Functional Characterization of the Candidate Tumor Suppressor Gene \( \text{NPRL2/G21} \) Located in 3p21.3C

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ABSTRACT

Initial analysis identified the \( \text{NPRL2/G21} \) gene located in 3p21.3C, the lung cancer region, as a strong candidate tumor suppressor gene. Here we provide additional evidence of the tumor suppressor function of \( \text{NPRL2/G21} \). The gene has highly conserved homologs/orthologs ranging from yeast to humans. The yeast ortholog, \( \text{NPR2} \), shows three highly conserved regions with 32 to 36% identity over the whole length. By sequence analysis, the main product of \( \text{NPRL2/G21} \) encodes a soluble protein that has a bipartite nuclear localization signal, a protein-binding domain, similar to the MutS core domain, and a newly identified nitrogen permease regulator 2 domain with unknown function. The gene is highly expressed in many tissues.

We report inactivating mutations in a variety of tumors and cancer cell lines, growth suppression of tumor cells with tet-controlled \( \text{NPRL2/G21} \) transgenes on plastic Petri dishes, and suppression of tumor formation in SCID mice. Screening of 7 renal, 5 lung, and 7 cervical carcinoma cell lines showed homozygous deletions in the 3' end of \( \text{NPRL2} \) in 2 renal, 3 lung, and 1 cervical (HeLa) cell line. Deletions in the 3' part of \( \text{NPRL2} \) could result in improper splicing, leading to the loss of the 1.8 kb functional \( \text{NPRL2} \) mRNA. We speculate that the \( \text{NPRL2/G21} \) nuclear protein may be involved in mismatch repair, cell cycle checkpoint signaling, and activation of apoptotic pathway(s). The yeast \( \text{NPR2} \) was shown to be a target of cisplatin, suggesting that the human \( \text{NPRL2/G21} \) may play a similar role. At least two homozygous deletions of \( \text{NPRL2/G21} \) were detected in 6 tumor biopsies from various locations and with microsatellite instability. This study, together with previously obtained results, indicates that \( \text{NPRL2} \) is a multiple tumor suppressor gene.

INTRODUCTION

Epithelial tumors are the most prevalent and lethal cancers in the world. For example, lung cancer alone kills >150,000 patients each year in the United States and more than a million around the world (1). Loss of heterozygosity involving several chromosome 3p regions, accompanied by chromosome 3p homozygous deletions, is a characteristic feature of most major epithelial carcinomas, such as lung, breast, cervical, oral cavity, ovary, and kidney (2, 3). These changes indicate the involvement of multiple tumor suppressor genes.

We have performed a comprehensive deletion survey of 3p on more than 400 major epithelial carcinoma cases (4–8), using a defined set of markers, combining conventional loss of heterozygosity with quantitative real-time PCR, and comparative genomic and Southern hybridizations. We identified two most frequently affected 3p21.3 regions, LUCA at the centromeric and AP20 at the telomeric border of 3p21.3. Aberrations of either region were detected in more than 90% of the studied tumors. These 3p21.3 aberrations were complex and, in addition to deletions, could involve gene amplifications as well. Homozygous deletions were detected in 10 to 18% of all of the tumors in both the LUCA and AP20 sites. The frequent chromosome aberrations in these regions suggest that they harbor multiple tumor suppressor genes (2, 3).

Analysis of 15 homozygous deletions in the LUCA region permitted us to establish the smallest homozygously deleted region in 3p21.3C, located between D3S1568 (\( \text{CACA2D2} \) gene) and D3S4604 (\( \text{SEMA3F} \) gene). It contains 17 genes that were defined previously as lung cancer candidate tumor suppressor genes. Mapping of 19 homozygous deletions in the 3p21.3T/\( \text{AP20} \) region resulted in localization of its smallest homozygous deletion to the region flanked by D3S1298 and D3S3623. It contains 4 genes, namely \( \text{APRGI, ITGA9, RBSP3, HYA22} \), and \( \text{VILL} \), which need to be analyzed (9).

In this study, we report the functional characterization of the \( \text{NPRL2/G21} \) gene from the LUCA region and its tumor suppressor genes-like features.

MATERIALS AND METHODS

Cell Lines and General Methods. Small cell lung carcinoma cell line U2020 was described earlier (10). The ACC-LC5 small cell lung carcinoma cell line that carried a deletion in 3p21.3 (11) was kindly provided by Dr. Yusuke Nakamura (University of Tokyo, Tokyo, Japan), and the GLC20 small cell lung carcinoma cell line with homozygous deletion in LUCA region was obtained from Dr. Charles H. Buys (12). A498, Caki1, and Caki2 renal clear cell carcinoma lines, 7 cervical carcinoma cell lines (HTB, C33A, HeLa, CaSki, C4–1, MS-75, and SiH2), and the N417 small cell lung carcinoma cell line were purchased from the American Type Culture Collection (Manassas, MD). Lymphoblastoid cell line CBMI-Ral-STO, osteosarcoma cell line Saos-2, non-small cell lung carcinoma line A549, and renal clear cell carcinoma lines KRC-Y, ACHN, TK164, HN4, TK10, and KH-39 were obtained from the MTC-KI (Stockholm, Sweden) cell lines collection (6).

KRC-Y, Caki 1, Caki 2, ACHN, TK10, and TK164 cell lines were karyotyped using trypsin-Giemsa banding, and different cell clones were found (data not shown). Heterogeneity of the small cell lung carcinoma U2020 and renal cell carcinoma KH39 cell lines was shown previously (10, 13).

Molecular cloning of the human \( \text{NPRL2/G21} \) gene was described previously (ref. 14; accession no. AF040707). The structures of \( \text{NPRL2} \) and PCR primers used in the study are shown in Fig. 1.
RESULTS AND DISCUSSION

Specific Aims and Experimental Design. The NPRL2 gene was one of the promising tumor suppressor gene candidates that we analyzed previously (14). Forced, uncontrolled expression of wild-type NPRL2/G21 transgenes from adenovirus vectors inhibited tumor cell growth by inducing apoptosis and cell cycle arrest of non-small cell lung carcinoma cell lines H1299 and A549. Also, intratumoral injection of a NPRL2 expressing adenovirus vector or systemic administration of protease-complexed vectors significantly suppressed the growth of H1299 and A549 tumor xenographs and inhibited A549 experimental lung metastases in nu/nu mice (19). Although suggestive, these results could not be interpreted unequivocally, because adenovirus-mediated overexpression was not controlled and could lead to artificial inhibition of tumor growth.

In this study, we reinvestigated the tumor suppressing function of NPRL2 under more physiologic conditions using the previously described tetracycline-controlled gene inactivation test (18, 20). This test is based on the functional inactivation of the analyzed genes in conjunction with tumor growth in SCID mice. Our hypothesis was that under selective pressure in vivo, the introduced tumor suppressor genes must be inactivated in growing experimental tumors (by deletion, mutation, and promoter methylation) as they are in naturally growing patient tumors.

Because the LUCA region is a frequent target of hemizygous and homozygous deletions in renal cancer (2, 3, 6, 8), we also analyzed NPRL2/G21 for mutations and/or loss of expression in renal cell carcinoma cell lines.

Growth Inhibiting Activity of NPRL2 In vitro and In vivo. To test for the growth suppressing effect of NPRL2/G21, we performed colony formation assays using the KRC/Y renal cell carcinoma cells that were used in our previous studies (3, 17, 18) and showed aberrant expression of NPRL2 (see below). Selection was done for expression

For amplification of RASSFIA gene, the following primers were used: forward, F2a 5’-GCTCCAAAGCC AGCGAAGCAC-3’; and reverse, F2m2 5’-ACCCAGGCAG CCCTCGAGAA-3’ (Fig. 1). The PCR primers were purchased from Invitrogen (Carlsbad, CA). The PCR was performed as described earlier (4). However, we used only 28 cycles for detection of homozygous deletions in NPRL2 gene in both cancer cell lines and tumor biopsies. This was done to reduce the effect of normal cell contamination in the case of biopsies and heterogeneity in the case of cancer cell lines.

PCR products were cloned, using the TOPO TA cloning kit for sequencing (Invitrogen).

All molecular biology and microbiology procedures were performed as described previously (15, 16). Plasmid DNA was purified using the R.E.A.L.-Prep kit (Qiagen, Valencia, CA). Sequencing was done using an ABI 310 Sequencer (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. Methylation studies were performed as described previously (17).

Southern and Northern transfer and hybridization were performed as described earlier (15, 16). To determine the expression pattern of NPRL2/G21 gene in normal tissues, Northern hybridizations with human multiple tissue Northern blots (Clontech, Palo Alto, CA) were performed.

Cell and tumor growth assays were done as described previously (17, 18). NPRL2/G21 open reading frames from PCR-TOPO clones (digested by EcoRI and blunt-ended by Klenow Fragment) were reintroduced into blunt-ended NheI sites of PETE (elimination test episomal plasmid) vectors (18).

Bioinformatics. DNA homology searches were performed using BLASTX and BLASTN programs at the National Center for Biotechnology Information server. Sequence assembling was done using DNASIS (HITACHI-Pharmacia, Freiburg, Germany). The BEAUTY Post-Processor was used with the BLASTP protein databases searches provided by the Human Genome Sequencing Center (Houston, TX). 9 Scanning of the PROSITE and the Pfam A protein families and domains was performed at the Swiss Institute for Bioinformatics. Sequence assembling was done using DNASIS (HITACHI-Pharmacia, Freiburg, Germany). The BEAUTY Post-Processor was used with the BLASTP protein databases searches provided by the Human Genome Sequencing Center (Houston, TX). 9 Scanning of the PROSITE and the Pfam A protein families and domains was performed at the Swiss Institute for Bioinformatics.

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of the Hgy gene, carried by the pETE vector. As a negative control, the empty pETE vector was used. Transfection with pETE containing the RASSF1A gene served as a positive control. Fig. 2 shows that NPRL2/G21 inhibited colony formation similarly to RASSF1A.

For the gene inactivation test assay, described previously (18, 20, 21), the pETE-Hyg vector (containing the complete open reading frame for NPRL2) was transfected in the presence of doxycycline into cells constitutively producing the tetracycline transactivator. This system permitted the modulation of gene expression both in vitro and in vivo. The transfected genes are expressed in the absence but not in the presence of tetracycline or its analog doxycycline. The system simulates physiologic expression levels.

Two tetracycline transactivator-producing cell lines were used: the non-small cell lung carcinoma line cell line A549 and the small cell lung carcinoma line U2020. The two clones chosen showed the best tetacycline regulation (at least 100-fold difference between induced and repressed state; see example in Fig. 3). For the U2020 cell line, pETE-NPRL2 clone 1 (UG1) was chosen and for A549, pETE-NPRL2 clone 1 (AG1).

The UG1 clone was assessed for growth on plastic Petri dishes in the absence or presence of doxycycline. The original cells were used as a control because, in our previous papers (see ref. 3, 17, 18, 20, 21), we showed that empty pETE vector, 3PK, MLH1, mutated RB1, and mutated RASSF1A didn’t exhibit any effect on cell growth. Fig. 4 shows the results. NPRL2/G21 expression suppressed cell growth in the absence of doxycycline (80 to 90% suppression from day 7). A partial growth inhibition effect was also seen in the presence of doxycycline. Leakage of expression and growth inhibition because of the doxycycline effect (22) are the most important reasons for the slower growth of UG1 cells in the presence of doxycycline.

UG1 cells (5 x 10^6) were inoculated into 10 SCID mice, and U2020 cells were used as a control. Previously, we have shown that tetracycline doesn’t affect the growth of parental cell lines (18, 20, 21). Five of the mice were then given 1 mg/ml tetacycline in their water, whereas the remaining five were not. Strong inhibition of tumor growth was seen in the mice, and only 3 large tumors have grown (Table 1; Fig. 4B). The one tumor that grew in a tetacycline-consuming SCID mouse exhibited only a slightly delayed growth rate, compared with the control U2020 cells. It is known that tetracycline is a weaker inhibitor of expression compared with doxycycline in the tetracycline transactivator system, and it is likely that expression leakage is stronger in vivo in SCID mice than in vitro. Even weak expression of the NPRL2 because of the leakage could have a strong growth inhibiting effect.

After 3 weeks, tumors were explanted and tested for the presence of transgenic NPRL2 by PCR. The transgenic NPRL2 was detected in all three samples. These three cases were additionally examined with Northern hybridization. In tumor T3, no expression was detected; although good expression was found in T1, and very weak expression was found in T2 (Fig. 5A). The transgenic NPRL2 gene was sequenced in T1 and T2, and in both cases missence mutations were discovered (in T1 Arg324Gly and in T2 His16Arg; see Fig. 5B). It is likely that these mutations could have led to faster growth of the T1 and T2 tumors after week 3.

In another experiment, AG1 cells were inoculated into 8 SCID mice, and no tumors were observed after 8 weeks (Fig. 6).

These experiments extended our previous observations. They demonstrated that NPRL2/G21 has true growth inhibiting activity both in vitro and in vivo, and not only in non-small cell lung carcinoma line but also in small cell lung carcinoma cell lines.

**Analysis of NPRL2 Gene in Renal Cell Carcinoma Cell Lines.** As described in the previous section, NPRL2/G21 strongly inhibited colony formation by KRC/Y cells. This confirmed our hypothesis that loss or inactivation of this gene may be important for the development of renal cell carcinoma and is the reason why we decided to study this gene in renal cell carcinoma lines more carefully.

We tested 8 renal cell carcinoma cell lines for the methylation of the 5' end of the NPRL2 gene using G21methylF and G21methylR primers (see Fig. 1). Direct bisulfite sequencing analysis did not reveal any methylation.

![Fig. 2. The effect of expression of NPRL2 on colony formation efficiency in KRC/Y cells. Efficiency of colony formation for the pETE vector is taken as 100%. RASSF1A was used as a positive control. Bars, ±SD.](image1)

![Fig. 3. Dox-dependent NPRL2 expression in vitro for transfected U2020 cell clones UG4 and UG1. Gel stained with ethidium bromide was used for loading control (lower panel).](image2)

![Fig. 4. Growth inhibition of U2020 cells by NPRL2 (clone UG1) gene in vitro (A) and in vivo (B). A. An average of three independent experiments is presented. B. The average size of 3 tumors for U2020 and of 2 tumors for UG1 without tetracycline (T1 and T2; see Table 1) is given. For UG1 with tetracycline, growth curve for T3 is shown.](image3)
Then we performed Northern analysis of 7 renal cell carcinoma cell lines. It is known that NPRL2 is expressed in multiple splice isoforms (see Fig. 7 A; ref. 14). The main transcript in normal kidney was found to be ~1.8-kb size. However, in all of the 7 tested renal cell carcinoma cell lines this transcript was absent, and only a ~7-kb transcript was detected (Fig. 7 B).

To understand the basis of these changes, we performed PCR with genomic DNA using different primers located in the NPRL2 gene (see Fig. 7 C and Fig. 1 for location of primers) and found frequent changes in the 3’ part of the gene. In 1 (HN4) of 6 renal cell carcinoma lines shown in Fig. 7 C, the 3’ part was completely absent. The same deletion was found in the renal cell carcinoma cell line ACHN (data not shown). We have found a similar situation in lung cancer cell lines: the 3’ part of NPRL2 was completely absent in U2020 and A549 (Fig. 7 C). One of the tested small cell lung carcinoma lines, GLC20, had the whole LUCA region homozygously deleted. One more homozygous deletion of the 3’ part of NPRL2 was detected in the H647 non-small cell lung carcinoma line (data not shown). Interestingly, the 3’ end of NPRL2 was also homozygously deleted in 1 of 7 (HeLa) cervical carcinoma cell lines. Thus, after screening 7 renal cell carcinoma, 3 small cell lung carcinoma (without GLC20), 2 non-small cell lung carcinoma, and 7 cervical carcinoma lines, we found the 3’ end of NPRL2 homozygously deleted in 6 of them. Additionally, homozygous deletions of NPRL2 were found in the prostate cancer cell line LNCaP and oral squamous carcinoma line SCC15 (data not shown). In all of the of these cell lines (except HeLa and LNCaP), the homozygous deletions included the RASSF1A gene (see Fig. 1 and Fig. 7 C; P2a-F2int2 primers). In HeLa and LNCaP, the homozygous deletions involved only the 3’ end of NPRL2 and did not involve the RASSF1 coding sequences. However, we cannot exclude the possibility that in these cases RASSF1A was inactivated because of deletion of noncoding or 5’ regulatory sequences. Deletions in the 3’ part of NPRL2 gene could result in improper splicing, leading to the loss of the functional 1.8-kb NPRL2 mRNA. Preliminary analysis of expressed sequence tag (EST) databases, as well, revealed the presence of nonsense mutations in NPRL2 clones obtained from tumor cells/tissues. For instance, deletion of 172-176nt downstream from ATG codon was found in lymphoma (BR061866), neuroblastoma (ALS526015), and retinoblastoma (BM413639) cells. A nonsense deletion was found also in small cell lung carcinoma BC008802, and missence mutations were found in uterine (A1884407), colon (BE867114), brain (A1933357, A1199457), and other tumors.

**Conclusions and Hypothesis.** In summary, we found that the NPRL2/G21 gene has growth inhibitory activity for renal cell carcinoma (KRC/Y), small cell lung carcinoma (U2020), and non-small cell lung carcinoma (A549) cell lines when tested under controlled physiologic conditions of gene expression (see ref. 2, 21) both in vitro and in vivo in SCID mice. We have also found mutations in experimental tumors and intragenic homozygous deletions in renal cell carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, and other cancer cell lines (cervical HeLa, prostate LNCaP, and oral squamous SCC15). Preliminary analysis of EST databases also revealed the occurrence of nonsense and missence mutations in NPRL2 clones obtained from lymphoma, neuroblastoma, retinoblastoma, uterine, colon, brain, and other tumors. All of these features are consistent with the conclusion that NPRL2/G21 is a multiple tumor suppressor gene. Its inactivation or loss may favor the development of breast and cervical carcinomas, as we have detected previously frequent homozygous deletions affecting the LUCA region in these tumors (7, 8).

**Fig. 5.** Analysis of SCID tumors. **A,** Expression of NPRL2 in xenografts tested with Northern hybridization. Gel stained with ethidium bromide was used for loading control (bottom panel). +, mouse received water with tetracycline. **B,** mutations in SCID tumors T1 and T2. The mutated positions of NPRL2 gene (accession no. AF040707) are indicated by arrows.

**Fig. 6.** Tumor growth inhibition of A549 cells by NPRL2 (clone AG1) in vivo in SCID mice. The average size of 4 tumors is shown for A549, and growth of 8 inoculates of AG1 is presented.
identity (53% similarity) over 152 amino acids; (2) with 36% identity (50% similarity) over 119 amino acids; and (3) with 36% identity (54% similarity) over 55 amino acids. By sequence analysis, the main product of NPRL2/G21 encodes a soluble protein that has a bipartite nuclear localization signal (residues 62-79), a protein-binding domain called granulin (residues 86-98), weak similarity to the MutS core domain (residues 273-315), and a newly identified domain with unknown function (residues 3-380), which was predicted by protein families database (PFAM) and is present in many proteins. The gene is highly expressed in many tissues, including lung and kidney tissues, with alternative splicing of all of the 11 exons and introns creating a variety of transcripts (altogether 31). These transcripts encode 24 different protein isoforms with different predicted cellular locations. This information suggests that the nuclear NPRL2/G21 protein may be involved in mismatch repair and signaling to cell cycle checkpoints that activate apoptotic pathway(s).

Recently, the yeast ortholog, NPR2 (accession no. P39923), has yeast nitrogen permease regulator activity, which suggests that NPRL2 might also have this activity. NPR2 is involved in posttranslational control of nitrogen permease (24). However, it could be involved in many other vital processes (23, 25). The exact function of the gene in different species should be established by additional experiments.

Inactivating homozygous deletion of the 3’ end of NPRL2 was obvious in breast (357T) and ovarian (585T) carcinoma (Fig. 8). One might suspect that renal cell carcinoma biopsy (347T) also contains a homozygous deletion. In all of the six cases, both 5F-1R and 6F-9R primer pairs produced bands with normal control DNA. Additionally, we have searched public databases with the primer sequences and didn’t find any mutations within these sequences, indicating they could not be a site of frequent polymorphism. To reduce the effect of normal cell contamination, we used only 28 cycles for PCR. These preliminary observations are consistent with the suggestion that NPRL2 could participate in mismatch repair; however, more detailed studies are needed to confirm this hypothesis.

The yeast ortholog, NPR2 (accession no. P39923), has yeast nitrogen permease regulator activity, which suggests that NPRL2 might also have this activity. NPR2 is involved in posttranslational control of nitrogen permease (24). However, it could be involved in many other vital processes (23, 25). The exact function of the gene in different species should be established by additional experiments.

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