer Res.* 28:97.
3. Colby TV, Koss M, Travis WD (1995) Tumors of the Lower Respiratory Tract. 3rd ed. Armed Forces Institute of Pathology: Washington, DC.
4. Ferlay J, Shin HR, Bray F, et al (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer.* 127:2893-917.
5. Hirsch FR, Franklin WA, Veve R, Varella-Garcia M, Bunn PA Jr (2002) HER2/neu expression in malignant lung tumors. *Semin Oncol.* 29:51-8.
6. Jemal A, Clegg LX, Ward E, Ries LA, Wu X, Jamison PM, et al (2004) Annual report to the nation on the status of cancer, 1975-2001, with a special feature regarding survival. *Cancer.* 101(1):3-27.
7. Langer CJ, Stephenson P, Thor A, Vangel M, Johnson DH (2004) Eastern Cooperative Oncology Group Study 2598. Trastuzumab in the treatment of advanced non-small-cell lung cancer: is there a role? Focus on Eastern Cooperative Oncology Group study 2598. *J Clin Oncol.* 22(7):1180-7.
8. Melville A, Eastwood A (1998) Management of lung cancer. *Qual Health Care.* 7(3):170-7.
9. Millikan RC, Hummer AJ, Wolff MS, Hishida A, Begg CB. HER2 codon 655 polymorphism and breast cancer: results from kin-cohort and case-control analyses. *Breast Cancer Res Treat* 2005;89(3):309-12.
10. Papewalis J, Nikitin AY, Rajewsky MF (1991) G to A polymorphism at amino acid codon 655 of the human erbB-2/HER2 gene. *Nucleic Acids Res.* 19:5452.
11. Takenaka M, Hanagiri T, Shinohara S, Kuwata T, Chikaishi Y, Oka S, et al (2011) The prognostic significance of HER2 overexpression in non-small cell lung cancer. *Anticancer Res.* 31(12):4631-6.
12. Wang L, Habuchi T, Takahashi T, Kamoto T, Zuo T, Mitsumori K, et al (2002) No association between HER-2 gene polymorphism at codon 655 and a risk of bladder cancer. *Int J Cancer.* 97(6):787-90.
13. Xie D, Shu XO, Deng Z, Wen WQ, Creek KE, Dai Q, et al. Population-based, case-control study of HER2 genetic polymorphism and breast cancer risk. *J Natl Cancer Inst* 2000;92(5):412-7.