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Missense Mutations of the *BRAF* Gene in Human Lung Adenocarcinoma[1]

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ABSTRACT

Mutations of the *BRAF* protein serine/threonine kinase gene have recently been identified in a variety of human cancers, most notably melanomas. We sought to determine the frequency of *BRAF* mutations in human lung cancer pathogenesis. Analysis of *BRAF* sequence from 127 primary human lung adenocarcinomas revealed mutations in two tumor specimens, one in exon 11 (G465V), and a second in exon 15 (L596R). These specimens belong to the same adenocarcinoma subgroup as defined by clustering of gene expression data. BRAF may provide a target for anticancer chemotherapy in a subset of lung adenocarcinoma patients.

INTRODUCTION

Lung carcinoma is the leading cause of cancer death in the United States and worldwide, claiming more than 150,000 lives each year in the United States alone (1). Current therapies for lung carcinoma remain inadequate; only an average of 15% of patients survive as long as 5 years from diagnosis. The development of targeted therapy for cancer-specific molecular alterations has led to significant clinical advances, such as the use of the c-Abl tyrosine kinase inhibitor, imatinib mesylate, for treatment of chronic myelogenous leukemia (2). Genome-wide screens for gene alterations in lung carcinoma should provide new therapeutic opportunities for this disease.

Adenocarcinoma is the most common histological class of lung carcinoma, and its relative incidence is increasing (3). Activating mutations of the *KRAS* proto-oncogene are known to occur in roughly 30% of human lung adenocarcinomas (4, 5). The pathway linking receptor tyrosine kinases to the Ras family to the Raf serine-threonine kinase to the mitogen-activated protein kinase cascade is critical for cell proliferation and is frequently activated in human cancers (6). Thus it is important to look for mutations of other members of this pathway in lung adenocarcinoma, in addition to *KRAS*.

A recent study revealed activating mutations in the *BRAF* kinase gene in over 60% of melanomas and a broad range of other human cancers (7). In particular, Davies *et al.* (7) found *BRAF* missense mutations in 4 of 35 lung adenocarcinoma cell lines tested (11%), but not in 14 primary lung cancers analyzed. Because of the possibility of therapeutic inhibition of the BRAF kinase in lung cancer, it is important to determine the frequency of *BRAF* mutations in primary lung adenocarcinomas.

We have recently analyzed a set of 127 human lung adenocarcinomas by gene expression profiling and unsupervised clustering (8). Here we report 2 cases with *BRAF* missense mutations of 127 lung adenocarcinomas tested.

MATERIALS AND METHODS

Specimens and RNA. Lung adenocarcinoma samples and RNA preparation methods have been described previously (8). We used 127 of 139 samples from the previous report, excluding 12 samples suspected to be metastases of extrapulmonary origin.

RT-PCR,[3] Genomic DNA PCR, and DNA Sequencing. Total RNA from lung cancer specimens was used to generate cRNA by *in vitro* transcription (8). cRNA samples were amplified by RT-PCR of *BRAF* exon 11 and exon 15 using specific primers [exon 11, TACCTGGCTCACTAACTAACGTG (forward) and CACATGTCGTGTTTTCCTGAG (reverse); exon 15, ACT GCACAGGGCATGGATTAC (forward) and AATTCATACAGAACAATC-CCAAA (reverse)]. PCR primers were designed to amplify target exons plus approximately 50-bp flanking exonic sequences in both upstream and downstream directions.

RT-PCR was performed using Superscript One-Step RT-PCR with Platinum Taq kit (Life Technologies, Inc., Gaithersburg, MD). A single 50-μl RT-PCR reaction mix contained 1 μg of cRNA, 3 mM $MgSO_4$, 100 pmol of each primer, and 1 μl of reverse transcriptase/Platinum Taq mix. RT-PCR was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) as follows: after 30 min of cDNA synthesis at 50°C and 2 min of denaturation at 94°C, samples were subjected to 35 cycles of amplification, consisting of 15 s at 94°C, 30 s at 61°C, and 1 min at 72°C, with a final additional extension step at 72°C for 7 min.

We also analyzed *KRAS* codon 12, 13, and 61 mutations using cRNA samples with RT-PCR. Amplification was done using specific primers (forward, CGGGAGAGAGGCCTGCTGA; reverse, CCACTTGTACTAGTAT-GCCTTAAGAA). Conditions are the same as those described above, except for an annealing temperature of 55°C.

For samples with mutations detected at the cDNA level, we isolated genomic DNA from frozen specimens of both tumor and uninvolved normal lung controls, using the QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Using primers designed to amplify *BRAF* exons 11 and 15 from genomic DNA (7), 100 ng of genomic DNA were amplified using standard PCR protocol.

RT-PCR and PCR products were visualized by 2% agarose gel electrophoresis and purified using QIA quick purification kit (Qiagen) according to the manufacturer's instructions. Purified products were subjected to primer extension sequencing (9) in both forward and reverse directions, by either the Molecular Biology Core Facility at Dana-Farber Cancer Institute or Seqwright Inc. (Houston, TX). Sequences were examined using Sequencher software (Gene Codes Corp., Inc.).

As positive and negative controls, we sequenced the *BRAF* gene in four lung carcinoma cell lines. We detected the missense mutations that were reported previously (7). The NCI-H1395 cell line has a G1403C (G468A) mutation in *BRAF* exon 11, whereas the NCI-H2087 cell line has a C1786G (L596V) mutation in exon 15. The other two cell lines, CaLu1 and NCI-H1437, did not have *BRAF* mutations in these exons.

Expression Analysis. Gene expression values were compared between the two *BRAF* mutant adenocarcinomas and the 57 lung adenocarcinomas known to be wild-type for *BRAF* and *KRAS* sequence, using the dChip program (10). We used the following criteria to select genes specific for the *BRAF* mutant tumors: (*a*) minimum fold change (*BRAF* mutant *versus* wild-type) >2.0; (*b*) minimum lower bound of fold change (90% confidence bound) >1.25; and (*c*) minimum mean difference in arbitrary Affymetrix expression units (8) >100.

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[3] The abbreviation used is: RT-PCR, reverse transcription-PCR.

RESULTS AND DISCUSSION

To detect mutations in the *BRAF* gene in primary lung adenocarcinomas, we sequenced exons 11 and 15 from the 127 lung adenocarcinomas characterized by gene expression analysis (8). These exons were chosen because all reported *BRAF* mutations were within them (7).

Among the primary lung adenocarcinomas, we detected a lower frequency of *BRAF* mutations than had been reported in the cell lines: 2 of 127 adenocarcinomas (1.6%). One human lung adenocarcinoma (AD210) has the missense mutation G1394T (G465V) in exon 11 (Fig. 1*A*); no mutation was detected in the corresponding normal lung tissue (Fig. 1*B*). In a second human lung adenocarcinoma (AD238), we found the missense mutation T1787G (L596R) in exon 15 (Fig. 1*C*); again, this sequence is wild-type in the uninvolved lung from the same patient (Fig. 1*D*). Both of these mutations had been previously reported in human cancer, G465V in a lung adenocarcinoma cell line and L596R in a primary ovarian cancer (7).

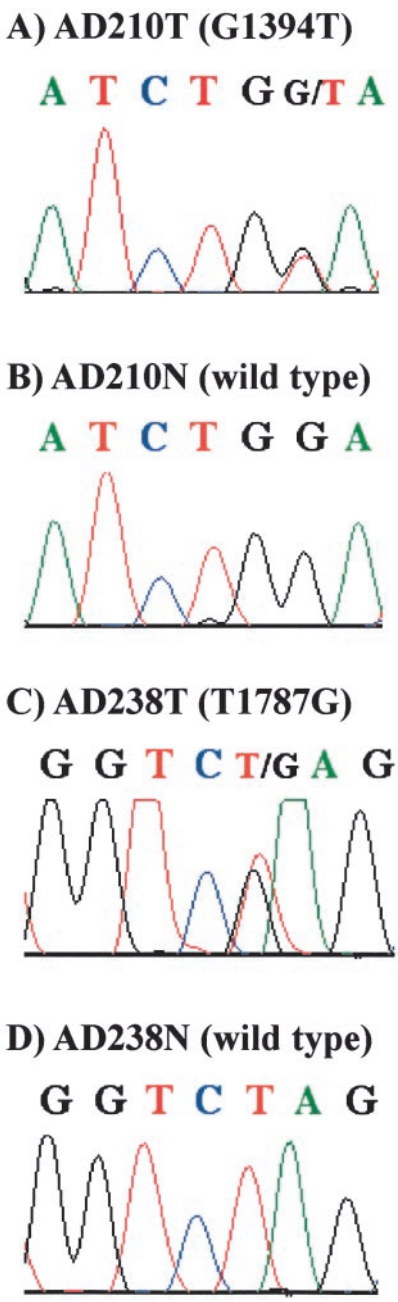

Fig. 1. Mutations in the *BRAF* gene. *A* and *B*, a point mutation was identified in exon 11 of one human lung adenocarcinoma sample, AD210T (G1394T/G465V), which is not present in a normal lung tissue sample from the same patient, AD210N. *C* and *D*, a point mutation was identified in exon 15 of sample AD238T (T1787G/L596R), which is not present in the corresponding normal lung tissue sample, AD238N.

Table 1 *BRAF and KRAS mutations in lung adenocarcinoma*

	No. of cases	*BRAF* mutation[a]	*KRAS* mutation[b]	Cluster[c]	Frequency
Total	127	2/127 (1.6%)	30/89 (33.7%)		
BRAF mutants	1	G1394T	WT[d]	Group I	
	1	T1787G	WT	Group I	
				Group I	2/19 (11%)
KRAS mutants	30	WT	MUT[e]		
				Group I	7/11 (64%)
				C1	1/8 (13%)
				C2	5/7 (71%)
				C3	5/11 (45%)
				C4	3/11 (27%)
				Others	9/39 (23%)
Others	57	WT	WT		

[a] Exons 11 or 15.
[b] Codons 12, 13, or 61.
[c] Clusters defined in Ref. 8.
[d] WT, wild-type sequence.
[e] MUT, mutant sequence.

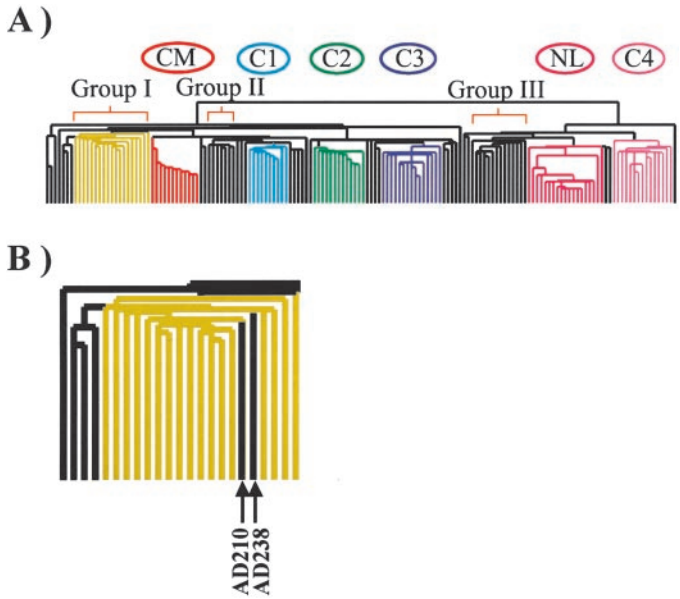

Fig. 2. Dendrogram of lung adenocarcinoma with reference to *BRAF* mutation. *A*, expression-defined adenocarcinoma subclasses (8). There are four major clusters (C1−C4) and three groups with weaker association (group I−III). Samples of colon metastasis (*CM*) and normal lung (*NL*) were not included in this *BRAF* mutation search. *B*, enlarged view of group I. The two samples with *BRAF* mutation (AD210 and AD238) were both included in group I. Please note that this figure is adapted from Ref. 8.

Because *KRAS* is frequently mutated in lung adenocarcinoma, we compared the frequency of *BRAF* mutations with *KRAS* mutations and with gene expression-defined tumor classes (8) for the same samples (Table 1). The frequency of *KRAS* mutations in the 89 samples sequenced for *KRAS* was 33.7%, similar to that reported in the literature; in these samples, *BRAF* and *KRAS* mutations were not present in the same tumor specimen.

Comparison with gene expression patterns revealed that both tumors with *BRAF* mutants were clustered in group I of our expression analysis (Fig. 2). Histologically, both tumors showed moderately differentiated adenocarcinoma, and both patients had stage I tumors. Whereas this clustering is intriguing, the sample size is too small at present to deter-

Table 2 *BRAF mutant-specific genes*[a,b]

Probe set	Description	Fold change[c]	Mean change[d]	Rank
40406_at	Macrophage stimulating, pseudogene 9	5.7 (4.0–8.5)	101	1
32115_r_at	Adenosine A2a receptor	4.2 (1.6–7.5)	169	2
1047_s_at	Human hepatocyte growth factor-like protein gene	3.6 (2.4–5.3)	246	3
40237_at	Tumor-suppressing subtransferable candidate 3	3.5 (1.7–5.9)	108	4
34094_i_at	Human immunoglobulin heavy chain variable region	3.3 (2.3–5.9)	510	5
37473_at	Keratin 16	3.1 (2.1–6.2)	179	6
34095_f_at	Human immunoglobulin heavy chain variable region	2.8 (2.0–4.0)	1041	7
35350_at	B-cell RAG-associated protein	2.3 (1.8–3.0)	162	8
40928_at	SOCS box-containing WD protein SWiP-1	2.3 (1.5–3.2)	705	9
2017_s_at	Cyclin D1	2.2 (1.5–3.0)	243	10
38028_at	Neuronal specific transcription factor DAT1	2.2 (1.8–2.8)	160	11
35566_f_at	Immunoglobulin heavy constant mu	2.1 (1.5–2.8)	1419	12
31888_s_at	Tumor-suppressing subtransferable candidate 3	2.1 (1.5–2.7)	324	13
37323_r_at	Hydroxyprostaglandin dehydrogenase 15-(NAD)	2.1 (1.3–2.9)	180	14
40317_at	Amiloride-sensitive cation channel 1	2.0 (1.8–2.3)	117	15
32919_at	*Homo sapiens*, clone IMAGE:3625286	2.0 (1.8–2.4)	184	16

[a] Comparison between 2 *BRAF* mutants and 57 WT (*KRAS* and *BRAF*).
[b] Genes were chosen by D-chip software (see "Materials and Methods").
[c] Fold change (increase in *BRAF* mutants) with 90% lower and upper confidence bounds in parentheses.
[d] Difference between means of groups in normalized Affymetrix arbitrary expression values.

mine whether *BRAF*-mutant tumors represent a distinct subset of lung adenocarcinomas. It is worth noting that analysis of oligonucleotide array gene expression data did not reveal any significant differences in *BRAF* expression between mutant and wild-type tumors. However, the *BRAF*-mutant tumors are characterized by high relative expression levels of multiple genes in this sample set (Table 2); these include the adenosine A2a receptor, several B lymphocyte and/or plasma cell genes, and cyclin D1. It is interesting to note that cyclin D1, itself a transforming oncoprotein, has been reported to be downstream of oncogenic Raf signaling pathways in several independent studies (11–15). Obviously, the sample size is small, and any conclusions about expression correlates of *BRAF* mutation must remain tentative.

In summary, we have found 2 cases with *BRAF* missense mutation in 127 human lung adenocarcinomas. Although the percentage of lung adenocarcinomas with *BRAF* mutation in lung cancer patients is relatively low compared with melanomas and colon carcinomas (7, 16), if BRAF inhibitors are found to be effective in these tumors, they should be also considered for treatment of *BRAF*-mutant lung adenocarcinomas. Furthermore, even 1.6% of lung adenocarcinoma would represent well over 1,000 patients per year in the United States, a significant group in terms of public health importance. Several Raf inhibitors have been recently discovered and entered into clinical trials (17–19).

Furthermore, the presence of both *KRAS and BRAF* mutations in lung adenocarcinoma suggests that other members or the receptor tyrosine kinase/Ras/Raf/mitogen-activated protein kinase cascade should be evaluated for mutations in these tumors.

REFERENCES

1. Jemal, A., Thomas, A., Murray, T., and Thun, M. Cancer statistics, 2002. CA Cancer J. Clin., *52:* 23–47, 2002.
2. Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C. L. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N. Engl. J. Med., *344:* 1031–1037, 2001.
3. Travis, W. D., Travis, L. B., and Devesa, S. S. Lung cancer. Cancer (Phila.), *75:* 191–202, 1995.
4. Rodenhuis, S., van de Wetering, M. L., Mooi, W. J., Evers, S. G., van Zandwijk, N., and Bos, J. L. Mutational activation of the K-ras oncogene. A possible pathogenetic factor in adenocarcinoma of the lung. N. Engl. J. Med., *317:* 929–935, 1987.
5. Kwiatkowski, D. J., Harpole, D. H., Jr., Godleski, J., Herndon, J. E. N., Shieh, D. B., Richards, W., Blanco, R., Xu, H. J., Strauss, G. M., and Sugarbaker, D. J. Molecular pathologic substaging in 244 stage I non-small-cell lung cancer patients: clinical implications. J. Clin. Oncol., *16:* 2468–2477, 1998.
6. Peyssonnaux, C., and Eychene, A. The Raf/MEK/ERK pathway: new concepts of activation. Biol. Cell, *93:* 53–62, 2001.
7. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. Mutations of the *BRAF* gene in human cancer. Nature (Lond.), *417:* 949–954, 2002.
8. Bhattacharjee, A., Richards, W. G., Staunton, J., Li, C., Monti, S., Vasa, P., Ladd, C., Beheshti, J., Bueno, R., Gillette, M., Loda, M., Weber, G., Mark, E. J., Lander, E. S., Wong, W., Johnson, B. E., Golub, T. R., Sugarbaker, D. J., and Meyerson, M. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc. Natl. Acad. Sci. USA, *98:* 13790–13795, 2001.
9. Sanger, F., and Coulson, A. R. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol., *94:* 441–448, 1975.
10. Li, C., and Wong, W. H. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc. Natl. Acad. Sci. USA, *98:* 31–36, 2001.
11. Liu, J. J., Chao, J. R., Jiang, M. C., Ng, S. Y., Yen, J. J., and Yang-Yen, H. F. Ras transformation results in an elevated level of cyclin D1 and acceleration of G_1 progression in NIH 3T3 cells. Mol. Cell. Biol., *15:* 3654–3663, 1995.
12. Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J. Biol. Chem., *271:* 20608–20616, 1996.
13. Kerkhoff, E., and Rapp, U. R. Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1. Mol. Cell. Biol., *17:* 2576–2586, 1997.
14. Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. Mol. Cell. Biol., *17:* 5598–5611, 1997.
15. Gille, H., and Downward, J. Multiple ras effector pathways contribute to G_1 cell cycle progression. J. Biol. Chem., *274:* 22033–22040, 1999.
16. Rajagopalan, H., Bardelli, A., Lengauer, C., Kinzler, K. W., Vogelstein, B., and Velculescu, V. E. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature (Lond.), *418:* 934, 2002.
17. Lyons, J. F., Wilhelm, S., Hibner, B., and Bollag, G. Discovery of a novel Raf kinase inhibitor. Endocr. Relat. Cancer, *8:* 219–225, 2001.
18. Cripps, M. C., Figueredo, A. T., Oza, A. M., Taylor, M. J., Fields, A. L., Holmlund, J. T., McIntosh, L. W., Geary, R. S., and Eisenhauer, E. A. Phase II randomized study of ISIS 3521 and ISIS 5132 in patients with locally advanced or metastatic colorectal cancer: a National Cancer Institute of Canada Clinical Trials Group Study. Clin. Cancer Res., *8:* 2188–2192, 2002.
19. Coudert, B., Anthoney, A., Fiedler, W., Droz, J. P., Dieras, V., Borner, M., Smyth, J. F., Morant, R., de Vries, M. J., Roelvink, M., and Fumoleau, P. Phase II trial with ISIS 5132 in patients with small-cell (SCLC) and non-small cell (NSCLC) lung cancer. A European Organization for Research and Treatment of Cancer (EORTC) Early Clinical Studies Group report. Eur. J. Cancer, *37:* 2194–2198, 2001.

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