行政院國家科學委員會補助專題研究計畫成果報告

非小細胞肺癌組織中經由差異表現法確認之 Transmembrane GTPase 之研究

計畫類別：個別型計畫

計畫編號：NSC90-2320-B-039-023

執行期間：90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：何恆堅 中國醫藥學院醫學系生化學科

共同主持人：鍾景光 中國醫藥學院醫學系微生物學科

葉坤土 彰化基督教醫院病理科

計畫參與人員：葉雅雯 中國醫藥學院醫學系生化學科

王毓芬 彰化基督教醫院病理科

執行單位：中國醫藥學院醫學系生化學科

中 華 民 國 九十 年 十二 月 十二 日

Abstract

The technique of differential display was previously used to profile the gene expression patterns of NSCLC and several genes differentially expressed were thus identified. In this report, we demonstrate that a DNA fragment of 347-bp length, up-regulated in tumor tissues, showed 100% sequence similarity to human cDNA FLJ20693 for a 370-residue protein. The gene product of cDNA FLJ20693 was postulated to be a shorter isoform of transmembrane GTPase, termed TG370, based upon the results of searching for sequence homology. The nucleotide sequence alignment also indicated that the cDNA FLJ20693 and the cDNA for 741-residue human mitofusin 1 (TG741) possibly resulted from the event of alternative splicing from which a 127-bp region was retained in the latter. Analysis of the genome sequence confirmed the speculation that both cDNAs were mapped to the same chromosomal position composing of 18 exons, of which the 127-bp region of TG741 constituted exon 11. The alternative splicing in all lung cancer cell lines was also observed to occur nearly in all tissue specimens examined. The up-regulated expression of transmembrane GTPase was subsequently found in tumor tissues from at least 5 out of 7 NSCLC patients. Also, a distinct PCR product was initially detected in cell line H520 and further sequence analysis identified the presence of the 86-bp region mapped to the genome sequence immediately followed by exon 10. To

evaluate the retention of 86-bp region, it was found that, besides the predicted 486-bp product, an unexpected 332-bp product was concomitantly observed and identified as the result of exon 8 deletion. The expression and subcellular localization of the full-length TG741 and other shorter isoforms were detected by flow cytometry using three polyclonal antibodies. It was concluded that the full-length TG741 located at plasma membrane with its N-terminal domain exposed extracellularly and the shorter isoforms retained at cytosol. Finally, the up-regulation of transmembrane GTPase in tumor tissues was further illustrated using immunohistochemical staining.

Introduction

Lung cancer has become the major cause of mortality, more than 5000 cases annually, in Taiwan (1). The five-year survival rate, 65-75%, for patients going through stage I NSCLC (non-small cell lung cancer), exhibits no significant change during the past twenty years (2). Actually, there have been very few well-documented prognostic factors to evaluate survival and supplement the stage designation. Therefore, it is extremely important to identify novel markers for detecting high-risk and early-stage patients, who could potentially benefit from more aggressive treatment approaches.

Advances in molecular biology have accelerated the illustrations that several differentially expressed genes were implicated in lung cancer tissues or cell lines using differential display technique developed by Liang et al. (3). They included a member of the NF2/ERM/4.1 superfamily (4), laminin beta3 and gamma2 chains (5), semaphorin E (6) and RAB5A (7). We also employed this approach to profile the gene expression patterns of NSCLC patients and thus identified several differentially expressed genes. Among these, the overexpression of dihydrodiol dehydrogenase 1 (DD1) in tumor tissues as a prognostic factor had been reported as the first illustration in lung cancer (8).

In this study, we demonstrate the identification of a novel gene, up-regulated in

lung cancer tissues, as transmembrane GTPase. Based upon the cDNA sequences reported by different groups and analysis of the genome sequence, two isoforms of transmembrane GTPase were speculated to result from the mRNA alternative splicing. The detail of this ubiquitous alternative splicing occurring in lung cancer tissues and different cell lines will be thoroughly described in the text. The study also indicates that the events of the alternative splicing will further generate several distinct cDNA isoforms. Moreover, detection and localization of the gene products is carried out by flow cytometry.

Materials and Methods

Tissue Specimens and Cell Lines. The primary cancer tissues and the pair-wise normal tissues were surgically resected and used for research purpose with the permission from all NSCLC patients. Nine cases of squamous cell carcinoma (SCC), used for mRNA differential display, were obtained from China Medical College Hospital within the time period of experimental design. Thirty-six cases of SCC and adenocarcinoma used for immunohistochemistry were collected from Changhua Christian Hospital, Changhua. Tissue specimens were immediately snap-frozen and stored in liquid nitrogen until use. All patients were also subjected to radical N2 lymph nodes dissection. Tumor size, lymph node number, differentiation, vascular invasion and mitotic number were also evaluated.

Several lung cancer cell lines were used in the study, including H23, H125, H226, H838, A549, H661 and H520, to illustrate the alternative splicing of cDNA. All cell lines examined were separately cultured in RPMI 1640 (Life Technologies) containing 10% FBS (fetal bovine serum) (Life Technologies) and 2% penicillin (10000 U/ml)-streptomycin (10 mg/ml). These cells were then placed into 75 cm^3 tissue culture flasks and grown at 37℃ under a humidified 5% CO_2 until they reached the amounts of $5x10^6$ cells for further use.

Primers. The primers, used for mRNA differential display, 3´ RACE, RT-PCR

and gene expression, were all listed in Table 1.

RNA Isolation, mRNA Differential Display and Gene Identification. Total RNA of tumor and the pair-wise normal tissue of NSCLC patient was isolated using TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the instruction manual. For mRNA differential display (RNAimage kit, GenHunter Corporation, Nashville, TN, USA), the conducting procedures were exactly the same as those suggested by the manufacturer and described previously (8). Arbitrary primer H-AP76 and reverse primer H-T_{11}A were used in the study. After being precipitated and filled in at both ends with T4 DNA polymerase, the re-amplified cDNA fragments were subcloned into the EcoRV site of the vector pZErO-2.1 (Invitrogen Corporation, San Diego, CA, USA). The cDNA insert was then sequenced using primers (5'-GTAAAACGACGGCCAG-3' and 5'-CAGGAAACAGCTATGAC-3'). [α-^{35}S]-dATP (10 mCi/ml, specific activity>1000 Ci/mmol) was from Amersham Pharmacia Biotech.

3´ RACE. Based upon the sequence of differentially expressed cDNA, two specific primers were designed to proceed 3´ RACE (rapid amplification of cDNA ends). Briefly, about 3.5 μg of total RNA, which was isolated from a tumor tissue of NSCLC patient designated 8T in Fig. 1, 10 pmoles of adapter primer and DEPC-treated water were combined into a microcentrifuge tube to a final volume of

12 μl. The subsequent procedures were conducted entirely as those in the instruction manual (3′ RACE system, Life Technologies). When amplifying the target cDNA, 20 pmoles of primer FP1-A76A, 10 pmoles of primer UAP, 1 μl of cDNA, 2 mM $MgCl_2$, 0.2 mM dNTP and 2.5 units of Ex. Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan) were included in 50 μl of reaction mixture. After preincubating the reaction at 95℃ for 5 min, thirty-five cycles of reaction were performed (95℃ for 30 s, 60℃ for 30 s and 72℃ for 30 s). The same conditions were also applied to the nested amplification reaction, except using primers FP2-A76A and AUAP and 1 μl of primary PCR product as template. The subsequent protocols for subcloning the secondary PCR product and sequencing were equivalent with those mentioned above.

Preparations of Recombinant Proteins for Immunization. Three recombinant proteins were prepared to immunize the mice in the study. Each primer pair, used in RT-PCR, was designed to incorporate a restriction enzyme site (BamHI or XhoI, Table 1) at their 5'-ends. For recombinant protein encompassing the complete 370 residues of shorter transmembrane GTPase, TG370 (GenBank accession number AK000700) (9), the primers used were B-AEPVSP (residues 2-7) and RFHVQ-X (residues 366-370). The PCR product, treated with BamHI and XhoI, was subcloned into the corresponding sites of the expression vector pET-29a$^+$ (Novagen Inc., Madison, WI, USA). After transforming the recombinant plasmid,

lacking of exon 11, into the expression host, *E. coli* strain BL21(DE3), the recombinant protein was introduced to over-express after 3-h induction in the presence of 1 mM IPTG. The isolated inclusion body was washed twice with distilled water and re-dissolved in 1% SDS overnight at room temperature. The prepared recombinant protein showed near homogeneity as judged by SDS-PAGE and was ready for immunization.

Similar methods were also applied to prepare the other two recombinant proteins encompassing the C-terminal 100 residues and the internal 226 residues of transmembrane GTPase, TG741 (10). In the former, primers B-FKQQFV (residues 642-647 of TG741) and SNEES-X (residues 737-741 of TG741) were used. In the latter, primers B-YSVEER (residues 368-373 of TG741) and LASVTS-X (residues 588-593 of TG741) were employed to amplify the coding region followed by a putative transmembrane region of residues 600-622 of TG741.

Preparations of Polyclonal Antibodies. The prepared recombinant proteins were used to immunize the 6-weeks-old female Balb/c mice. First of all, each mouse was initially injected with 0.5 ml of pristane. About 100 μg of antigen, mixed with equal volume of complete Freund's adjuvant, was applied subcutaneously after 10-15 days. Equal amounts of antigen, emulsified with incomplete Freund's adjuvant, was injected intraperitoneally after 10-20 days and boosted again after another 15-20 days.

Finally, the serum-free myeloma cells ($0.5\text{-}1x10^6$) in PBS were injected intraperitoneally into the mouse. The ascite fluids, normally accumulated after one week, were collected daily for about 5-8 days.

Detection of Transmembrane GTPase by Flow Cytometry. The expression of transmembrane GTPase of the lung cancer cell line A549, at cell surface or intracellularly, was illustrated by flow cytometry (FCM), using the three prepared polyclonal antibodies mentioned above (11, 12). For detecting the surface antigen, cells were washed twice, resuspended with the given antibody and incubated at 4℃ for 35-min staining. After being washed three times with 10% FBS in RPMI 1640 with 0.1 % sodium azide, cells were stained with FITC-labeled secondary antibody (goat anti-mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 4℃ for 35 min. The cells were then washed three times, resuspended in PBS and analyzed by flow cytometry.

For detecting the intracellular antigen, cells were initially washed twice, resuspended in 100 μl of ice-cold 1% formaldehyde for 5 min, and mixed with 100 μl of ice-cold 99% methanol for 30 min. Then the cells were washed three times with 0.1% BSA in PBS and mixed with 100 μl of 0.1% Triton X-100 in PBS with 0.1% sodium citrate on ice for 45 min. After being washed three times with the same buffer, the cells were incubated with polyclonal antibody at 4℃ for 35-min staining

and then washed three times with 0.1% BSA in FBS. The subsequent procedures were equivalent to those for detection of surface antigen.

Immunohistochemistry. Polyclonal antibody for the C-terminal 100 residues of TG741 was used in the immunohistochemistry. Four-micrometer-thick paraffin-embedded tissue sections on poly-L-lysine coated slides were deparaffinized. After quenching endogenous peroxidase with 3% H_2O_2 in methanol, the sections were hydrated with gradient alcohol and PBS, then incubated with 10 mM citrate buffer and, finally, heated at 100℃ for 20 minutes in PBS. After being exposed to 50-fold-diluted antibody for 30 minutes at room temperature, slides were incubated with a HRP/Fab polymer conjugate (Zymed, PicTure Polymer Kit, South San Francisco, CA, USA) for the same time. The sections were thoroughly washed with PBS at each interval. The sites of peroxidase were visualized with 3,3'-diaminobenzidine tetrahydrochloride. Hematoxylin was used for counterstaining. Appropriate positive and negative controls were also included.

Statistical Analysis. Statistical comparisons were carried out using the Fisher's exact test to determine the significance of the association between different variables. The level of significance was set at 0.05.

Results

Identification of Putative Transmembrane GTPase in NSCLC. After making a trial to profile the difference of gene expression patterns using arbitrary primer H-AP76 and reverse primer H-T_{11}A, it was found that a DNA fragment of 347-bases length, termed A76A, is up-regulated in most of the tumor tissues examined (Fig. 1). The DNA sequence of this fragment, except the primer sequences at both ends, showed 100% similarity to that of nucleotides 2767-3084 of human cDNA FLJ 20693 (GenBank accession number AK000700) (9). A76A was undoubtedly amplified from the cDNA FLJ 20693, based upon the sequence analysis of the nested PCR product of 3' RACE possessing near 200-bp extension at 3’ end. The 3148-bp cDNA FLJ20693 encoded a 370-residue protein whose function was not understood. A search for sequence homologies was subsequently performed based upon the 370-residue sequence. Significant sequence similarities were observed in the N-terminal 370 residues of human mitofusin 1 (98%, 362/370) (10) and mouse putative transmembrane GTPase (90%, 333/370) (GenBank accession number AK018181) (13). Therefore, it was speculated that the gene product encoded by cDNA FLJ20693 represented a shorter form of transmembrane GTPase and is termed TG370 herein to distinguish it from TG741 of 741-residue human mitofusin 1.

The high sequence similarity was also reflected in the coding regions of cDNA

for TG370 and TG741. The difference is the presence of an extra 127-bp region in nts 1098-1224 of cDNA coding region for TG741 (10) listed as: GCATTATTCAGTGGAAGAGAGGGAAGACCAAATTGATAGACTGGACTTTAT TCGAAACCAGATGAACCTTTTAACACTGGATGTTAAGAAAAAAATCAAGG AGGTTACCGAGGAGGTGGCAAACAAA. It seemed likely that both cDNAs resulted from the alternative splicing, i. e. such a 127-bp sequence constituted an exon. Therefore, we analyzed the genome sequence and, as expected, both cDNAs were mapped to localize at the chromosome 3q25.1-25.33 (14). The genomic DNA structure for TG370/TG741, spanning over 45 kb, was made up of 18 exons, in which all intron excisions followed the GT-AG rules shown in bold and exon 2 encoded the initiation codon (Table 2). In the case of cDNA for TG370, exon 11 was spliced out and the reading frame was in-frame with the stop codon TGA in exon 12.

Alternative Splicing in Lung Cancer. As the identified 347-bp DNA fragment was mapped to the 3' untranslated region of cDNA FLJ20693 for TG370 (9), two specific primers FP0-A76A (residues 180-186 of TG370) and RFHVQ-X (residues 366-370 of TG370) were designed to evaluate the occurrence of alternative splicing in lung cancer. As shown in Fig. 2A, two PCR products of 714- and 587-bp length, in which the former was the predominant, were simultaneously observed in all lung cancer cell lines. Due to the inadequate amounts of RNA, the tumor tissue and

the pair-wise normal lung tissue of patients, numbering 6 and 7 in Fig. 1, were not included to evaluate. The alternative splicing was also observed and nearly occurred in all tissues examined (Fig. 2B). Moreover, the up-regulated expression of transmembrane GTPase was implicated in at least 5 out of 7 tumor tissues. Such observation was also consistent with the result of differential display in Fig. 1. Another distinct PCR product migrating between those two major ones was significantly, however, observed in cell line H520 (Fig. 2A). Sequence analysis showed that the striking feature of the 673-bp product was the presence of an extra 86-bp region lying between exons 9 and 10 (Fig. 2C). Based upon the genome sequence, the additional 86-bp sequence was mapped to which was followed immediately by exon 10. Primers RP5-A76A, in the 86-bp region, and FP0-A76A were further used to interpret the retention of such a sequence in the splicing process. The observation of the 486-bp product showed its universal occurrence in most tumor tissues and cell lines (Fig. 2D).

Prior to the illustration of cDNA for TG741, another human cDNA for putative transmembrane GTPase (GenBank accession number U95822) had been described by Fuller and Hales (15). The partial 493-residues gene product exhibited the lack of 111 residues, which exactly constituted exons 13 and 14, between residues Ser^{443} and Leu^{555} of TG741. It was initially concluded that the alternative splicing would result

in the formation of cDNA encoding at least three gene products with different residue numbers. However, the retention of the 86-bp region preceding exon 10 complicated the alternative splicing as illustrated in Fig. 2. Moreover, another type of exon deletion was also observed. As shown in Fig. 2D, besides the predicted 486-bp product, a distinct 332-bp product was detected in all tumor tissues and cell lines. Sequence analysis identified the unique deletion of exon 8. Equivalent result was also obtained using primers RP5-A76A and B-AEPVSP from which, besides the predicted 1028-bp product, the 874-bp PCR product, lacking of exon 8, was also derived (data not shown). Actually, there were still distinct splice variants to be identified in the experiment. As an example, the splicing pattern of exons 1-18 was illustrated using primers FP6-A76A, in exon 1, and RP3-A76A, in exon 18, from which a splice variant, lacking of exons 12-14, was thus identified in tumor tissues (data not shown).

Locating Transmembrane GTPase at Cell Surface and Cytosol. The results of locating transmembrane GTPase by flow cytometry are presented in Fig. 3. It indicates that, using antibody raised against TG370, the proportion of cells expressing antigens at the surface and intracellularly was 78% and 99% respectively, as illustrated in Fig. 3A and 3B. About 73% of cells expressed the antigen at the surface using antibody raised against the internal 226 residues preceding the

transmembrane region of TG741 (Fig. 3C). After increasing the antibody concentration, the proportion of cells being stained increased only to about 80%. It may be that some cells were not mature enough to produce the surface antigen. In contrast, it was observed that low percentages of cells, comparable to the control group, were stained for cytoplasmic antigen expression using the same antibody (Fig. 3D). On the other hand, the intracellular antigen expression was significantly detected in 92% of cells, using antibody for the C-terminal 100 residues of TG741 (Fig. 3F). In this case, antigen was not detected at the surface (Fig. 3E). Combined with these results, the following two facts were concluded. First, transmembrane GTPase was destined to cell surface or cytosol. Depending on the length of polypeptide chain, the shorter form(s) might retain at cytosol and that with transmembrane region was translocated to plasma membrane. Second, the full-length TG741 was anchored at cell surface in which its N-terminal domain was exposed extracellularly.

Immunohistochemical studies. The expression of transmembrane GTPase in NSCLC was further illustrated immunohistochemically using antibody raised for the C-terminal 100 residues of TG741. The results showed that differential expression of transmembrane GTPase in tumor tissues was revealed in 13 out of 18 cases of adenocarcinoma (72.2%) and 5 out of 18 cases of SCC (27.8%) ($p=0.018$). The

staining results of representative case of each tumor type, accompanied with adjacent normal lung tissue from the same patient, were shown in Fig. 4. As observed in most cases of adenocarcinoma and SCC examined in the experiment, the expression of transmembrane GTPase was detected to a significant lower extent in type 2 pneumocytes and alveolar macrophages in the peripheral lung.

Discussion

In our experiment, the alternative splicing obviously resulted from the retention/deletion of exon 8, the 86-bp region and exon 11. The putative shorter isoforms and deduced C-terminal unique sequences are listed at Table 3. If exon 8 was spliced out, a stop codon TGA or TAA would be encountered in exon 10 in the absence or presence of the 86-bp region, i.e., the predicted gene products of 282 and 322 residues were not relevant to the exon 11. In the case of retention of exon 8 and the 86-bp region, an in-frame stop codon TAA in the 86-bp region caused the predicted protein to have the C-terminal unique sequence $LQTASFCV^{333}$.. Therefore, four putative shorter isoforms of transmembrane GTPase, including TG370, theoretically existed. Also, the multiplicity of shorter isoforms may be appropriate to account for the detection of intracellular antigen using antibodies for TG370 (Fig. 3B). In the future, synthetic peptide, its design based upon the C-terminal unique sequence, will be used to prepare the antibody to identify the existence of the individual shorter isoform by flow cytometry.

Protein sequence analysis of TG741 revealed the presence of a transmembrane region of $I^{600}IVGGVIWKTIGWKLLSVSLTMY^{622}$ but no putative signal peptide or mitochondrial prosequence. From the results of flow cytometry in Fig. 3, TG741 was assigned to locate at the plasma membrane with the orientation of its N-terminal

domain exposed extracellularly. However, Fuller et al. demonstrated that human mitofusin 1, equivalent with TG741, was destined to mitochondrial membrane and implicated in mitochondrial fusion (10) and the homologues of fruit fly (Fzo) (15) and yeast (Fzo1p) (16) played the same roles. There is yet no reasonable explanation for the controversy of locating TG741 destination. Actually, increasing members of putative transmembrane GTPase family displayed similar structures of polypeptide chain length, P-loop near the N-terminus and transmembrane region near the C-terminus (13, 15-19). There were no reports to reveal the presence of the shorter isoforms in other species. Distinct kinds of transmembrane GTPase, which showed no sequence similarity to TG741, were also previously described, including interleukin-1 receptor (20) and beta subunit of SRP (signal recognition particle) receptor in ER membrane (21). This provided an insight that TG741, whose precise functions were still under-established, served as a membrane-anchored receptor to participate in the signal transduction pathway.

On the other hand, the shorter isoforms, all having no transmembrane regions, were experimentally concluded to be soluble in cytoplasm. It may be suitable to refer to the shorter isoforms of transmembrane GTPase as TG-related GTPase, which still retained the P-loop of $GR^{83}TSSGK^{88}S$, like that of TG741, and showed no significant sequence similarities to the members of the Ras-related GTPase

superfamily. These shorter isoforms might be postulated to be functional based upon the fact that the GTP-binding domain of TG-related GTPase was conserved in several nucleotide-binding proteins. These candidates were widely distributed in human (MMR_HSR1 and NPG1_HUMAN) (22, 23), yeast *Schizosaccharomyces pombe* (YAWG_SCHPO and T39037) (24, 25), *Mycoplasma pneumoniae* (Y442_MYCPN) (26), *Aquifex aeolicus* (ENGA_AQUAE) (27), *Synechocystis* PCC6803 (ENGA_SYNY3) (28), *Buchnera aphidicola* (ENGA_BUCAP) (29) and *Chlamydia trachomatis* (ENGA_CHLTR) (30). The aligned conserved domains are shown below:

TG	RHMKVAFFGRTSSGKSSVINAMLWDKVLP-SGIGHITNCFLSVEGT-DGDKAYLM-TEGSDEKKSVK^{137}
MMR_HSR1	KSIRVGVIGYPNVGKSSLINALVKKKRAIVSNRPGTTRDIQEVKLV-KDKKIYLIDTPGIRFPSSVD^{174}
YAWG_SCHPO	TKMTFGLVGYPNVGKSSTINALVGSKKVSVSSTPGKTKHFQTINL---SEKVSLLDCPGLVFPSFAT^{363}
NGP1_HUMAN	KQISVGFIGYPNVGKSSVINTLRSKKVCNVAPIAGETKVWQYITL---MRRIFLIDCPGVVYPS-ED^{371}
T39037	KQISVGLIGFPNAGKSSIINTLRKKKVCNVAPIPGETKVWQYVAL---MKRIFLIDCPGIVPPSSND^{372}
Y442_MYCPN	HQFRLAVIGMPNVGKSSLINLLLNKNHLQVANRAGVTKSMSWNQI---SSEFYLSDTPGVFFKRIDE^{179}
ENGA_AQUAE	IKVAFIGRPNVGKSSLVNAILKDERVIVSPIAGTTRDAIEIPFRWKDKNFILIDTAGVRRPSNVE^{239}
ENGA_SYNY3	IKVAIVGRPNVGKSSLLNALTGEQRAIVSPISGTTRDAIDMVVERNGQKYRLIDTAGIRRKKNVD^{241}
ENGA_BUCAP	NSVKIACIGKPNVGKSTLINSLLMKKRMITSNKAGTTLDTVLVPIKYNYKNYIFIDTAGMSKKKSKT^{252}
ENGA_CHLTR	RPLKVALIGHPNVGKSSIINALLKEERCITDNSPGTTRDNIDVAYTHNNKEYVFIDTAGLRKTKSIK^{291}

Additionally, the similar sequences of the P-loop have also been found in cystic fibrosis transmembrane conductance regulator (GRTGSGKS^{1221}) (31), sulfonylurea receptor 2B (GRTGSGKS^{1349}) (32), ATP-binding cassette transporter abc1 (GSTGSGKS^{621} and GRTGSGKS^{1221}) (33), multidrug resistance protein (GSTGSGKS^{674} and GRTGSGKS^{1327}) (34) and the 1beta dynein heavy chain

(GRTGSGKS^{2134}) (35). Several reports have revealed the identification and characterization of various GTPase-interacting or -activating proteins (36-39). In perspective, we need to investigate the target proteins interacting with or activating TG741/TG-related GTPase for further illustrations of their physiological functions.

The up-regulated expression of transmembrane GTPase might be involved with the tumorigenesis process. As shown in Fig. 4, transmembrane GTPase was differentially expressed in tumor tissues, especially in adenocarcinoma. Most cases of adenocarcinoma were clinically destined to peripheral lung. It had been established that this tumor type was derived from type 2 pneumocytes of alveolar sac and Clara cells of the bronchiole. Therefore, transmembrane GTPase may have the potential to serve as a useful tumor maker for detecting primary lung adenocarcinoma.

References

1. Health and Vital Statistics. Department of Health, the Executive Yuan, Taiwan ROC 1972-1989.
2. Moore, D. F. and Lee, J. S. Staging and prognostic factor: non-small cell lung cancer. *In:* H. I. Pass, J. B. Mitchell, D. H. Johnson, and A. T. Turrisi (eds.), Lung Cancer: Principle and Practice, pp. 481-494. Philadelphia: Lippincott-Raven Publishers, 1996.
3. Liang, P. and Pardee, A. B. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science, *257:* 967-970, 1992.
4. Tran, Y. K., Bogler, O., Gorse. K. M., Wieland, I., Green, M. R., and Newsham, I. F. A novel member of the NF2/ERM/4.1 superfamily with growth suppressing properties in lung cancer. Cancer Res., *59:* 35-43, 1999.
5. Manda, R., Kohno, T., Niki, T., Yamada, T., Takenoshita, S., Kuwano, H., and Yokota, J. Differential expression of the LAMB3 and LAMC2 genes between small cell and non-small cell lung carcinomas. Biochem. Biophys. Res. Commun., *275:* 440-445, 2000.
6. Martin-Satue, M. and Blanco, J. Identification of semaphorin E gene expression in metastatic human lung adenocarcinoma cells by mRNA differential display. J. Surg. Oncol., *72:* 18-23, 1999.

7. Yu, L., Hui-chen, F., Chen, Y., Zou, R., Yan, S., Chun-xiang, L., Wu-ru, W., and Li, P. Differential expression of RAB5A in human lung adenocarcinoma cells with different metastasis potential. Clin. Exp. Metastas., *17:* 213-219, 1999.

8. Hsu, N. Y., Ho, H. C., Chow, K. C., Lin, T. Y., Shih, C. S., Wang, L. S., and Tsai. C. M. Overexpression of dihydrodiol dehydrogenase as a prognostic factor of non-small cell lung cancer. Cancer Res., *61:* 2727-2731, 2001.

9. Sugano, S., Suzuki, Y., Ota, T., Obayashi, M., Nishi, T., Isogai, T., Shibahara, T., Tanaka, T., and Nakamura, Y. EMBL/GenBank/DDBJ data banks accession number AK000700, 2000.

10. Santel, A and Fuller, M. Control of mitochondrial morphology by a human mitofusin. J Cell Sci., *114:* 867-874, 2001.

11. Jacobberger, J. W., Fogelman, D., and Lehman, J. M. Analysis of intracellular antigens by flow cytometry. Cytometry, *7:* 356-364, 1986.

12. Francis, C. and Connelly, M. C. Rapid single step method for flow cytometric detection of surface and intracellular antigens using whole blood. Cytometry, *25:* 58-70, 1996.

13. The RIKEN Genome Exploration Research Group Phase II Team and FANTOM Consortium. Functional annotation of a full-length mouse cDNA collection. Nature, *409:* 685-690, 2001.

14. Worley, K. C. EMBL/GenBank/DDBJ data banks accession number AC007823, 2000.

15. Hales, K. G. and Fuller, M. T. Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. Cell, *90:* 121-129, 1997.

16. Hermann, G. J., Thatcher, J. W., Mills, J. P., Hales, K. G., Fuller, M. T., Nunnari, J., and Shaw, J. M. Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. J. Cell Biol., *143:* 359-373, 1998.

17. Nagase, T., Seki, N., Ishikawa, K., Ohira, M., Kawarabayasi, Y., Ohara, O., Tanaka, A., Kotani, H., Miyajima, N., and Nomura, N. Prediction of the coding sequences of unidentified human genes. VI. The coding sequences of 80 new genes (KIAA0201-KIAA0280) deduced by analysis of cDNA clones from cell line KG-1 and brain. DNA Res., *3:* 321-329, 1996.

18. Adams, M. D., Celniker, S. E., Gibbs, R. A., Rubin, G. M., and Venter, C. J. EMBL/GenBank/DDBJ data banks accession number AAF46161, 2000.

19. Latreille, P. EMBL/GenBank/DDBJ data banks accession number T34496, 1995.

20. Hopp, T. P. Evidence from sequence information that the interleukin-1 receptor is a transmembrane GTPase. Protein Sci., *4:* 1851-1859, 1995.

21. Miller, J. D., Tajima, S., Lauffer, L., and Walter, P. The beta subunit of the

signal recognition particle receptor is a transmembrane GTPase that anchors the alpha subunit, a peripheral membrane GTPase, to the endoplasmic reticulum membrane. J. Cell Biol., *128:* 273-282, 1995.

22. Vernet, C., Ribouchon, M. T., Chimini, G., and Pontarotti, P. Structure and evolution of a member of a new subfamily of GTP-binding proteins mapping to the human MHC class I region. Mamm. Genome, *5:* 100-105, 1994.

23. Racevskis, J., Dill, A., Stockert, R., and Fineberg, S. A. Cloning of a novel nucleolar guanosine 5'-triphosphate binding protein autoantigen from a breast tumor. Cell Growth Differ., *7:* 271-280, 1996.

24. Murphy, L., Harris, D., Barrell, B. G., Rajandream, M. A., and Walsh, S. V. EMBL/GenBank/DDBJ data banks accession number Q10190, 1996.

25. Gentles, S., Churcher, C. M., Barrell, B. G., Rajandream, M. A., and Wood, V. EMBL/GenBank/DDBJ data banks accession number T39037, 1997.

26. Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B. C., and Herrmann, R. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. Nucleic Acids Res., *24:* 4420-4449, 1996.

27. Deckert, G., Warren, P. V., Gaasterland, T., Young, W. G., Lenox, A. L., Graham, D. E., Overbeek, R., Snead, M. A., Keller, M., Aujay, M., Huber, R., Feldman, R. A., Short, J. M., Olson, G. J., and Swanson, R. V. The complete genome of the

hyperthermophilic bacterium *Aquifex aeolicus*. Nature, *392:* 353-358, 1998.

28. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res., *3:* 109-136, 1996.

29. Clark, M. A., Baumann, L., and Baumann, P. Sequence analysis of a 34.7-kb DNA segment from the genome of *Buchnera aphidicola* (endosymbiont of aphids) containing groEL, dnaA, the atp operon, gidA, and rho. Curr. Microbiol., *36:* 158-163, 1998.

30. Stephens, R. S., Kalman, S., Lammel, C. J., Fan, J., Marathe, R., Aravind, L., Mitchell, W. P., Olinger, L., Tatusov, R. L., Zhao, Q., Koonin, E. V., and Davis, R. W. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science, *282:* 754-759, 1998.

31. Hart, P., Warth, J. D., Levesque, P. C., Collier, M. L., Geary, Y., Horowitz, B., and Hume, J. R. Cystic fibrosis gene encodes a cAMP-dependent chloride channel in heart. Proc. Natl. Acad. Sci. (U.S.A.), *93:* 6343-6348, 1996.

32. Tanemoto, M., Abe, T., and Hebert, S. C. EMBL/GenBank/DDBJ data banks accession number T46645, 1997.

33. Christensen, P. U., Davis, K., Nielsen, O., and Davey, J. Abc1: a new ABC transporter from the fission yeast *Schizosaccharomyces pombe*. FEMS Microbiol. Lett., *147:* 97-102, 1997.

34. Ouellette, M., Fase-Fowler, F., and Borst, P. The amplified H circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. EMBO J., *9:* 1027-1033, 1990.

35. Perrone, C. A., Myster, S. H., Bower, R., O'Toole, E. T., and Porter, M. E. Insights into the structural organization of the I1 inner arm dynein from a domain analysis of the 1beta dynein heavy chain. Mol. Biol. Cell, *11:* 2297-2313, 2000.

36. Brondyk, W. H., McKiernan, C. J., Fortner, K. A., Stabila, P., Holz, R. W., and Macara, I. G. Interaction cloning of Rabin3, a novel protein that associates with the Ras-like GTPase Rab3A. Mol. Cell. Biol., *15:* 1137-1143, 1995.

37. Haataja, L., Groffen, J., and Heisterkamp, N. Identification of a novel Rac3-interacting protein C1D. Int. J. Mol. Med., *1:* 665-670, 1998.

38. Cuif, M. H., Possmayer, F., Zander, H., Bordes, N., Jollivet, F., Couedel-Courteille, A., Janoueix-Lerosey, I., Langsley, G., Bornens, M., and Goud, B. Characterization of GAPCenA, a GTPase activating protein for Rab6, part of

which associates with the centrosome. EMBO J., *18:* 1772-1782, 1999.

39. Hoffenberg, S., Liu, X., Nikolova, L., Hall, H. S., Dai, W., Baughn, R. E., Dickey, B. F.., Barbieri, M. A., Aballay, A., Stahl, P. D., and Knoll, B. J. A novel membrane-anchored Rab5 interacting protein required for homotypic endosome fusion. J. Biol. Chem., *275:* 24661-24669, 2000.

Table 1. Sequences of the primers used in the study

Primer	Sequence (5′→3′)[a]	Orientation[b]
B-AEPVSP	CACCCGGATCCGCAGAACCTGTTTCTCCA	S
RFHVQ-X	GGTCCCTCGAGTCATTGCACATGAAACCT	A
B-FKQQFV	CACCCGGATCCTTTAAACAGCAGTTTGTA	S
SNEES-X	GGTCCCTCGAGTTAGGATTCTTCATTGCT	A
B-YSVEER	CACCCGGATCCTATTCAGTGGAAGAGAGG	S
LASVTS-X	GGTCCCTCGAGTCAAGATGTAACGGACGCCAA	A
FP0-A76A	CCAGGCACAGATGTCACTAC	S
FP1-A76A	CAGCTTTGCTCCCATT	S
FP2-A76A	GGAACGCTTTCCTAGTGCA	S
FP4-A76A	TGTTTAAATTCCTGAAAATGTTGCG	S
FP6-A76A	GTTGCCGGGTGATAGTTGGAG	S
RP3-A76A	ACAGCCCCACCCCTCAGGGG	A
RP5-A76A	CATGGCAACATTTTCAGGAATTTAA	A
Adapter primer	GGCCACGCGTCGACTAGTACT$_{17}$	A
UAP	CUACUACUACUAGGCCACGCGTCGACTAGTAC	A
AUAP	GGCCACGCGTCGACTAGTAC	A
H-AP76	AAGCTTGTTATAG	S
H-T$_{11}$A	AAGCTTTTTTTTTTA	A

[a]The underlined sequences GGATCC, CTCGAG and AAGCTT represented the sites recognized by restriction enzymes BamHI, XhoI and HindIII, respectively.

[b]S, sense; A, antisense.

Table 2. Genomic organization of TG (transmembrane GTPase) gene

Exon		Exon/intron junction		Intron
Number	Size (bp)	5′donor	3′acceptor	size (kb)
1	76	AGCGGAGACT**gt**gagtg	cctct**ag**TAGCATAATG Met	1.0
2	119	TTTGTTGAAG**gt**tagtt PheValGluA---	tttgc**ag**CAACATATAA ---laThrTyrLy	2.9
3	136	TTTTTGGCAG**gt**aatta hePheGlyAr---	tcttc**ag**GACAAGCAGT ---gThrSerSer	6.8
4	163	GAGTGTGAAG**gt**atgat sSerValLys---	gcttt**ag**ACAGTTAATC ---ThrValAsnQ	3.4
5	125	TAGTAGACAG**gt**aaaat euValAspSe---	tcatt**ag**TCCAGGCACA ---rProGlyThr	1.8
6	109	AATGAATACG**gt**aggat uMetAsnThr---	ttttc**ag**GAAAAACACT ---GluLysHisP	0.7
7	108	TATGGAAGAC**gt**aagtt rMetGluAsp---	tctgt**ag**GTACGCAGAC ---ValArgArgG	2.2
8	154	CCAGAAAGTG**gt**atgca ProGluSerG---	gttac**ag**GTGTGGCACT ---lyValAlaLe	0.4
9	68	AATCTTTGAG**gt**aggaa nIlePheGlu---	ctcgc**ag**GAGTGTATCT ---GluCysIleS	7.1
10	122	AAGATAAAAG**gt**atgag luAspLysAr---	gaaat**ag**GCATTATTCA ---gHisTyrSer	1.7
11	127	GGCAAACAAG**gt**gggta lAlaAsnLys---	ttttc**ag**GTTTCATGTG ---ValSerCysA	0.2
12	105	ATATAAAAGT**gt**aagtt eTyrLysSer---	ttaac**ag**GAATTAAATA ---GluLeuAsnL	0.9
13	103	GAAATTATTG**gt**aatat GluIleIleG---	tttct**ag**AAAATTTGAA ---luAsnLeuLy	0.1
14	230	TATCTTTCAG**gt**atgta oIlePheGln---	ttggc**ag**CTCCCTAGAT ---LeuProArgS	6.8
15	153	TGGAGGAGTG**gt**aagaa lGlyGlyVal---	cttac**ag**ATTTGGAAAA ---IleTrpLysT	0.7
16	197	AAGTAAAACA**gt**aagt lnValLysGl---	tttta**ag**ACAAATAGCT ---nGlnIleAla	3.4
17	135	AGCTCTTAAG**gt**attt ysLeuLeuAr---	taatt**ag**AAATAAAGCT ---gAsnLysAla	1.8
18	1238 Poly(A)$^+$			

Table 3. Putative shorter isoforms of transmembrane GTPase

Isoforms	Exon 8	Extra 86 bp	Exon 11	C-terminal sequences[a]
TG370	+	-	-	L[360]AAEDKRFHVQ[370]
TG333	+	+	+/-	N[319]FEQIFELQTASFCV[333]
TG282	-	-	+/-	R[268]ILNKSLRSVSRSQQ[282]
TG322	-	+	+/-	R[268]ILNKSLSYKQQVFVFKFL-KMLRWNYSNVLRLGPLAGV-YLAVSSENKVRTAHYQS[322]

[a]The C-terminal unique sequence of each shorter isoform is underlined.

FIGURE LEGENDS

Fig.1. The gene expression patterns of NSCLC are profiled by differential display using the arbitrary primer H-AP76 and reverse primer H-T_{11}A. The up-regulated DNA fragment of 347-bp length termed A76A in most tumor tissues is indicated by arrowhead. The symbols 'N' and 'T' represent non-tumor and pair-wise tumor fractions of surgical resections of 9 NSCLC patients.

Fig. 2. Occurrence of alternative splicing and up-regulated expression of cDNA for transmembrane GTPase in lung cancer cell lines and NSCLC patients. A. Presence or absence of exon 11 is determined using primers FP0-A76A and RFHVQ-X. The two predicted PCR products of 714- and 587-bp length, in A and B, and an additional distinct PCR product of 673-bp length are shown at right. Lung cancer cell lines used in the study are indicated at the top of each lane. The left lane is 100-bp DNA ladder. B. To evaluate the alternative splicing and up-regulated expression, 7 out of 9 NSCLC patients in Fig. 1, which are indicated at top of each lane, are used in the experiment. The symbols 'N' and 'L' following the patient number are normal and lung cancer tissues. The left lane is 100-bp DNA ladder. C. DNA sequence of the 673-bp product is shown and the extra 86-bp region preceding exon 10 is typed in

boldface. The exon/intron boundaries are denoted under the DNA sequence. D. Universal presence of the extra 86-bp region is illustrated using primers FP0-A76A and RP5-A76A and the predicted 486-bp product is indicated. The unexpected 332-bp product is derived from the deletion of exon 8 and described in the Results.

Fig. 3. Flow cytometry (FCM) histograms of transmembrane GTPase expression in lung cancer cell line A549 using antibodies for surface and cytoplasmic staining. The X-axis and Y-axis in each histogram represent fluorescence intensity and cell number. After being stained by a primary antibody, the cells were then stained by a fluorescence-labeled secondary antibody and analyzed by FCM as described in Materials and Methods. The left (A, C and E) and right (B, D and F) panels indicate the detection of surface and cytoplasmic antigens, respectively. The antibodies used were raised for TG370 (A and B), for the internal 226 residues of TG741 (C and D) and for the C-terminal 100 residues of TG741 (E and F).

Fig. 4. Immunohistochemical staining of transmembrane GTPase in representative examples of adenocarcinoma and SCC of NSCLC. A and B. Tumor tissues of adenocarcinoma and SCC. C and D. Adjacent normal lung tissues of adenocarcinoma and SCC (original magnification x 400). The up-regulated

expression of transmembrane GTPase is detected in tumor tissues (A and B), whereas type 2 pneumocytes and alveolar macrophages show weak staining and type 1 pneumocytes appear negative in the pair-wise normal lung tissues (C and D).

Fig. 1

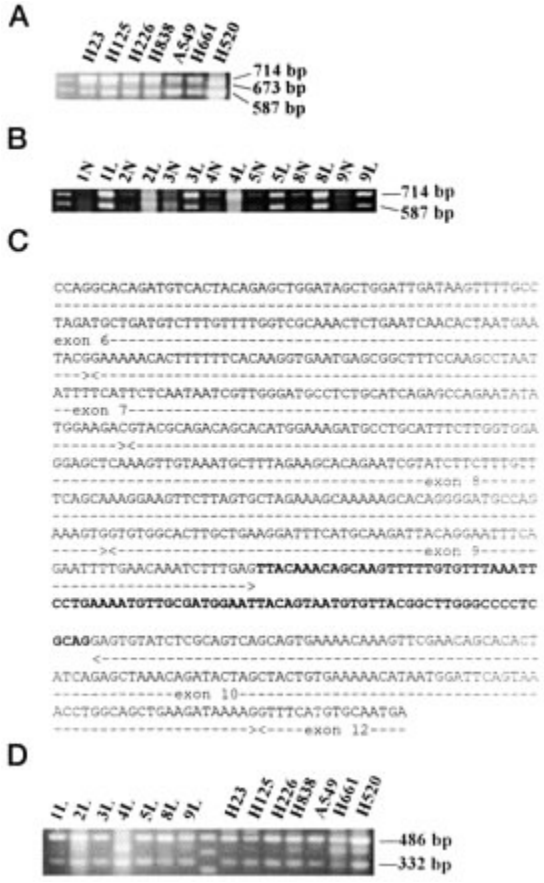
A
H23
H125
H226
H838
A549
H661
H520
714 bp
673 bp
587 bp
B
1N
1L
2N
2L
3N
3L
4N
4L
5N
5L
8N
8L
9N
9L
714 bp
587 bp
C
CCAGGCACAGATGTCACTACAGAGCTGGATAGCTGGATTGATAAGTTTTGCC
TAGATGCTGATGTCTTTGTTTTGGTCGCAAACTCTGAATCAACACTAATGAA
exon 6
TACGGAAAAACACTTTTTTCACAAGGTGAATGAGCGGCTTTCCAAGCCTAAT
ATTTTCATTCTCAATAATCGTTGGGATGCCTCTGCATCAGAGCCAGAATATA
--exon 7
TGGAAGACGTACGCAGACAGCACATGGAAAGATGCCTGCATTTCTTGGTGGA
GGAGCTCAAAGTTGTAAATGCTTTAGAAGCACAGAATCGTATCTTCTTTGTT
exon 8
TCAGCAAAGGAAGTTCTTAGTGCTAGAAAGCAAAAAGCACAGGGGATGCCAG
AAAGTGGTGTGGCACTTGCTGAAGGATTTCATGCAAGATTACAGGAAATTTCA
exon 9
GAATTTTGAACAAATCTTTGAGTTACAAACAGCAAGTTTTTGTGTTTAAATT
CCTGAAAATGTTGCGATGGAATTACAGTAATGTGTTACGGCTTGGGCCCCTC
GCAGGAGTGTATCTCGCAGTCAGCAGTGAAAACAAAGTTCGAACAGCACACT
ATCAGAGCTAAACAGATACTAGCTACTGTGAAAAACATAATGGATTCAGTAA
exon 10
ACCTGGCAGCTGAAGATAAAAGGTTTCATGTGCAATGA
exon 12
D
1L
2L
3L
4L
5L
8L
9L
H23
H125
H226
H838
A549
H661
H520
486 bp
332 bp

Fig. 2

Fig. 3

Fig. 4