Assessment of K–ras Gene Mutation after Neoadjuvant Chemotherapy in Patients with Stage ⅢA non–small Cell Lung Cancer

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Abstract **Objective** To assess the state of K-ras gene mutation after neoadjuvant chemotherapy in patients with stage ⅢA non-small cell lung cancer (NSCLC). **Methods** Twenty four patients with stage ⅢA NSCLC received treatment of operation after two cycles neoadjuvant chemotherapy. Histopathologic response in resection specimens was observed. Assessment of K-ras genes mutation in resection specimens was performed by polymerase chain reaction single strand conformation polymorphism (PCR- SSCP) analysis. **Results** Clinical effectine rate of chemotherapy was 41.7% (10/24). Surgery resection rate was 95.8% (23/24). The rate of marked histopathologic response (tumor regression grade Ⅳ-Ⅲ) for chemotherapy was 43.5%. Nine mutation (39.1%) of K-ras gene in resection specimens of 23 patients were found. The rate of K-ras mutation (20.0%) in patients who had tumor regression grade Ⅳ-Ⅲ was significantly lower than those (53.8%) in patients with histologic response grade Ⅱ - Ⅰ for chemotherapy (P<0.05). The rate of K-ras mutation was 20.0% in patients who achieved complete resection of tumor, as compared with 75.0% in patients who had incomplete resection of tumor (P<0.05). **Conclusion** The lower rate of K-ras gene mutation in patients who had marked histologic response (grade Ⅳ-Ⅲ) for chemotherapy may be a result of effective neoadjuvant chemotherapy, eliminating tumor compartments with mutation K-ras gene. The presence of K-ras gene mutation may not be of impact on effect of neoadjuvant chemotherapy and patients prognosis. Farther study of state of K-ras gene mutation after neoadjuvant chemotherapy in patients with NSCLC is needed.

Key Words Non-small cell lung cancer; Neoadjuvant chemotherapy; K-ras gene; Mutation

Approximately 25% to 30% of non-small cell lung cancer (NSCLC) patients present with stage Ⅲ disease, which is defined as locally advanced tumor confined to the chest without distant metastasis. Stage ⅢA patients undergoing surgery had a 5-year survival of 15%~20%[1]. Extrathoracic disease recurrence makes the possibility of surgical cure unlikely in this group. Combined-modality therapy employing systemic (chemotherapy) and local (surgery or radiotherapy) approaches has shown favorable results in patients with stage Ⅲ disease. The results of numerous randomized trials demonstrate that neoadjuvant (induction) chemotherapy followed by surgery may improve outcome compared to that achieved with surgery alone[2-5]. A randomized phase Ⅲ trial publised by Rosell et al shown that the low survival rate in the surgery-alone group may be associated with the higher incidence of K-ras gene point mutation in this group (42%) vs the neoadjuvant chemotherapy group (15%)[2]. Hence, it is possible that K-ras gene mutation in the group of surgery alone is responsible for the observed outcome differences rather than a beneficial effect of the neoadjuvant chemotherapy itself. This study was designed to treat the patients with stage ⅢA NSCLC by neoadjuvant chemotherapy followed surgery, then, to examined the mutational state of K-ras gene in resection specimens. The purpose of the study was to assess the possible impact of K-ras gene mutation on neoadjuvant chemotherapy in patients with stage ⅢA NSCLC.

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MATERIAL AND METHODS

Patients

During the period from February 1999 to April 2002, 24 patients with stage ⅢA NSCLC were entred into our study. Among them, 16 were males, 8 females, mean age 58.4±10.3 years old ranging from 48 to 69 years. All patients were diagnosed by histologic examination of biopsy material or cytologic examination of percutaneous needle aspiration specimen or bronchoscopic brushing or washings. Among them 13 were adenocarcinoma, 8 squamous cell carcinoma, 2 adenosquamous cell carcinoma, large cell carcinoma 1. Initial clinical evaluation included a medical history, physical examination, chest X-ray, CT scan of chest, brain and abdomen, pulmonary function, electrocardiogram, measurement of blood biochemical analysis. All patients were staged ⅢA NSCLC according to the International

Staging System for Lung Cancer, 1997 revision[6]. Eligibility criteria included a favorable medical condition (a karnofsky performance score of 80 or higher); sufficient bone marrow reserve (leukocytes>4000×10^9 and thrombocytes>100000×10^9); normal renal and hepatic function, adequate pulmonary function(a predicted postoperative FEV1 greater than 1.0L).

Treatment, response assessment, and follow-up

All patients received neoadjuvant chemotherapy regimen consisting of mitomycin (8mg/m2 day 1), vindesine (3mg/m2 days 1, 8), and cisplatin (60 mg/m2 day 1), or etopside (80 mg/m2 days 1-4) and cisplatin (60 mg/m2 day 1) at three to four weeks intervals for two cycles. Intensive antiemetic therapy and hydration was administered as required.

Approximately 4 weeks after completion of neoadjuvant chemotherapy, clinical response was evaluated with a second CT scan of chest. Conventional response definitions were used for this CT scan-based clinical response assessment. A complete response (CR) was defined as the total disappearance of all radiographic evidence of tumor. A partial response (PR) was defined as any response less than complete, but with greater than 50% reduction in the sum of the products of the crossed diameters of all measurable lesions. Patients with less tumor shrinkage were considered stable disease (SD). Progressine disease (PD) was defined as greater than a 25% increase in the sum of the products of the crossed diameters of all measurable lesion. A thoracotomy was performed in four to five weeks after 2 cycles neoadjuvant chemotherapy. After postoperative recovery, patients were referred for adjuvant chemotherapy. A chemotherapy regimen identical to the pretreatment regimen was given for 4 to 5 cycles. Chemotherapy-related toxicity was observed and recorded.

Follow-up examination were taken every 3 months for the first 2 years and all 6 months thereafter.

Histopathologic assessment

The degree of histopathologic response was determined by assessing the extent of tumor necrosis, as described by Junker et al[7], somewhat revised by us. Grade Ⅳ: complete tumor regression with no evidence of vital tumor tissue in the section of surgical specimen; Grade Ⅲ: morphologic evidence of therapy-induced tumor regression with < 20% residual tumor cell in the section of surgical specimen presenting focal disease; Grade Ⅱ: tumor regression with at least 20% residual tumor cell in the section of specimen presenting more than focal microscopic disease; Grade Ⅰ: no tumor regression or only spontaneous tumor regression in the section specimen.

Detection of K-ras gene mutation with PCR-SSCP

DNA preperation Resection tumor specimens were finely minced and suspended in 500μl DNA lysis solution. The mixtures were incubated at 55℃ for 2h, heated at 95℃ for 10min, and centrifuged briefly. After phenol /choroform extraction, the DNAs were precipitated with 50μl isopropanol. DNAs were washed once with 70% ethanol and resuspended in 100μl of filtered H_2O. Finally, DNA content were determined by fluorimetry.

PCR-SSCp and nucleotide sequencing PCR-SSCP sequencing analysis was performed according to the technique described by Cho et al[8]. DNA was amplified by polymerase chain reaction(PCR). Reaction were generally performed in a volume of 50μl and were comprised of 0.2μg DNA sample, 200μM dNTPS, 5μl PCR reaction buffer, 1.5mM $MgCl_2$ and 2.5μ Taq polymerase, two primers (K-ras / 12, 13; 5' - ATTATAAGGCCTGCTG - 3', 5' - CCTGCACCAGTAATATGC - 3'), the samples were overlaid with paraffin oil and subjected to 35 cycles of amplification. The standard temperature profile of the cycles was as followed: denaturation at 94℃ for 1min, annealing at 55℃ for 1 min, and extension at 72℃ for 30 seconds. The SSCP analysis was performed as followed: PCR products were diluted in loading buffer (95% formanide, 20mM EDTA, 10mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol blue), boiled for 5 minutes, and rapidly cooled on ice. Samples were run at 150 Volt for 5h on 6% acrylamide gels with 10% glycerol at 4℃. Autoradiography against radiographic film with intensifying screens was performed at - 70℃.

Statistical analysis

Comparisons between patients with K-ras mutution and without K-ras mutation on the median survival were made on the student t test. Difference in the K-ras mutation and 3 - year surrival rate were evaluated by the Chi square test. A p ralue of less than 0.05 was considered as statistically significant.

RESULTS

Clinical response and toxicity

All 24 patients received the planned 2 cycles of neoadjuvant chemotherapy. Among them, one patient (4.2%) had CR, 9 (37.5%) had PR, and 13 patients (54.2%) demonstrated SD, only 1 patient had evidence

of PD. Total response rate (CR+PR) was 41.7%. The main chemotherapy-related toxicity was gastrointestinal reactions (45.8%), such as nausea, vomiting and diarrhea, neutropenia (25.0%), thrombocytopnea (16.7%), and abnormal hepatic function tests (12.5%). It was entirely manageable to chemotherapy-related toxicity without treatment interruption.

Surgical result

All 24 patients underwent operation in 4 weeks after chemotherapy. Twenty three patients (95.8%) received surgical resection, 15 cases (62.5%) had complete resection, 8 (33.3%) had incomplete resection, in one case, the tumor was not resectable because of encasement of the great vessels. Operation included lobectomy (14 cases), lobectomy plus other segmental lobe resection (3 cases), lobectomy plus bronchoplastic sleeve resection (3 cases), and complete pneumonectomy (3 cases).

Histopathological assessment tumor specimens in 23 patients who received surgical resection shown that 1 (4.3%) was histologic response grade Ⅳ, 9 (39.1%) were grade Ⅲ, 10 (43.5%) shown grade Ⅱ. In 3 specimens (13.0%), no evidence of chemotherapy-induced tumor histological response (grade Ⅰ) was found. The rate of marked histopathological response (grade Ⅳ-Ⅲ) was 43.5% (10/23), among them, adenocacinoma were 4, squamous cell carcinoma 5, adenosquamou cell carcinoma 1. All 10 patients with histopathologic response grade Ⅳ and Ⅲ achieved complete resection. Only 5 of 13 patients with tumor regression grade Ⅱ-Ⅰ had complete resection.

Detection of K-ras gene mutation.

PCR-SSCP analysis is based on the fact that the electrophoretic mobility of single stranded nucleic acids depends not only on size but also on its sequence in nondenaturing polyacrylamide gels. In 23 resection tumor specimens, 9 K-ras mutation (39.1%) were confirmed by direct nucleotide sequencing. All 9 mutation were at codon 12 of the K- ras gene. Mutation were present in 6 of 12 adenocarcinomas (50.0%), 2 of 8 squamous cell carcinomas (25.0%), 1 of 2 adenosquamous cell carcinomas (50.0%). Rate of K-ras mutation was 20.0% (2/10) in tumor regression grade Ⅳ-Ⅲ as compared to 53.8% (7/13) in histopathologic response grade Ⅱ-Ⅰ ($p<0.05$) (table 1). Rate of K-ras mutation in patients who achieved complete resection was 20.0% (3/12), compared with 75.0% (6/8) in patients who had incomplete resection ($p<0.05$) (table 2).

Table 1. Comparison of K-ras gene mutation between tumor regression grade Ⅳ-Ⅲ and grade Ⅱ-Ⅰ.

K-ras mutation status	n	grade Ⅳ-Ⅲ (%)	grade Ⅱ-Ⅰ (%)
K-ras mutation	9	2(20.0)	7(53.8)*
no K-ras mutation	14	8(80.0)	6(46.2)

$p<0.05$, vs grade Ⅳ-Ⅲ

Table 2 Comparison of K-ras mutation between patients with complete resection and incomplete resection.

K-ras mutation status	n	complete resection (%)	incomplete resection (%)
K-ras mutation	9	3(20.0)	6(75.0)*
no K-ras mutation	14	12(80.0)	2(25.0)

$p<0.05$, vs complete resection

Follow-up and survival

After neoadjuvant chemotherapy and surgery, all 23 patients were followed up. The median follow-up period was 40.5 months, the median survival duration were 23.5 months, 3-year survival rate was 30.4%. The median survival duration for patients with K-ras gene mutation were 15.6 months as compared to 35.8 months in without K-ras gene mutation ($p<0.05$). The 3-year survival rate was 22.2% (2/9) for patients with K-ras gene mutation, 35.7% (5/14) for patients without K-ras gene mutation, the difference of 3-year survival rate between these patients were not statistically significant ($p>0.05$).

DISCUSSION

The concept of neoadjuvant chemotherapy, surgical resection performed after chemotherapy, has gained widespread acceptance for patients with ⅢA NSCLC because the results from numberous trials have demonstrate the feasibility of this approach and have suggest that survival rates are improved [1-5]. Neoadjuvant chemotherapy is considered as a cytoreductive therapy administered prior to surgical resection. The intent of cytoreductive therapy is to downstage primary tumor and thence increase the resectability rate[9]. Early administration of chemotherapy may eradicate micrometastasis, decrease relapse, improve overall survival, and allow accurate pathologic assessment of response to chemotherapy. Two randomized trials had indicated that the survival rate of patients with stage $ⅢA$ disease is significantly better in association with neoadjuvant chemotherapy and surgical resection than that with resection alone[2-5]. In a trial Rosell et al performed by mutation K-ras gene was found in 3 out of 20 patients (15%) in the chemotherapy-surgery group as opposed to 10 out of 24 (42%) in the surgery alone group[2]. We

want to known whether K-ras mutation status influences the effect of chemotherapy or chemotherapy could reverse the expression of K-ras gene, and prognostic impact of a K-ras gene mutation on patients with stage ⅢA NSCLC receiving chemotherapy follow by surgery.

The ras gene family comprise three genes: K-ras, H-ras and N-ras encoding membrancebound proteins $p21^{ras}$. By a point mutation in codons 12 (80% of all K-ras mutation), 13 and 60, the oncogene acquires transforming potential. The amino acid change alters the resulting $P21^{ras}$ protein configuration and stimulate cell growth and differentiation autonomously[10]. Early studies suggested that K-ras gene mutations were an important negative prognostic factor, and were associated with tumor progression and shortened survival in patients with NSCLC. However, several more recent and larger studies cast doubt on this[11]. Therefore, these analyses on the prognostic impact of K-ras gene mutation are still controversy.

Our study showed that K-ras gene mutation occured more frequently in patients with a minor extent of histopathologic response (grade Ⅱ-Ⅰ) compared with patients will histopathologic response grade Ⅳ-Ⅲ, and in patients with incomplete resection as compared to patients with complete resection. It seems to be difficulty to confirms that an inhomogeneous distribution of mutated K-ras gene have multidrug resistance contributed to these different result, or the lower K-ras mutation rate in patients with marked histopathologic respone (grade Ⅳ-Ⅲ) was a result of effective neoadjuvant chemotherapy, eliminating tumor compartment with mutated K-ras gene. Experimental study found that several NSCLC cell lines were tested for resistance against different cytotoxic agents without any association between ras gene mutation and resistance[12]. In our study, absence of tumor cell or the presence of significant areas of tumor necrosis were seen throughout resection specimens in patients with histopathologic response grade Ⅳ-Ⅲ. Hence, lower rate of K-ras gene mutation in patient with tumor regression grade Ⅳ-Ⅲ could be a result of effective chemotherapy eliminating tumor cells with mutated K-ras gene. Shorter median survival in patients with K-ras gene mutation in addition to could be impact of K-ras gene mutation itself, other important factor was the rate of surgical complete resection in patients without K-ras mutation were markedly higher than that in patients with K-ras mutation. The survival benefit in patients with complete resection after neoadjuvant chemotherapy had been confirmed[9].

In conclusion, our study data and the results of the current analysis have indicated that the neoadjuvant chemotherapy is acceptable to eligible patients with stage ⅢA NSCLC. The rate of K-ras gene mutation in surgical specimens with tumor regression grade Ⅳ-Ⅲ was significantly lower than that with histophathologic response grade Ⅱ-Ⅰ, it may be a result of effective preoperative chemotherapy eliminating tumor cell with mutated K-ras gene. There are no sufficient data to conclude that K-ras gene mutation make an impact on neoadjuvant chemotherapy in patients with NSCLC. The further evaluation of K-ras gene mutation in study on neoadjuvant chemotherapy is needed.

REFERENCES

1. Belani CP and Ramanthan RK. Combined-modality treatment of locally advanced non-small cell lung cancer. Chest, 1998, 113 (suppl 1): 53s - 60s.
2. Rosell R, Gomez-Codina J, Camps C, et al. A randomized trial comparing preoperative chemotherapy plus surgery with surgery alone in patients with non-small-cell cancer. N Engl J Med, 1994, 330 (3): 153-158.
3. Roth JA, Fossella F, Komake R, et al. A randomized trial comparing perioperative chemotherapy and surgery with surgery alone in resectable stage Ⅲa non-small cell lung cancer. J Natl Cancer Inst, 1994, 86(2): 673-680.
4. Rosell R, Gomez-Codina J, Camps C, et al. Preresetional chemotherapy in stage ⅢA non-small cell lung cancer: a 7-year assessment of a randomized controlled trial. Lung Can cer, 1999, 26(1): 7-14.
5. Roth JA, Atkinson EN, Fosella F, et al. Long-term follow-up of patients enrolled in a randomized trial comparing perioperative chemotherapy and surgery with surgery alone in resectable stage Ⅲa non-small cell lung cancer. Lung Cancer, 1998, 21 (1): 1-6.
6. Mountain CF. Revisions in the international system for staging lung cancer. Chest, 1997, 111(5): 1710~1717.
7. Junker K, Langner K, Klinke F, et al. Grading of tumor regression in non-small cell lung cancer. Chest, 2001, 120 (5): 1584-1591.
8. Cho JY, Kim JH, Lee YH, et al. Correlation between K-ras gene mutation and prognosis of patients with nonsmall cell lung carcinoma. Cancer, 1997, 79(3): 462-467.
9. Strauss GM. Role of chemotherapy in stage Ⅰ to Ⅲ non-small cell lung cancer. Chest, 1999, 116 (suppl 6): 509s-516s.
10. Bos JL. The ras gene family and human carcinogenesis. Mutal Res, 1988, 195(3): 255~262.
11. Graziano SL, Gamble GP, Newman NB, et al. Prognostic signification of K - ras codon 12 mutations in patients with resected stage Ⅰ and Ⅱ non-small-cell lung cancer. J Clin Oncol, 1999, 17(2): 668-675.
12. Tsai CM, Chang KT, Perng Rp, et al. Correlation of intrinsic chemoresistance of non-small cell lung cancer cell lines with HER-2 /neu gene expression but not with RAS gene mutation. J Natl Cancer Inst, 1993, 85: 897-901.