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The relationship of erbB family genes and chemosensitivity of Pemetrexed treated non-squamous NSCLC patients

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Abstract

To investigate the relationship between the expression of the ErbB family genes and the sensitivity of non-squamous NSCLC by Pemetrexed treatment, 93 non-squamous NSCLC patients diagnosed as clinical stage ⅢB or Ⅳ and received pemetrexed-platinum chemotherapy enroll in this research. The tumor tissue and peripheral blood samples were collected for detecting ErbB family genes (EGFR\HER2\ErbB3\ErbB4) expression by immunohistochemical method and RT-PCR, and the therapeutic effect of the treatment was analyzed. The EGFR expression in tumor tissues is positively correlated with that in peripheral blood ($p=0.037$), while no significant correlation was observed between HER2/ErbB3 expression in tumor tissues and that in peripheral blood ($p>0.05$). The expression of EGFR/HER2/ErbB3/ErbB4 shows no statistical significance between PD group and PR plus SD group (all>0.05). The mRNA level of EGFR/HER2 in blood may serve as potential indicators of chemosensitivity to pemetrexed in patients with advanced lung adenocarcinoma.

1. Background

Lung cancer has become the leading cause of cancer-related death in the world. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer patients, more than 70% of patients with non-small cell lung cancer at the time of diagnosis are in advanced stage (stage III or Ⅳ), most of them lost the opportunity to operation, and chemotherapy is often the first-line treatment for these patients [1].

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Pemetrexed, a new kind of multitarget antifolate metabolite anticancer drug, is an inhibitor of thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyl transferase (GARFT) and has broadspectrum activity in multiple tumor types. In August 2004, the U.S. food and drug administration (FDA) approve pemetrexed to treat locally advanced or metastatic NSCLC as second-line treatment. Various large phase Ⅲ clinical trial show pemetrexed has better curative effect for non-squamous non-small cell lung cancer than squamous cell carcinoma [2-3]. Based on the above research results, pemetrexed and cisplatin combination chemotherapy approved for first-line treatment of non-squamous NSCLC by FDA [2]. However, in clinical application and scientific research, pemetrexed shows obvious individual curative effect differences in non-squamous NSCLC. It is inadequate only by the pathological types to meet clinical individualized treatment. The previous researches of Pemetrexed resistance focused on enzymes in the folate metabolic pathway primarily, and some researches have already indicated that over-expression of TS and DHFR is associated with insusceptibility of Pemetrexed. The identification of resistance mechanisms remains critical to improving the effectiveness of Pemetrexed.

The ErbB receptor family is involved in cell proliferation and differentiation [4-6], and is one of the most extensively studied signal-transduction networks. It consists of four receptor tyrosine kinases: EGFR (ErbB1), HER2 (ErbB2), ErbB3, and ErbB4 [6-7]. In normal cells, the ErbB receptor pathway plays critical roles in the regulation of cell growth, tissue homeostasis, differentiation and motility. Aberrant activation of the ErbB receptors or cognate ligands by gene amplification, overexpression or mutation is as sociated with tumor growth and metastasis [8–10].

In our previous study we detected the binding of the Pemetrexed with ErbBs recptors at lowest energy, hydrogen bonding and space shape, by using computeraided drug design molecular docking techconiquche. In the in vitro experiments we established a Pemetrexed-resistant lung adenocarcinoma cell line and investigated the relationship between ErbB family genes expression and PEM-resistance. The result showed some differences of the biological characteristics compared to the parental strain. ErbB family genes expression appeared to be associated with resistance to Pemetrexed in lung adenocarcinoma. Based on this finding, we hypothesis that the ErbBs family genes may influence the chemosensitivity of Pemetrexed on non-squamous NSCLC.

2. Materials and methods

2.1 Samples

Ninety-three patients with a confirmed histologically diagnosis of non-squamous NSCLC who had not undergone chemo or radiotherapy were recruited between 2011 and 2013 from The Affiliated Hospital of Medical College Qingdao University. All patients signed a consent form. The project was approved by the Ethic Committees.

From those patients 3 ml of peripheral blood were collected, total RNA was extracted using the TRIzolW (Invitrogen, USA) reagent following the protocol described by the manufacturer. All RNA samples were quantified by spectrophotometry and their integrity was evaluated by formaldehyde-agarose gel electrophoresis. The quality of the RNA samples was determined by the ratio of the 28S, 18S and 5.8S ribosomal RNA bands.

Thirty-five biopsies of percutaneous lung biopsy tissue fixed in formaldehyde and embedded in paraffin were previously analyzed by immunohistochemical (IHC) method.

2.2 Quantitative reverse transcriptasepoly-merase chain reaction (qRT-PCR)

Expression of mRNA for ErbBs family was assayed by qRT-PCR. Total RNA was reverse transcribed into cDNA using a standard oligo-dT RT protocol. cDNA samples were used as template for qRT-PCR analysis. SYBRII (Takara

Bio, Tokyo, Japan) was used according to the manufacturer's instructions. The EGFR primers forward: 5'-GAGGGTGAGCCAAGGGAGTTTG-3', reverse: 5'-GGCAGGTCTTGACGCAGTGG-3'. HER2 primers forward: 5'- TGTGACTGCCTGTCCCTACAA-3', reverse: 5'-CCAGACCATAGCACACTCGG-3'; ErbB3 primers forward: 5'-GACCCAGGTCTACGATGGGAA-3', reverse: 5'-GTGAGCTGAGTCAAGCGGAG-3'; ErbB4 primers forward: 5'-GCAGATGCTACGGACCTTACG-3', reverse: 5'- GACACTGAGTAACACATGCTCC-3'. The housekeeping reference gene GAPDH primers GAPDH, forward primer, 5'-CTGCACCACCAACTGCTTAG-3', and reverse primer, 5'-TGAAGTCAGAGGAGACCACC-3'. The protocol was 95℃ for 1 min, then 40 cycles of 95℃ for 15sec, 60℃ for 60 sec and 72℃ for 1 min.

After the reaction, ErbB family genes mRNA expression was normalized by the expression of GAPDH. The mRNA relative quantitation was done using the ΔCt method. The parameter Ct (threshold) was defined as the number of cycles in which the fluorescence exceeded the previously set threshold. The difference (ΔCt) between the average (three experiments) of the gene of interest and the housekeeping gene (GAPDH) was calculated using the software Microsoft Excel.

2.3 Immunohistochemical(IHC)

Immunohistochemistry was performed on paraffin sections of 35 Non-squamous NSCLC cases to assess ErbB family protein expression.

EGFR, HER2 and ErbB3 Tissue samples were fixed in 10% neutral formalin and desiccated and embedded in paraffin and sectioned to 3μm. The antibody dilution and process of staining were performed according to the instructions. PBS was used as a negative control to primary antibodies. The results were observed under microscope.

EGFR and HER2 protein expression were considered positive if only cell membrane staining, the staining of ErbB3 were mainly localized in cytoplasma. Cells were categorized according to the positive rate: negative(-), the number of positive cells < 5%, weak positive (+), pale brown particles, the number of positive cells 5%-25%, positive (++), brown particles, the number of positive cells 25%-50%, strong positive (+++), dark brown particles, the number of positive cells > 50%.

2.4 Treatment and Efficacy

All of patients received the standard treatment consisting of pemetrexed and cisplatin. Treatment was repeated every three weeks. Treatment was continued until disease progression, the appearance of unacceptable toxicity and patient's withdrawal of consent, for a maximum of 4 cycles. Patient's responses to treatment were assessed after two cycles of chemotherapy by Response Evaluation Criteria in Solid Tumors [10], which classified the response into four categories. Complete response (CR) was defined as the disappearance of all target lesions; partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameter of target lesions, taking as reference the baseline sum longest diameter; stable disease (SD) was defined as neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease, taking as reference the smallest sum longest diameter since the treatment started; and progressive disease (PD) was defined as at least a 20% increase in the sum of the longest diameter of target lesions, taking as reference the smallest sum longest diameter recorded since the treatment started, or the appearance of one or more new lesions.

2.5 Statistical analysis

For data analysis, complete response and partial response were combined as responders, and stable disease and progressive disease were grouped as non-responders.

The mRNA and protein expression levels in the responders and non-responders were compared and analyzed by the nonparametric Mann-Whitney U test. The relationship of mRNA and protein expression was analyzed by the Spearman's correlation test. Differences were judged as statistically significant when p-values

(two-tailed) were < 0.05. All statistical analyses were performed with SPSS 19.0 software (USA).

3. Result

3.1 Study population

Nighty-nine patients, mostly males (63.4%), with a median age of 63 years at diagnosis, were included in the present analysis. As shown in Table 1, the sensitivity of pemetrexed-platinum was not related to age, sex, and smoking status (all > 0.05).

Table 1 shows key baseline clinicopathologic variables. In brief, all patients had adenocarcinomas; and were confirmed as EGFR-wild-typemost. [n (%)]

Clinical parameter	No	PR	SD	PD
Total	93	18(19.4)	52(55.9)	23(24.7)
＜60	37	9(24.3)	21(56.8)	7(18.9)
⩾60	56	9(16.1)	31(55.4)	16(28.5)
Male	59	11(18.6)	32(54.2)	16(27.1)
Female	34	7(20.6)	20(58.8)	7(20.6)
Ever	48	8(16.7)	28(58.3)	12(25.0)
Never	45	10(22.2)	24(53.3)	11(24.4)
ⅢB	20	5(25.0)	11(55.0)	4(20.0)
Ⅳ	73	13(17.8)	41(56.2)	19(26.0)

3.2 The expression levels of blood EGFR/ HER2/ErbB3 in negative group and sensitive group detected by qRT-PCR

Using GADPH as an internal reference, the expression level of EGFR/HER2/ErbB3 was detected by qRT-PCR, while ErbB4 was not detected from all of samples. The quantitative PCR amplification and melting curves reveal the expression levels of ErbB family genes and GADPH (Figure 1). The results show that the Ct value of GADPH is about 22, the Ct value of EGFR/HER2/ErbB3 are about 32/29/27, and the Tm is about 85℃. Impure or abnormal broaden peaks do not appear in the figure, indicating that there is no contamination, primer dimmers, or nonspecific amplification.

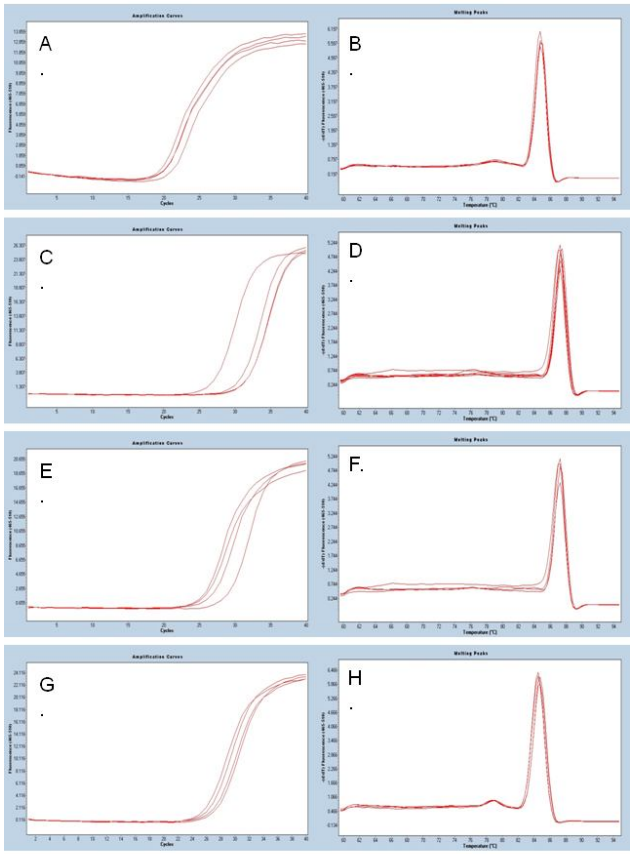
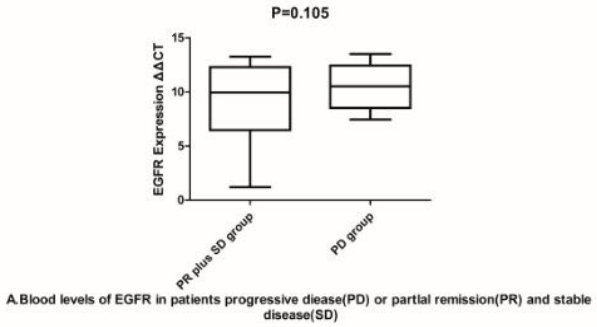


Figure 1 The amplification and melting curves of EGFR/HER/ErbB3

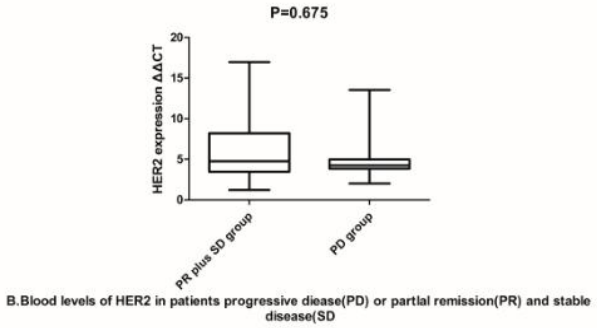
A, amplification curve of GADPH; B, melting curve of GADPH; C, amplification curve of EGFR; D, melting curve of EGFR; E, amplification curve of HER2; F, melting curve of HER2; G, amplification curve of ErbB3; H, melting curve of ErbB3. Quantitative fluorescence amplification curves show the relationship between fluorescence and the number of cycles. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportionate to the amount of target nucleic acid in the sample: the lower the Ct level, the greater

the amount of target nucleic acid in the sample. Melting curve is the quality control of amplification. It is used to analyze the homogeneity of PCR products.

3.3 Relationship between EGFR/HER2/ ErbB3 expression of blood and chemotherapeutic efficacy



A.Blood levels of EGFR in patients progressive diease(PD) or partlal remission(PR) and stable disease(SD)



B.Blood levels of HER2 in patients progressive diease(PD) or partlal remission(PR) and stable disease(SD

Figure 2 Blood level of EGFR/HER2/ErbB3 in patients with progressive diease (PD) or partlal remission (PR) and stable disease (SD)

Expression levels of the EGFR/HER2/ErbB3 are normalized to GADPH. Statistically differences were determined using the Mann-Whitney U test. A. Blood level of EGFR trend to be higher in PD samples than in PR plus SD samples (P=0.105). The quantity of EGFR in blood may be a potential biomarker for predicting platinum-pemetrexed chemosensitivity of NSCLC. B. Blood level of HER2 was no statistical significance between PD group and PR plus SD group (P=0.675).

Ninety-three patients with advanced NSCLC who underwent 2 to 4 cycles of platinum-pemetrexed chemotherapy were followed up: 18 had PR, 52 had SD, 23 had PD (six patients only received 2 cycles chemotherapy because of disease progression), and none achieved CR after chemotherapy. The expression of EGFR shows the trend of being higher in the PD group than in the PR plus SD group (P= 0.105), whereas no statistical significance was found in EGFR/HER2/ ErbB3 between PD group and PR plus SD group (all> 0.05) (Figure2), suggesting that EGFR expression may correlate with sensitivity to chemotherapy in NSCLC. In view of limitations of sample size, large-scale clinical studys are necessary to validate our results.

3.4 The protein expression of tumor tissue of EGFR/HER2/ErbB3 detected by immune-histochemical

Results of IHC staining were obtained for 35 of tumor samples for three markers (EGFR, HER2 and ErbB3). The pattern of immunostaining for EGFR and HER2 was predominantly membranous and EGFR was moderate to strong positive in 57.1% of cases (Figure 3A), while HER2 was moderate to positive in 22.9% of cases (Figure 3B). On the contrary, ErbB3 immuno-staining was mostly cytoplasmic (Figure 3C), and was absent in 88.6% of cases. These findings urged us to further explore the EGFR membranous IHC expression in relation to clinicopathological variables.

3.5 Correlation of EGFR/HER2/ErbB3 IHC expression with mRNA levels

We observed a significant trend for co-expression of EGFR membranous staining in tumor tissues and EGFR mRNA levels in blood, as evaluated with the Spearman's correlation test (p= 0.037), while there is non-significant trend for co-expression of HER2/ErbB3 membranous or cytoplasmic staining in tumor tissues and HER2/ErbB3 mRNA levels in blood (p= 0.234/ 0.784).

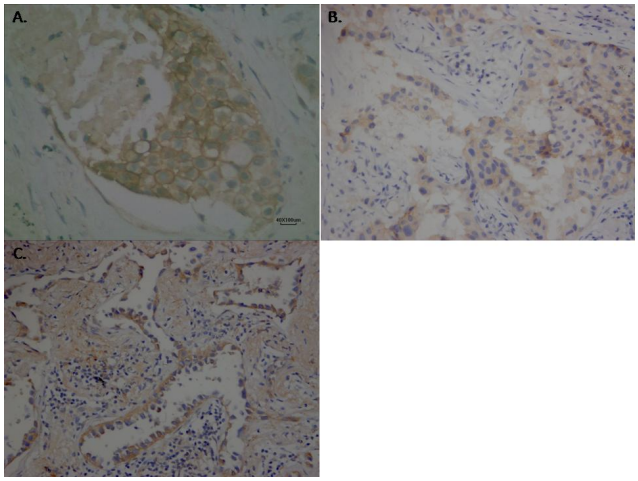


Figure 3. Illustrative examples of immunehisto-chemical expression. A. EGFR strong membraneous staining in cancer cells; B. HER2 strong membraneous staining in cancer cells; C. ErbB3 cytoplasmic staining in cancer cells. All pictures: magnification X 40.

4. Discussion

In order to identify predictors for sensitivity or resistance against pemetrexed, various researches have correlated the antitumor activity with expression data of genes well known in the molecular pathways of the compound. Several researches showed that low levels of mRNA expression of TS, GARFT, and DHFR correlated significantly with chemosensitivity to pemetrexed.

Although previous retrospective data suggest that TS and FPGS expression might be potential markers of Pemetrexed efficacy in many types of cancer, Lustgarten DE's data indicate TS and FPGS lack sufficient predictive value in individual patients and should not be used to guide therapeutic decisions in the absence of prospective studies [11]. The present study shows that lung cancer multidrug resistance involves a variety of mechanisms, any single mechanism cannot reasonably explain the primary and secondary drug resistance of lung cancer.

The human epidermal growth factor receptor (HER) family of tyrosine kinases is deregulated in multiple cancers either through amplification, overexpression, or mutation. It plays a critical role in normal development, forming homodimers or heterodimers, and triggering downstream signaling cascades controlling proliferation, cell survival, and apoptosis [12].The gene polymorphisms or mutations might also influence the response to Pemetrexed. Although the patients with EGFR mutations respond to TKIs dramatically, the second-site point mutation of the EGFR is major cause of acquired resistance to TKIs [13, 14]. Now many scholars began to focus on the relationship between EGFR gene copy and efficacy of TKIs treatment.

In this study, we examined the influence of ErbB family genes on the response to platinum-pemetrexed chemotherapy for advanced NSCLC patients. We found that patients with lower expression of ErbB family genes tended to show a better response to platinum-pemetrexed chemotherapy NSCLC in the recessive model. Cappuzzo et al. [15] suggested that EGFR gene copy increase might be more valuable than EGFR mutations in prognosis of NSCLC with TKIs treatment. Another research showed that gefitinib treatment leaded to increase of ErbB3 mRNA levels [16]. Hsu YC et al. [17] mentions that cells with lower expression of genes, such as EGFR and ITGA3, tend to be more resistant to tubulin-binding agents but they also tend to be more sensitive to the three-target therapy drugs. Some clinical studies, investigated the influence of HER2 expression on curative effect of cisplatin-based chemotherapy in advanced NSCLC patients, showed reduced median overall survival and a lower response to cisplatin-based chemotherapies in HER2-positive patients in comparison to the HER2-negative patients, although cisplatin-based combination chemotherapies are the standard treatment for NSCLC [18]. These results suggest that EGFR family genes status seem to be both a predictive and a prognostic factor for chemotherapy response and disease survival.

High expression of EGFR/HER2 correlated with the inhibition effect of DNA repair pathway activity, down-regulation of apoptosis and ultimately giving rise to multidrug resistance. The major signal transduction pathways that ultimately result in proliferative signals to the cell nucleus include: Ras/Raf/MEK/ERK/MAPK, PI3K/PDK1 /Akt (PKB), PLC-γ and JAK/STAT pathways [19, 20]. The phosphatidyrlinositol 3-kinase/protein

base B (PI3K/Akt) is involved in a variety of tumor biological markers as a classic survival signaling pathway of anti-apoptotic and promoting survival. This pathway plays a major role not only in tumor development but also in the tumor potential response to chemotherapy. It is in recent studies that sustained activation of the PI3K/Akt pathway is related to acquire resistance to chemotherapy [21]. The over- expressions of ErbB family genes and activity of these signaling pathways might play a critical role in the resistance of lung adenocarcinoma cells to Pemetrexed.

In Norimasa Miura' research [22], it showed the expression of EGFR mRNA in tumor tissue was positively correlated with EGFR protein in plasma. In our research, we found the EGFR mRNA levels in blood have a closely relative correlation with the expression of ErbB family genes in tumor tissue, while there is non-significant trend for co-expression of HER2/ErbB3 mRNA levels in blood and tumor tissue. ErbB family genes' mRNA is derived from tumor tissues or circulating tumor cells in the peripheral blood. Detection of ErbB family genes' mRNA in peripheral blood may replace that in tumor tissue, providing a quicker and easier examination method for the patients lacking biopsy tissue.

Overall, preclinical data and clinical studies provide vast quantities of evidences that EGFR and HER2 play a possible role in chemoresistance of lung cancer. Although there is barely known about the role of ErbB3 and ErbB4 in chemoresistance. Because the sample size is less, in this study, the mRNA levels of EGFR family genes have no obvious difference between effective and negative group. It's still necessary to enlarge sample size for further study. Moreover, the mechanism of up-regulation the expressions of EGFR family genes requires further investigations. In view of limitations of experiments, further clinical studies are necessary to validate our results.

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