A fully integrated, automated and rapid detection system for *KRAS* mutations

NORIO URESHINO[1,2], NAOKO SUEOKA-ARAGANE[1], TOMOMI NAKAMURA[1], AKEMI SATO[1], KAZUTOSHI KOMIYA[1], KENTARO IWANAGA[1,2], MASAHIRO MITSUOKA[3], YUJI TAKEDA[3], SHINICHIRO HAYASHI[1], EISABURO SUEOKA[4] and SHINYA KIMURA[1]

[1]Division of Hematology, Respiratory Medicine and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, Saga 849-8501; [2]Department of Medical Oncology, Saga Prefectural Hospital, Koseikan, Saga 840-8571; [3]Department of Thoracic Surgery, Faculty of Medicine, Saga University, Saga; [4]Department of Transfusion Medicine, Saga University Hospital, Saga 849-8501, Japan

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Abstract. *KRAS* mutations are detected in tumors of various organs, and they are also markers of resistance for epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors and monoclonal antibodies against the EGFR. Thus, the accurate and rapid detection of *KRAS* mutations is crucial, not only for screening, but also for the prediction of the efficacy of molecular-targeted therapy. The aim of the present study was to establish a novel automated detection system for *KRAS* mutations. One hundred and thirty-six lung adenocarcinoma patients were genotyped for *KRAS* mutations with both the conventional direct sequence (DS) method and with the newly developed quenching probe (QP) method that obtains data automatically within 60 min. The detection limit of the QP method using a control plasmid containing the *KRAS* mutation was 50 copies, and 10% mutant plasmid was detected in the mixture of wild-type and mutants. The results obtained by the QP and DS methods were identical in all but two of the 136 cases. The two differentially identified samples, which consisted of substantially fewer lung cancer cells, were positive according to the QP method but negative as determined by DS for *KRAS* mutations. These findings characterize the QP method as an accurate and rapid detection system for *KRAS* mutations.

Introduction

The *KRAS* gene encodes a GTP-binding protein that contributes to cell proliferation, angiogenesis, and tumor progression (1). Mutations of *KRAS* are active, and GTP-bound forms of RAS and hot spots are present in codons 12 and 13 (1-4). *KRAS* mutations are observed in tumors of various organs such as the pancreas, colon and lung; the prevalence of the mutations is 60-90, 40-50 and 13-30%, respectively (3-6). Thus, *KRAS* mutations are appropriate targets for detection and screening of cancers. Recently, *KRAS* mutations have been reported to be markers of resistance for epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) and monoclonal antibodies against the EGFR (6,7). In lung cancer, EGFR-activating mutations are frequently observed in non-smoker women with adenocarinoma, and *KRAS* mutations are exclusively detected with *EGFR* mutations (5). Treatment with EGFR-TKIs achieve a favorable response in lung cancer patients with *EGFR*-activating mutations in exons 18, 19 and 21, whereas they have no effect on patients with *KRAS* mutations (8-10). Monoclonal antibodies against EGFR, cetuximab and panitumumab, significantly prolong overall survival and progression-free survival in colon cancer patients with wild-type *KRAS*, but patients with *KRAS* mutations do not benefit from cetuximab (6,11). These results indicate that detection of *KRAS* mutations is useful for the prediction of the efficacy of molecular-targeted therapy as well as for the screening of cancers of various organs.

To investigate *KRAS* mutations in a large number of cancer patients, current methods such as direct sequence (DS), denaturing high-performance liquid chromatography, real-time polymerase chain reaction (PCR), TaqMan PCR single nucleotide polymorphism genotyping, pyrosequencing, and allele-specific PCR applied for the detection of point mutations are insufficient in terms of convenience, cost, and sensitivity. Thus, the demand has grown for a detection method that combines high sensitivity, simplicity, ease of use, and low cost.

As previously reported, the quenching probe (QP) method is extremely effective in detecting *JAK2* V617F, which contributes to the pathogenesis of myeloproliferative neoplasms (12). This method detects target genes using fluorescence quenching. Through the addition of a QProbe and a primer set, a gene amplification response is generated

Correspondence to: Dr Naoko Sueoka-Aragane, Division of Hematology, Respiratory Medicine and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan
E-mail: sueokan@cc.saga-u.ac.jp

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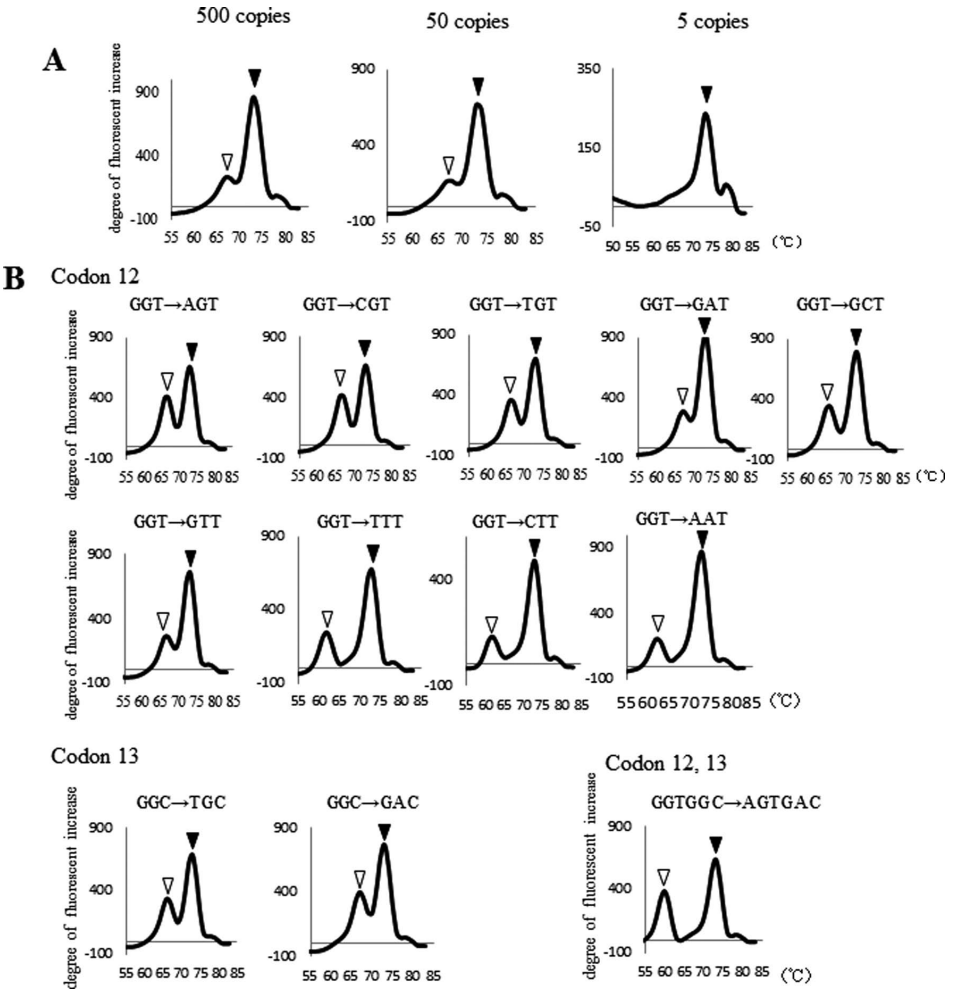

Figure 1. Detection limit of *KRAS* mutations. (A) The detection limit of the QP method using a control plasmid containing the *KRAS* mutation, codon 12 (GGT→GAT). Wild-type *KRAS* (▼), and KRAS mutation (▽) are shown. (B) Wild-type *KRAS* and 10% of the twelve different types of mutants were mixed, and *KRAS* mutants were determined using the QP method.

that is used to detect the specific gene arrangement quickly and easily using fluorometry. The present study reports the utility of the QP method compared with DS for the detection of *KRAS* mutations in lung adenocarcinoma patient samples.

Materials and methods

Human samples. Tissue samples were obtained from surgical specimens of 135 lung adenocarcinoma patients. The study patients underwent surgery at Saga Medical School Hospital but received no chemotherapy or thoracic irradiation. The population was a consecutive series of patients treated between 2004 and 2008. Pathological stage of the cancer was determined according to criteria of the International Union Against Cancer, 6th edition. The study protocol was approved by the Clinical Research Ethics Committee of Saga University, and all patients provided informed consent for collection of the surgical specimens according to the Declaration of Helsinki.

Detection of KRAS mutations by DS. Genomic DNA was isolated from 135 specimens using the QIAamp® DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *KRAS* mutations of codon 12/13 of exon 2 were determined by PCR-based DS using previously described primers for *KRAS* as follows: sense, 5'-catgttcta atatagtcaca-3' and antisense, 5'-aacaagatttacctctattg-3' (13). PCR amplification was performed in a 20-μl volume using Discoverase™ DHPLC DNA polymerase (Invitrogen Inc., Carlsbad, CA) for 40 cycles at 94˚C for 1 min, 55˚C for 1 min, 72˚C for 5 min, and a final extension at 72˚C for 10 min. The amplified product was isolated using Microcon YM-50 (Millipore Inc., MA) and sequenced directly using the Applied Biosystems PRISM dye terminator cycle sequencing method with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Detection of KRAS mutations by the QP method and preparation of control plasmid. The insert sequence of *KRAS*-WT (320-bp DNA fragment, corresponding to Accession no. NG_007524, 10380-10699) was obtained by PCR from human genomic DNA, purified, and subcloned into the pT7Blue T-Vector. The twelve types of *KRAS* mutations in codon 12/13 were introduced by using the QuickChange Site-Directed Mutagenesis kit (Stratagene) (14). The presence of the mutations was confirmed by direct sequencing.

Using the fully automated genotyping system I-densy (Arkray Inc., Kyoto, Japan), 135 specimens were genotyped for *KRAS* codon 12/13 by the QP method. Before testing, the

Table I. Comparison of the Quenching probe method and direct sequencing of *KRAS* mutations in codon 12/13.

DS		QP	DS		QP	DS		QP	DS		QP
Codon 12	Codon 13	*KRAS*	Codon 12	Codon 13	*KRAS*	Codon 12	Codon 13	*KRAS*	Codon 12	Codon 13	*KRAS*
-	-	-	+	-	+	-	-	-	-	-	-
-	-	-	+	-	+	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
+	-	+	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
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-	-	-	+	-	+	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
+	-	+	+	-	+	-	-	-	-	-	-
+	-	+	+	-	+	+	-	+	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
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-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	+[b]	-	-	-	-	-	-
-	-	+[a]	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	+	-	+
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-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	+	-	+
-	-	-	-	-	-	-	-	-	-	-	-
			-	-	-	-	-	-	-	-	-

[a]Case no. 1; [b]case no. 2. DS, direct sequencing; QP, Quenching probe method.

requisite number of tips, reaction tubes, reagent packs, and purified DNA were set in their designated places. The forward and reverse PCR primers for *KRAS* codon 12/13 amplification were 5'-aaggcctgctgaaaatgactg-3' and 5'-ggtcctgcaccagtaatat gca-3', respectively. The guanine quenching probe QProbe (J-Bio21, Tokyo, Japan) was designed to be perfectly complementary to the wild-type *KRAS* codon 12/13. The sequence of QProbe was 5'-(TAMRA)-ctcttgcctacgccaccagctccaact-3'. The QP method using QProbe can detect whether a sample harbors wild-type *KRAS* or a *KRAS* mutation in codon 12/13, but is unable to distinguish between codons 12 and 13. In I-densy, PCR was automatically performed with initial denaturation for 1 min at 95˚C before 50 cycles of denaturation at 95˚C for 1 sec and annealing at 60˚C for 15 sec. After the PCR was complete, melting temperature (Tm) analyses were automatically performed. Mutations were identified by the difference in Tm (Fig. 1) (15).

Results and Discussion

We first evaluated the detection limit of the QP method using a control plasmid containing *KRAS* mutation, codon 12 (GGT→GAT). The detection limit was 50 copies (Fig. 1A). Next, we prepared 12 different types of mutants, which were reported to be detected in cancer tissues of various organs (4). When each mutant and wild-type were mixed, 10% mutant plasmid was detected in all mutant types examined (Fig. 1B).

Results obtained by the DS or QP method are summarized in Table I. In 135 lung adenocarcinoma patients, *KRAS* mutations were detected in 13 cases (9.6% of all tested) by DS

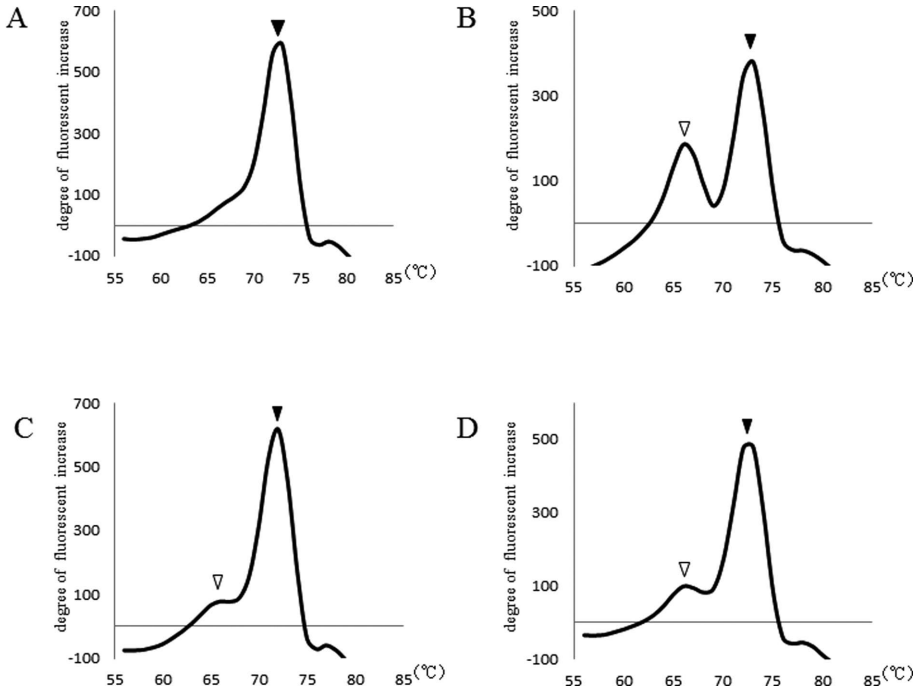

Figure 2. Detection of the *KRAS* mutation by the Quenching probe (QP) method. Lung adenocarcinoma patient specimen revealing wild-type *KRAS* (A, ▼) or *KRAS* mutation (B, ▽). Although negative for the *KRAS* mutation according to the DS method, specimens of cases 1 (C) and 2 (D) display a faint *KRAS* mutation peak.

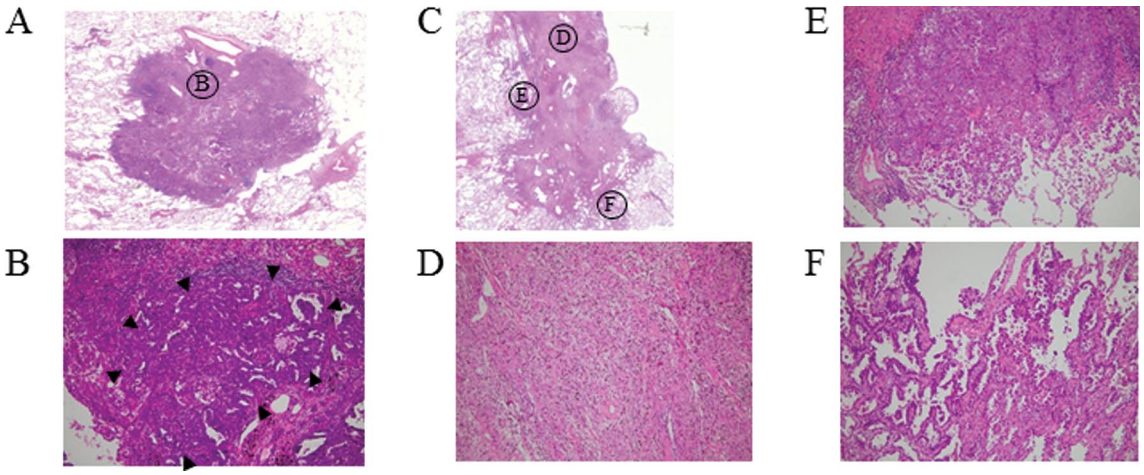

Figure 3. Histological analysis of specimens obtained from cases 1 and 2. (A) Loupe view of the case 1 specimen with H&E staining. Open circle indicates the small cancer lesion. (B) The lesion in the case 1 specimen (surrounded by ▽) contains a small number of cancer cells. (C) Loupe view of the case 2 specimen. (D) Most of the area of the case 2 specimen is replaced by necrotic tissue. (E) Small lesion reveals mucinous bronchioloalveolar carcinoma (BAC). (F) Small lesion reveals BAC. Original magnification, x100 in B, D-F.

(codon 12, 12 cases; codon 13, 1 case). The incidence of *KRAS* mutations (9.6%) was less than that reported previously in Caucasians (8) but almost the same as that reported in a Japanese cohort (5,16). *KRAS* mutations were detected in 15 cases by the QP method. Notably, two samples (cases 1 and 2, indicated by a and b in Table I, respectively) were positive as determined by the QP method (Fig. 2C and D) but negative according to DS, suggesting that the QP method was more sensitive than DS or provided false-positives (Table I).

To investigate why these two samples were missed by DS, the histology of the tumors was reexamined comprehensively. The specimen of case 1 (a in Table I) mainly contained inflammatory cells and fibrosis (Fig. 3A), and only contained a very small portion of cancer cells (Fig. 3B). The specimen of case 2 (b in Table I) also contained a small tumor lesion (Fig. 3C) and was dominated by necrotic tissue (Fig. 3D). In addition, two different histological types, mucinous bronchioloalveolar carcinoma (BAC) (Fig. 3E) and BAC (Fig. 3F), were mixed in the small cancer lesion. *KRAS* mutations are more common in mucinous BAC (~80%), while they are rare in BAC (17,18). The presence of these other histological types may have induced a false-negative by DS in case 2. The

consistency of the *KRAS* status in 133 of the 135 lung adenocarinoma patients, and the positivity of the *KRAS* mutation with the QP method in two samples that contained few cancer cells, indicate the increased accuracy and sensitivity of the QP method compared with DS. This finding was similar to that obtained for the detection of the *JAK2* mutation (12).

DS requires three steps, including DNA extraction from a sample, amplification of the target area on the extracted DNA, and sequencing of the target mutation analysis on the *KRAS* gene in the amplified product, therefore it usually provides results within approximately a week. In contrast, the QP method using I-densy detects mutations automatically within 60 min after DNA extraction. Therefore, the QP method is superior to the DS method in terms of convenience and running costs, including personnel expenses, resulting in easy access to mutation analysis for a large number of patients.

In conclusion, the QP method with its high sensitivity, effectiveness, and speed allows the determination of *KRAS* gene mutations in clinical settings. With its own adjustable PCR primers and QProbe, the QP method can be adapted to any point mutation possibly effecting the efficacy of molecular-targeted therapy. Consequently, this method may be a driving force for personalized medicine (12,15).

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References

1. Malumbres M and Barbacid M: RAS oncogenes: the first 30 years. Nat Rev Cancer 3: 459-465, 2003.
2. Jiang Y, Kimchi ET, Staveley-O'Carroll KF, *et al*: Assessment of K-ras mutation: a step toward personalized medicine for patients with colorectal cancer. Cancer 115: 3609-3617, 2009.
3. Parsons BL and Meng F: K-RAS mutation in the screening, prognosis and treatment of cancer. Biomark Med 3: 757-769, 2009.
4. Kiaris H and Spandidos DA: Mutations of ras genes in human tumours. Int J Oncol 7: 413-429, 1995.
5. Kosaka T, Yatabe Y, Endoh H, *et al*: Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 64: 8919-8923, 2004.
6. Karapetis CS, Khambata-Ford S, Jonker DJ, *et al*: K-ras mutation and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 359: 1757-1765, 2008.
7. Riely GJ, Pao W, Pham D, *et al*: Clinical course of patients with non-small cell lung cancer and epidermal growth factor receptor exon 19 and exon 21 mutations treated with gefitinib or erlotinib. Clin Cancer Res 12: 839-844, 2006.
8. Eberhard DA, Johnson BE, Amler LC, *et al*: Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small cell lung cancer treated with chemotherapy alone and in combination with erlotinib. J Clin Oncol 23: 5900-5909, 2005.
9. Pao W, Wang TY, Riely GJ, *et al*: KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med 2: e17, 2005.
10. Massarelli E, Varella-Garcia M, Tang X, *et al*: KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Cancer Res 13: 2890-2896, 2007.
11. Amado RG, Wolf M, Peeters M, *et al*: Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26: 1626-1634, 2008.
12. Tanaka R, Kuroda J, Stevenson W, *et al*: Fully automated and super-rapid system for the detection of JAK2V617F mutation. Leuk Res 32: 1462-1467, 2008.
13. Suzuki T, Nakagawa T, Endo H, *et al*: The sensitivity of lung cancer cell lines to the EGFR-selective tyrosine kinase inhibitor ZD1839 ('Iressa') is not related to the expression of EGFR or HER-2 or to K-ras gene status. Lung Cancer 42: 35-41, 2003.
14. Samowitz WS, Curtin K, Schaffer D, *et al*: Relationship of Ki-ras mutations in colon cancers to tumor location, stage, and survival: a population-based study. Cancer Epidemiol Biomarkers Prev 9: 1193-1197, 2000.
15. Matsumoto N, Kakihara F, Kimura S, *et al*: Single nucleotide polymorphism genotyping of CYP2C19 using a new automated system. Anal Biochem 370: 121-123, 2007.
16. Sasaki H, Okuda K, Kawano O, *et al*: Nras and Kras mutation in Japanese lung cancer patients: genotyping analysis using LightCycler. Oncol Rep 18: 623-628, 2007.
17. Finberg KE, Sequist LV, Joshi VA, *et al*: Mucinous differentiation correlates with absence of EGFR mutation and presence of KRAS mutation in lung adenocarcinomas with bronchioloalveolar features. J Mol Diagn 9: 320-326, 2007.
18. Hata A, Katakami N, Fujita S, *et al*: Frequency of EGFR and KRAS mutations in Japanese patients with lung adenocarcinoma with features of the mucinous subtype of bronchioloalveolar carcinoma. J Thorac Oncol 5: 1197-200, 2010.