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Amplification, up-regulation and over-expression of C3G (CRK SH3 domain-binding guanine nucleotide-releasing factor) in non-small cell lung cancers

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Abstract The Ras-CRK-Rap1 cellular signal-transduction system is regulated by guanine nucleotide exchange factors (GEFs). Transcription of C3G on chromosome 9q34 and a key member of the GEF gene family is activated by the CRK-adaptor protein; the C3G product is a CRK SH3 domain-binding guanine nucleotide-releasing factor. We document here the amplification of C3G in five of 18 primary non-small cell lung cancers examined and its increased expression in 18 of 28 tumors in comparison to corresponding non-cancerous lung tissues. Immunohistochemical staining revealed prominent C3G protein in the cytoplasm of cancer cells, associated with faint staining at the nucleolar membrane, but C3G was not detectable in normal bronchial mucoepithelial cells or in bronchoalveolar cells of the bronchial/bronchiolar ducts or alveoli. These data indicate that amplification and increased expression of the C3G gene may play some role in human lung carcinogenesis through derangement of the CRK-Rap1 signaling pathway.

Keywords Gene amplification · Allelic imbalance · mRNA expression · Immunohistochemistry · NSCLC, non-small cell lung cancer · C3G, CRK SH3 domain-binding guanine nucleotide-releasing factor

Introduction

Ras-family G proteins play important roles in signal-transduction cascades for cell growth and differentiation. The pathway is mediated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins via protein-protein interactions. GEFs switch proteins between GDP-bound, inactive forms and active GTP-bound forms. Binding of GTP induces conformational changes that permit interaction with downstream effectors (Campbell et al. 1998; Downward 1992). Thus, GEFs play essential roles in the pathway linking cell-surface receptors to activated Ras molecules.

Human C3G encodes a nucleotide-releasing factor with multiple proline-rich regions that bind to the CRK SH3 domain, specifically in the middle of the molecule (Bourne 1990; Tanaka et al. 1994). C3G functions as a GEF for Rap1 protein, which antagonizes Ras and thereby inhibits Ras-dependent activation of mitogen-activated kinase (MAPK) through blocking of cRaf1 (Ohba et al. 2000; Vossler 1997). The CRK-C3G-Rap1 signaling cascade activates MAPK signal-transduction through B-Raf as well as other MAPK proteins, such as JNK, that may play important roles in cell fates, apoptosis, and integrin-mediated signal transduction (Ohba et al. 2001; Chengbiao et al. 2001), and Ras-dependent activation of JNK was described to be critical for transformation of NIH 3T3 cells by v-CRK (Mochizuki et al. 1999). Thus, factors involved in CRK-C3G-Rap1 signaling cascade appeared to be fascinating candidates that might present with abnormality in human cancers. In the present work, we studied amplification, mRNA expression, and immunohistochemical staining of C3G

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in human primary non-small cell lung cancers and corresponding non-cancerous lung tissues. Our data tend to implicate aberrant expression of C3G in lung tumorigenesis.

Materials and methods

Specimens

Tumors and corresponding normal tissues were obtained with prior informed consent from 28 patients who underwent surgery for primary non-small cell lung cancers at hospitals affiliated with the Nippon Medical School from 1999 to April 2002. Fourteen of the 28 tumors were adenocarcinomas, and 14 were squamous-cell carcinomas. Dissected samples were frozen immediately and stored at -80°C . DNA was extracted from 18 paired tumor and normal frozen tissues according to methods described previously (Sato et al. 1990). Total RNA was extracted from each specimen using TRIzol (Life Technologies, Inc.) according to the manufacturer's instructions.

Seven lung cancer cell lines were analyzed for expression of C3G: RERF-LC-AI, PC-14, A549, EBC-1, Lu65, Lu99 and LK-2 (JCRB). These lines were either provided by the Japanese Cell Resource Center for Biomedical Research at the Institute of Development and Aging or purchased from the American Type Culture Collection (ATCC). Each cell line was cultured in the optimized conditions recommended by its respective distributor.

Microsatellite analysis coupled with differential PCR

Microsatellite analysis coupled with differential PCR that use bi-allelic microsatellite marker coupled with single-copy control PCR as an internal control of quantitative PCR, had been conventionally used to detect amplification/multiplication of a human genome region, as described (Tsukamoto et al. 1999; Yokota et al. 1999). Consistency between the method and the traditional Southern method were verified on the distal part of chromosome 8q (Fujiwara et al. 1993; Yokota et al. 1999). DNA from 18 of the primary non-small cell lung cancers was examined for allelic gain or loss at the C3G locus using a highly polymorphic microsatellite marker (C3G BAC). Primers for amplifying this BAC (CG3BAC-F, 5'-CGATGAGACAGACGTCAG-3' and C3G BAC-R, 5'-AGGCACTCAGGGCTTCTG-3') were designed to amplify the CA-repeat sequence present on a genomic clone, BAC-PR11-40A7 (GDB: AL160276), that contains the entire C3G gene. The interleukin-1 beta gene was chosen as a single-copy, quantity-control marker in order to obtain a reference for equalizing normal and tumor DNAs, since its chromosomal locus (2q13-q21) was not amplified in our panel of lung carcinomas. Primers for amplifying that

gene were IL1B.PCR2.1 (5'-TGG CAT TGA TCT GGT TCA TC-3') and IL1B.PCR2.2 (5'-GTT TAG GAA TCT TCC CAC TT-3') [7-9].

The microsatellite was coamplified in each sample with the single-copy control in PCR experiments using 20 ng of genomic DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, 200 mM dNTPs, 2.5 pmol each of [γ - ^{32}P]ATP-end-labeled primer and nonlabeled primer, 0.8-2.5 pmol each of [γ - ^{32}P]ATP-end-labeled IL1B.PCR.2.1 and non-labeled IL1B.PCR2.2, and 0.25 U of Taq polymerase, in volumes of 10 μl . The amount of end-labeled control primer was adjusted to roughly equalize signal intensities of both target and control markers. Cycle conditions were 94°C for 7 min, then 25-30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension step of 5 min at 72°C , in a GeneAmp PCR 9600 System (Perkin-Elmer Corp. Instruments, Norwalk, CT, USA). PCR products were electrophoresed at 2,000 V for 2-4 h in 0.3-mm-thick denaturing 6% polyacrylamide gels containing 36% formamide and 8 mM urea. Gel patterns were transferred to filter papers, dried at 80°C for 2-3 h, and exposed to autoradiographic film at room temperature for 16-20 h.

Determination of allelic dosage

Methods for measuring changes in allelic copy-number using polymorphic microsatellite markers were described previously (Fujiwara et al. 1993; Yokota et al. 1999). In brief, signal intensities of the control marker and each polymorphic allele of the microsatellite were quantified by a Hoefer GS-300 scanning densitometer; peak areas corresponding to each signal were calculated by electronic integration using the GS-370 electrophoresis data system (Hoefer Scientific Instruments, San Francisco, CA, USA). To analyze allelic dosage we compared signal intensities of four bands, two derived from the polymorphic alleles on 1p36 in normal DNA and the other two derived from the same pair of alleles in tumor DNA. All were normalized to the signal intensity of the control marker to adjust for any subtle differences in efficiency of amplification between normal and tumor samples. The extent of increase in an allele that had gained signal was calculated by dividing the intensity of the increased allele by that of the normal allele. When the results exceeded 1.8, the allele was considered to be amplified; when the result was less than 0.5, the allele was considered to be lost.

Semiquantitative RT-PCR

A 5- μg aliquot of each total RNA was treated with DNase I (Epicentre Technologies) and reverse-transcribed to single-stranded cDNA using oligo(dT) $^{12-18}$ primer with ReverscriptII reverse transcriptase (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Each

single-stranded cDNA was diluted for subsequent PCR amplification by monitoring beta-actin as a quantitative control. All PCRs were carried out in 30- μ l volumes of 1 \times PCR buffer for 2 min at 94°C for initial denaturing followed by 25–35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension step of 3.5 min at 72°C in the GeneAmp PCR system 9600 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The primers used for RT-PCR were C3GF (5'-TGA TTT TAC TGC TCC TGA GTC AAC C-3') and C3GR, (5'-CGA CTC CAG AGC ATC TGG TGA CTT T-3'), beta-actin F (5'-CCT CGC CTT TGC CGA TCC-3'), and beta-actin R (5'-GGA TCT TCA TGA GGT AGT CAG TC-3'). The PCR products were electrophoresed in 2% agarose gels.

Immunohistochemical analysis of C3G protein

Formalin-fixed, paraffin-embedded tissues from 20 lung tumors were stained with rabbit anti-C3G polyclonal antibody (purchased from Santa Cruz Biotechnology, USA) using the streptavidin biotin peroxidase complex (sABC) method. After heat treatment, peroxidase activity was suppressed with 10% goat serum for 10 min and 1% hydrogen peroxidase, respectively. The primary antibody was applied to the slides and incubated overnight at 4°C. After washing in phosphate-buffered saline (PBS), C3G protein was detected after incubation for 30 min with biotinylated goat anti-rabbit immunoglobulins (Dako code #E0432). After three washes in PBS, each

sample was incubated with 125 μ g/ml streptavidin (Dako code #K0377A) for 20 min, again washed three times in PBS, and treated for 3 min with 0.05% 3-3' diaminobenzidine (DAB) containing 0.01% hydrogen peroxide for 7 min. Finally, the slides were rinsed with distilled water, dehydrated in ethanol, and mounted under cover-glasses.

Results

Amplification of the C3G locus in non-small cell lung cancers

Table 1 presents with clinicopathological data including gender, age, histological subclass, stage, and TNM classification, of all 28 patients analyzed. Using a differential PCR method, we examined paired normal and tumor DNAs from 18 patients with primary non-small cell lung cancers for abnormalities in DNA copy number at the C3G locus on 9q34. Representative autoradiograms are presented in Fig. 1, where a CA-repeat marker for C3G revealed increased intensity of both alleles in tumor DNAs compared to their normal counterpart DNAs. Densitometry showed that the intensity of those bands in tumor DNA was increased about 5 to 10-fold in comparison to normal DNA, although the intensities of the single-copy control marker in tumor DNAs were almost equal to those of normal DNA samples. The signal intensities of C3G alleles were increased in approximately one third of the tumors we examined

Table 1 Clinical findings of patients

Case	Gender	Age	Subclass ^a	Stage ^b	T	N	M
2	F	73	Ad	IIIA	2	2	0
18	M	52	Sq	IA	1	0	0
19	M	66	Ad	IIIB	4	0	0
40	F	75	Sq	IA	1	0	0
43	M	55	Ad	IA	1	0	0
46	M	90	Sq	IA	1	0	0
51	M	69	Sq	IA	1	0	0
62	F	74	Ad	IIB	2	1	0
63	M	75	Sq	IB	2	0	0
64	M	73	Sq	IIIA	3	2	0
67	M	66	Ad	IA	1	0	0
74	F	45	Ad	IIIA	2	2	0
76	M	73	Sq	IB	2	0	0
78	M	66	Sq	IIB	2	1	0
97	M	69	Ad	IA	1	0	0
98	M	68	Sq	IA	1	0	0
99	M	65	Sq	IB	2	0	0
104	M	65	Sq	IB	2	0	0
109	M	62	Sq	IIIA	2	2	0
110	M	70	Sq	IIB	2	1	0
111	M	72	Sq	IB	2	0	0
114	M	44	Ad	IB	2	0	0
116	M	69	Ad	IIIA	2	2	0
129	M	84	Ad	IA	1	0	0
133	M	68	Ad	IIIB	4	2	0
134	M	78	Ad	IA	1	0	0
135	F	62	Ad	IA	1	0	0
136	M	67	Ad	IB	2	0	0

^aAd, adenocarcinoma; Sq, squamous-cell carcinoma.

^bTNM classification of malignant tumors, 6th edition, 2002

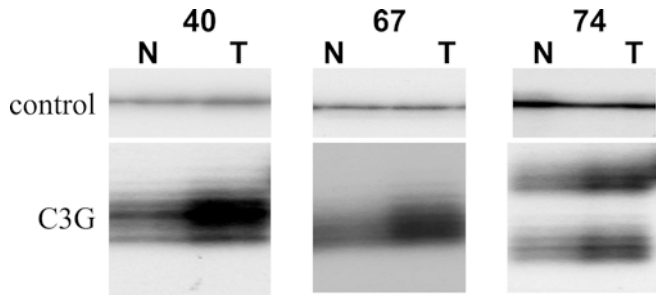


Fig. 1 Representative autoradiographs revealing amplification of DNA on chromosome 9q34 in primary lung carcinomas. *N* and *T*: DNA samples isolated from normal and tumor tissues, respectively. Case numbers are indicated above each pair of lanes. C3G BAC, polymorphic marker adjacent to the C3G gene. IL1B (Interleukin 1 beta): single-copy control marker from chromosome 2q13 to q21

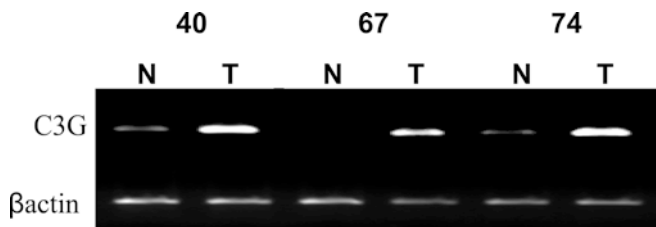


Fig. 2 Expression of C3G mRNA in representative lung cancer tissues (*T*) and corresponding normal lung (*N*). Case numbers are indicated above each pair of lanes. Beta-actin for quantitative control of PCR reaction

(5/18), even though LOH was observed only infrequently.

Over-expression of C3G mRNA in non-small cell lung cancers

To examine whether amplification of the *C3G* locus was associated with over-expression of the gene, we analyzed fresh-frozen normal and tumor tissues from 28 patients with primary lung cancers for abnormalities in levels of *C3G* mRNA using total RNAs for semiquantitative RT-PCR experiments. The concentration of each RT product was normalized to that of beta-actin, a housekeeping gene chosen as a quantitative control. Representative results are shown in Fig. 2, where increased expression of *C3G* is evident in tumors 129 and 134. *C3G* mRNA was over-expressed in nearly two thirds of the primary non-small cell lung cancers examined when tumor tissues were compared to their corresponding noncancerous tissues (18 of 28; 65%). Densitometric quantification showed that the intensities of the bands in tumor samples were increased 2.1 to 10-fold in comparison to bands representing normal lung tissues. Over-expression of *C3G* was also observed in six of the seven non-small cell lung cancer cell lines examined (data not shown). Attempts to correlate any of the clinicopathological

factors with status of gene amplification or transcript expression of *C3G* gene did not provide statistically significance (data not shown). Future immunological investigation in a larger study design is warranted to clarify such relationships.

Immunohistochemical staining of C3G in non-small cell lung cancers

We examined the *C3G* product by immunohistochemical staining of primary lung tumor specimens; representative results are shown in Fig. 3. *C3G* antigens were negative in staining among normal mucoepithelial and bronchoalveolar cells of the bronchial/bronchiolar ducts or alveoli shown in Fig. 3a,b, as well as in the noncancerous region shown in Fig. 3c,d. Normal smooth-muscle tissues (shown in Fig. 3e,f) showed positive staining, as had been described previously (Tanaka et al. 1994). *C3G* protein was prominent in the cytoplasm of cancer cells, associated with faint staining at the nucleolar membrane. We observed enhanced immunohistochemical staining of *C3G* antigen in 17 of 20 primary non-small cell lung cancers examined in this way.

Discussion

Non-small cell lung cancer accounts for more cancer-related deaths than any other malignancy in Western countries (Alberg and Samet 2003) and Japan. In the United States, non-small cell lung cancer remains the leading cause of death in both men and women in spite of well-characterized and widely reported risk factors. Far and away the most important cause of non-small cell lung cancer is exposure to tobacco smoke through active or passive smoking (Alberg and Samet 2003). Several lines of evidence implicate atypical adenomatous hyperplasia (AAH) as an initial morphologic stage in multistep lung tumorigenesis (William 2000). More than 50% of lung adenocarcinomas show mutations in codon 12 of *K-ras*, usually at an early stage of the disease. Point mutations of *p53* and/or activation of *ras* genes also are often present in initial stages of non-small cell lung cancer (Rodenhuis and Slebos 1992; Li et al. 1994; Kumar et al. 1990).

Ras-family G proteins play important roles in signal-transduction cascades for cell growth and differentiation (Campbell et al. 1998). This signaling, via protein-protein interactions, is mediated by GEFs and GTPase-activating proteins whose activities serve to link cell-surface receptors to activated Ras molecules. GEFs convert GDP-bound, inactive enzymes to GTP-bound, active forms; binding of GTP induces conformational changes that permit interaction with downstream effectors (Bourne 1990; Downward 1992). *C3G* protein is a nucleotide-releasing factor with multiple proline-rich regions that bind to the CRK SH3 domain, specifically

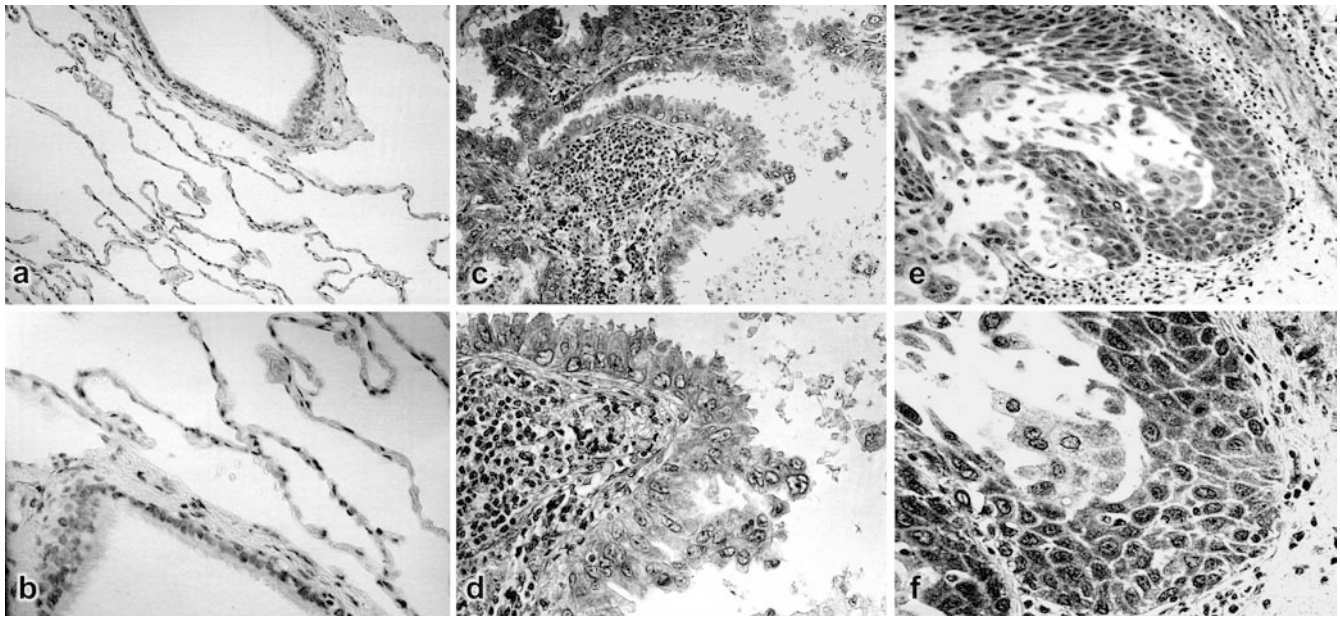


Fig. 3a-f Immunohistochemical staining. **a,b** Noncancerous bronchial mucoepithelial and bronchioalveolar cells are not stained. **c,d** Strong staining of well-differentiated adenocarcinomas, especially bronchioalveolar cell carcinomas. **e,f** Squamous-cell carcinomas, well stained in all cases

in the middle of the molecule (Knudsen et al. 1994; Tanaka et al. 1994). By associating with the CRK-adaptor protein, C3G functions as a GEF for Rap1, which has been linked definitively to the tyrosine-kinase signaling pathway; Rap1 also stimulates R-Ras and TC21 (Ohba et al. 2000). Another function of Rap1 is to antagonize Ras, thereby inhibiting the Ras-dependent activation of MAPK through blocking of c-Raf-1 (Kitayama et al. 1989; Sakoda et al. 1992). On the other hand, Rap1 activates MAPK cascades via B-Raf, as does Ras (Vossler 1997; York et al. 1998; Ohba et al. 2001).

In the present study, we detected amplification of the *C3G* gene in five of 18 primary non-small cell lung cancers examined for this anomaly. Since the distinction between allelic gain and allelic loss for allelic imbalance is not always straightforward in conventional microsatellite analyses, we designed multiplex PCR experiments using a control marker to assist evaluation of allelic status (allelic gains or losses) or dosage abnormalities. This improvement allowed quantitative estimation of gene dosage, and we were able to distinguish allelic changes by detecting subtle abnormalities in the copy number of the *C3G* locus in the tumor DNAs. Our experiments revealed that most of the genomic alterations with respect to this gene were attributable to amplification rather than to LOH; LOH in the *C3G* region occurred in less than 10% of tumors, most likely a reflection of random genetic changes in tumor genomes. Since contamination of tumor specimens by normal cells would decrease the chance of detecting amplification, our study may even have underestimated the frequency of multiplication events. *C3G* mRNA was

over-expressed in approximately two thirds (18 of 28) of the non-small cell lung cancers we examined by semi-quantitative RT-PCR experiments using RNAs from paired cancerous and non-cancerous lung tissues from each case. Over-expression of mRNA often accompanies gene amplification, although transcriptional activation through hypomethylation of promoter regions or transactivation by other cellular factors may account for over-expression in some instances. We have shown here that amplification of *C3G* is likely to cause over-expression of its mRNA in at least some lung tumors, because amplification and over-expression of the gene were observed together in the same tumors that showed genomic or transcriptional abnormalities of *C3G*. Up-regulation of the *C3G* gene in primary non-small cell lung cancers was further evidenced by increased amounts of C3G protein in lung cancer cells, shown by immunohistochemical staining with anti-C3G antibody.

C3G has been shown to form complexes with CRK in response to stimulation by growth factors (Chengbiao et al. 2001). The data presented here support the view that over-expression of *C3G* activates Rap1 and subsequently MAPK signal transduction through B-Raf. Moreover, the CRK-C3G-Rap1 signaling cascade also activates other MAPK proteins, such as JNK, that may play important roles in cell fates, apoptosis, and integrin-mediated signal transduction (Ohba et al. 2001; Chengbiao et al. 2001). Activated Rap1 itself induces cell adhesion by triggering an inside-out signal of integrin and cell spreading and suppresses cell migration. On the other hand, over-expression of *C3G* increases cell migration (Uemura and Griffin 2000). However, Mochizuki et al. have reported a pathway involving CRK activation of JNK via *C3G* and R-Ras but not Rap1, and others have noted that R-Ras-dependent activation of JNK is critical for transformation of NIH 3T3 cells by v-CRK (Mochizuki et al. 1999). Others have reported

that the signaling-adaptor protein CRK is up-regulated in human cancers (Nishihara et al. 2002). We suggest that up-regulation of C3G may affect the signaling pathway to c-Jun that is associated with up-regulation of CRK.

In conclusion, the results reported here suggest that amplification and up-regulation of the C3G gene are involved in lung carcinogenesis, especially in acceleration of tumor growth. This information might contribute to development of a molecular-targeted therapy for non-small cell lung cancers that show aberrant expression of C3G.

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