The t(1;3) breakpoint-spanning genes *LSAMP* and *NORE1* are involved in clear cell renal cell carcinomas

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Summary

By positional cloning, we identified two breakpoint-spanning genes in a familial clear cell renal cell carcinoma (CCRCC)-associated t(1;3)(q32.1;q13.3): *LSAMP* and *NORE1* (*RASSF1* homolog). Both genes are downregulated in 9 of 9 RCC cell lines. While the *NORE1A* promoter predominantly presents partial methylation in 6 of the cell lines and 17/53 (32%) primary tumors, the *LSAMP* promoter is completely methylated in 5 of 9 cell lines and in 14/53 (26%) sporadic and 4 familial CCRCCs. Expression of LSAMP and NORE1A proteins in CCRCC cell lines inhibited cell proliferation. These characteristics indicate that LSAMP and NORE1A may represent new candidate tumor suppressors for CCRCC.

Introduction

Renal carcinoma is known to have different histological types with distinct genetic profiles (Storkel et al., 1997). Worldwide, approximately 150,000 people are diagnosed with renal carcinoma, resulting in 78,000 deaths annually (Zbar et al., 2003). The most common type is clear cell renal cell carcinoma (CCRCC). Studies of familial CCRCC have led to the identification of important tumor suppressor genes such as *VHL* (Latif et al., 1993). Recently, position cloning also resulted in the discovery of other kidney cancer-related genes, *BHD*, *FH*, and *HRPT2* (Nickerson et al., 2002; Tomlinson et al., 2002; Carpten et al., 2002). While hereditary CCRCCs are mainly attributed to *VHL* mutations, there are known CCRCC families and a significant proportion of sporadic CCRCCs that are not associated with the *VHL* (Teh et al., 1997; Woodward et al., 2000), thus pointing to the existence of other CCRCC-related genes. Because some CCRCC families are associated with balanced chromosomal translocations, the translocation breakpoint-spanning genes are likely CCRCC-related candidate genes. The first CCRCC family with a balanced chromosomal translocation t(3;8)(p14;q24) was described by Cohen et al. (1979). To date, at least eight such hereditary CCRCC-related chromosomal translocation families have been reported (Cohen et al., 1979; Kovacs and Hoene, 1988; Kovacs et al., 1989; Koolen et al., 1998; Podolski et al., 2001; Kanayama et al., 2001). Interestingly, translocation in all these CCRCC families is linked to chromosome 3, making constitutional chromosome 3 translocation a predisposing factor (van Kessel et al., 1999; Bodmer et al., 1998, 2002c). The subsequent observation of the loss of translocation derivative chromosome 3 (der(3) chromosome) and somatic *VHL* mutations in a proportion of familial tumors led to the proposal of a three-step model of CCRCC tumorigenesis (Schmidt et al., 1995; Bodmer et al., 1998, 2002c): initial constitutional chromosome 3 translocation, subsequent somatic loss of the der(3) chromosome leading to the loss of a copy of *VHL*, and a third hit in the form of random somatic mutation in the second *VHL* allele. However, loss of the der(3) chromosome was observed only in a subset of the examined samples. Most of the analyzed familial tumors with loss of the der(3) did not carry *VHL* mutations. Furthermore, neither der(3) loss nor *VHL* mutations were observed in several tumor biopsies in the affected families (Eleveld et al., 2001;

SIGNIFICANCE

Identification of familial CCRCC genes such as the *VHL* tumor suppressor gene (TSG) has provided important insights into the pathogenesis of hereditary and sporadic tumors. Here we demonstrate that *LSAMP* and *NORE1* are the breakpoints of a familial CCRCC-related t(1;3) and that the expression of these genes is downregulated in RCC cell lines and sporadic CCRCCs. LSAMP has no known activities in cancer, but Nore1 is a Ras effector. Further investigations of LSAMP and NORE1 may lead to the elucidation of novel mechanisms of tumorigenesis.

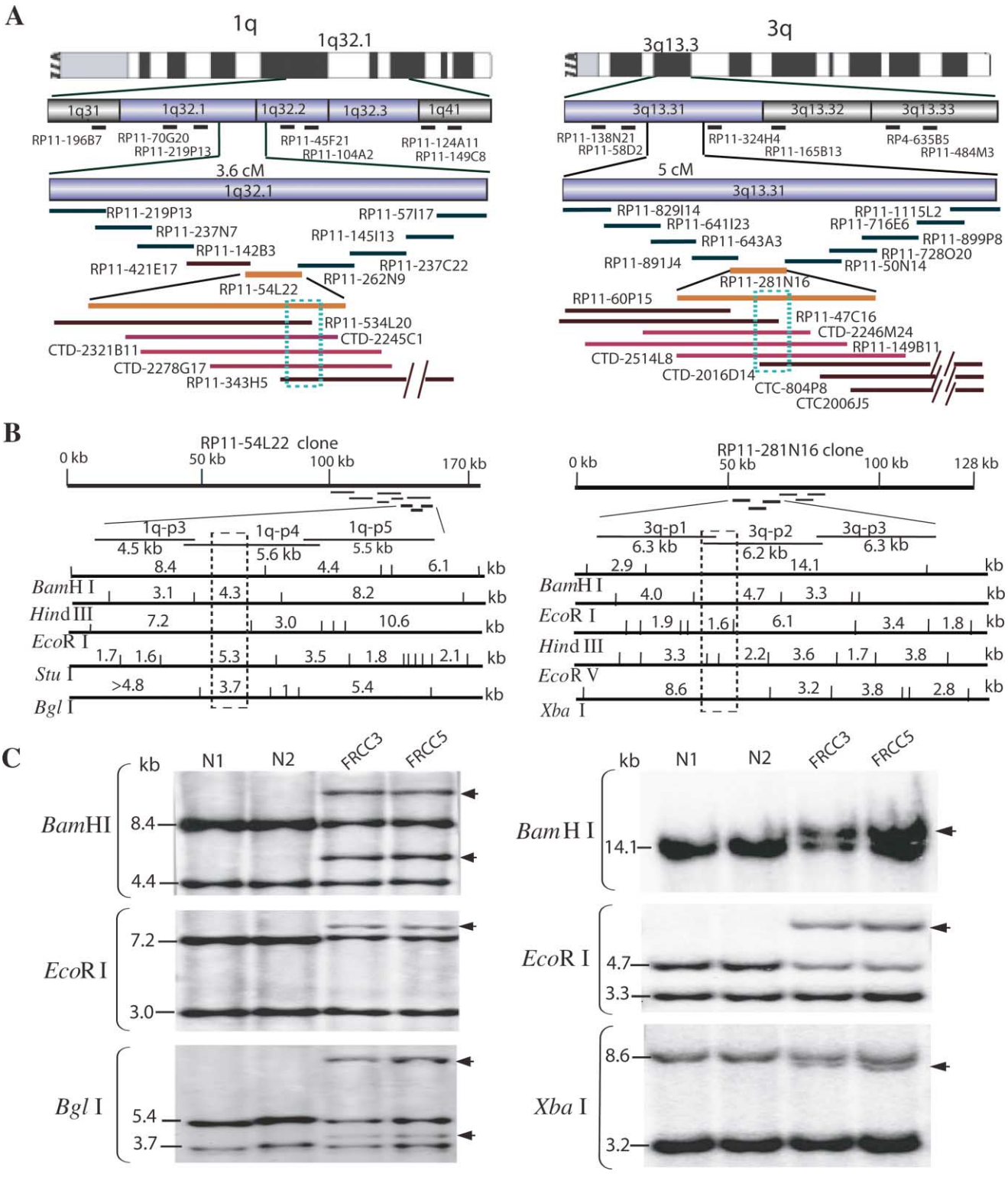


Figure 1. Mapping of the t(1;3) breakpoints on chromosomes 1q32.1 and 3q13.3 by FISH and Southern blot analysis

A: A contig of nine BAC clones in a 3.6 cM region of 1q32.1 was constructed (left panel). The RP11-54L22 was first found to span the breakpoint by FISH split assay. Overlapped BAC clones (CTD-2245C1, -2321B11, and -2278G17) also showed split signals and confine the der(1) breakpoint region to about 30 kb (dotted box). Similarly, a contig of ten BAC clones within a 5 cM region of 3q13.3 was established (right panel); and the breakpoint was found within RP11-281N16 and was further mapped to an about 30 kb region (dotted box) using overlapping clones (RP11-149B11, CTD-2246M24, and -2514L8).

B: Fine mapping of the 1q32.1 and 3q13.3 breakpoints by Southern blot analysis and restriction mapping. Fifteen specific DNA probes (4–10 kb) flanking the 1q and 3q breakpoints were synthesized by long-range PCR with specific primers from known sequence RP11-54L22 and -281N16. Southern blot analyses showed that a 5.6 kb 1q-p4 probe (left panel) and a 6.2 kb 3q-p2 probe (right panel) span the respective 1q32.1 and 3q13.31 breakpoints, which narrowed

Bodmer et al., 2002b). These observations suggest that the breakpoint-spanning genes in the familial RCC-associated chromosome 3 translocations are also likely implicated in RCC tumorigenesis or act synergistically in the above model in the form of genetic and/or epigenetic alterations.

Analysis of the constitutional t(3;8)(p14;q24) translocation associated with familial CCRCC led to the identification and extensive investigation of the breakpoint-spanning gene *FHIT* (fragile histidine triad) on 3p14 (Ohta et al., 1996). *FHIT* is thought to be a putative tumor suppressor gene, and aberrant *FHIT* transcripts and *FHIT* genomic lesions were observed in a variety of primary tumors and tumor-derived cell lines (Ohta et al., 1996; Siprashvili et al., 1997; Druck et al., 1997). The partner breakpoint-spanning gene *TRC8* on the chromosome 8 shows high homology to the *Drosophila* patched (*PTCH*) gene and probably also functions as a tumor suppressor (Gemmill et al., 2002). Also, another two breakpoint-spanning genes, *DIRC1* on chromosome 2q33 and *DIRC2* on 3q21, disrupted, respectively, in t(2;3)(q33;q21) and t(2;3)(q35;q21) breakpoints, have been identified (Druck et al., 2001; Bodmer et al., 2002a). The role of these genes in CCRCC tumorigenesis remains to be determined.

Here we described the positional cloning of the t(1;3)(q32.1;q13.3) chromosomal breakpoints and the identification of two breakpoint-spanning genes, *LSAMP* (limbic system-associated membrane protein gene) on 3q13.3 and *NORE1* on 1q32.1 in a previously reported Japanese hereditary CCRCC family (Kanayama et al., 2001). *LSAMP* encodes a neuronal surface glycoprotein that belongs to the IgLONs (immunoglobulin LSAMP, OPCML/OBCAM, and neurotrimin) family and is distributed in cortical and subcortical regions of the limbic system (Pimenta et al., 1996). To date, very little is known about *LSAMP* and its biological role remains unclear. However, its family partner gene *OPCML/OBCAM* on 11q25 was recently found to be epigenetically inactivated and was regarded as a candidate TSG in epithelial ovarian cancer (Sellar et al., 2003). *NORE1* was recently identified as a homolog of the tumor suppressor gene *RASSF1* at 3p21.3, which is frequently inactivated via promoter hypermethylation in a variety of human tumors (Dammann et al., 2000; Tommasi et al., 2002). The mouse counterpart Nore1 is a Ras effector (Vavvas et al., 1998).

Results and Discussion

We have previously mapped the constitutional t(1;3)-associated breakpoints to bands 1q32.1 and 3q13.3 in a family with four cases of CCRCC (Kanayama et al., 2001). In this study, we cloned the breakpoints of t(1;3)(q32.1;q13.3) by using a strategy that combined FISH, Southern blot, long-range PCR, and DNA sequencing. FISH experiments enabled us to narrow the breakpoint regions to a 20 to 30 kb range on both affected chromosomes (Figure 1A). These were further refined via Southern blot analyses and restriction mapping to approximately 2 kb (Figures 1B and 1C). Assisted by information from human genome sequence databases and BAC clone databases, we designed several sets of specific primers around the breakpoints and performed long-range PCR to amplify the breakpoint fragments (Figure 2A). A 2.15 kb der(1) breakpoint and a 3.25 kb der(3) breakpoint were amplified and subcloned into TA-cloning vector (Invitrogen) (Figure 2A). Subsequent DNA sequencing of the breakpoint fragments resulted in the identification of both breakpoints (Figure 2; also see Table S1 of the Supplemental Data at http://www.cancercell.org/cgi/content/full/4/5/405/DC1).

The cloning of the breakpoints led to the identification of two breakpoint-spanning genes, *NORE1* on 1q32.1 and *LSAMP* on 3q13.3 (Figure 2B). To investigate whether fusion proteins resulting from the chromosome translocation are involved in tumorigenesis of CCRCC, we carried out Northern blot analysis and RT-PCR to detect any fusion transcript of *NORE1* and *LSAMP* (see Table S1 on *Cancer Cell* website). No detectable fusion transcripts were found in the FRCC3 and FRCC5 cell lines from two patients in the t(1;3) family. We also tested the possible sequence combination from *NORE1* and *LSAMP*. Because *NORE1* lies in the positive DNA strand and *LSAMP* in the reverse strand, there is little likelihood for them to form any *NORE1-LSAMP* or *LSAMP-NORE1* fusion proteins.

Given the association between chromosome 3 translocations and CCRCC susceptibility (van Kessel et al., 1999; Bodmer et al., 2002c), we next investigated the gene *LSAMP. LSAMP* is composed of seven exons and is disrupted in intron 2 by the breakpoint (Figure 2B). To elucidate whether genetic changes in *LSAMP* play a role in CCRCC, we performed *LSAMP* mutation analysis in 9 CCRCC cell lines and in 53 sporadic and 4 familial tumors. No *LSAMP* mutation was detected. However, epigenetic silencing in association with hypermethylation, the most common form of inactivation for many tumor suppressor genes (Jones and Baylin, 2002), could still occur. First, by RT-PCR, we found that LSAMP was downregulated in all 9 RCC cell lines (Figure 3A). We further demonstrated that the *LSAMP* promoter was methylated in 7/9 CCRCC cell lines (78%), 14/53 sporadic CCRCCs (26%), and all 4 familial CCRCCs tumors from the t(1;3) family (Figure 3B). In association with the promoter-methylation status, LSAMP expression in ten examined tumors with *LSAMP*-promoter methylation was also downregulated (Figure 3A). Of the *LSAMP*-promoter-methylated cell lines and tumors, all presented complete methylation except two cell lines and one sporadic tumor. Furthermore, in the four familial tumors (FT1 to FT4), one *LSAMP* allele was breakpoint disrupted followed by the loss of the der(3) chromosome shown in our previous study (Kanayama et al., 2001), and the other copy was hypermethylated (Figure 3B), implying that *LSAMP* may undergo bi-allelic inactivation. These observations suggest that *LSAMP* may be involved in CCRCC, though further functional studies are needed to elucidate its mechanism.

The 1q32.1 breakpoint-disrupted gene, *NORE1*, also appeared to be an excellent candidate CCRCC suppressor gene. *NORE1* undergoes alternative splicing, resulting in two isoforms, *NORE1A* and *NORE1B*. The breakpoint disrupted both *NORE1A*

both breakpoint regions to approximately 6 kb. Restriction mapping refined the 1q and the 3q breakpoints to about 1.5 kb (left panel) and 2 kb regions, respectively (right panel).

C: Representative Southern blot analyses from both chromosomes showing distinct aberrant bands (indicated by arrowheads) after restriction digestion. DNA from two normal controls (N1, N2) and two patients (FRCC3 and FRCC5) were completely digested and subjected to DNA hybridization analysis.

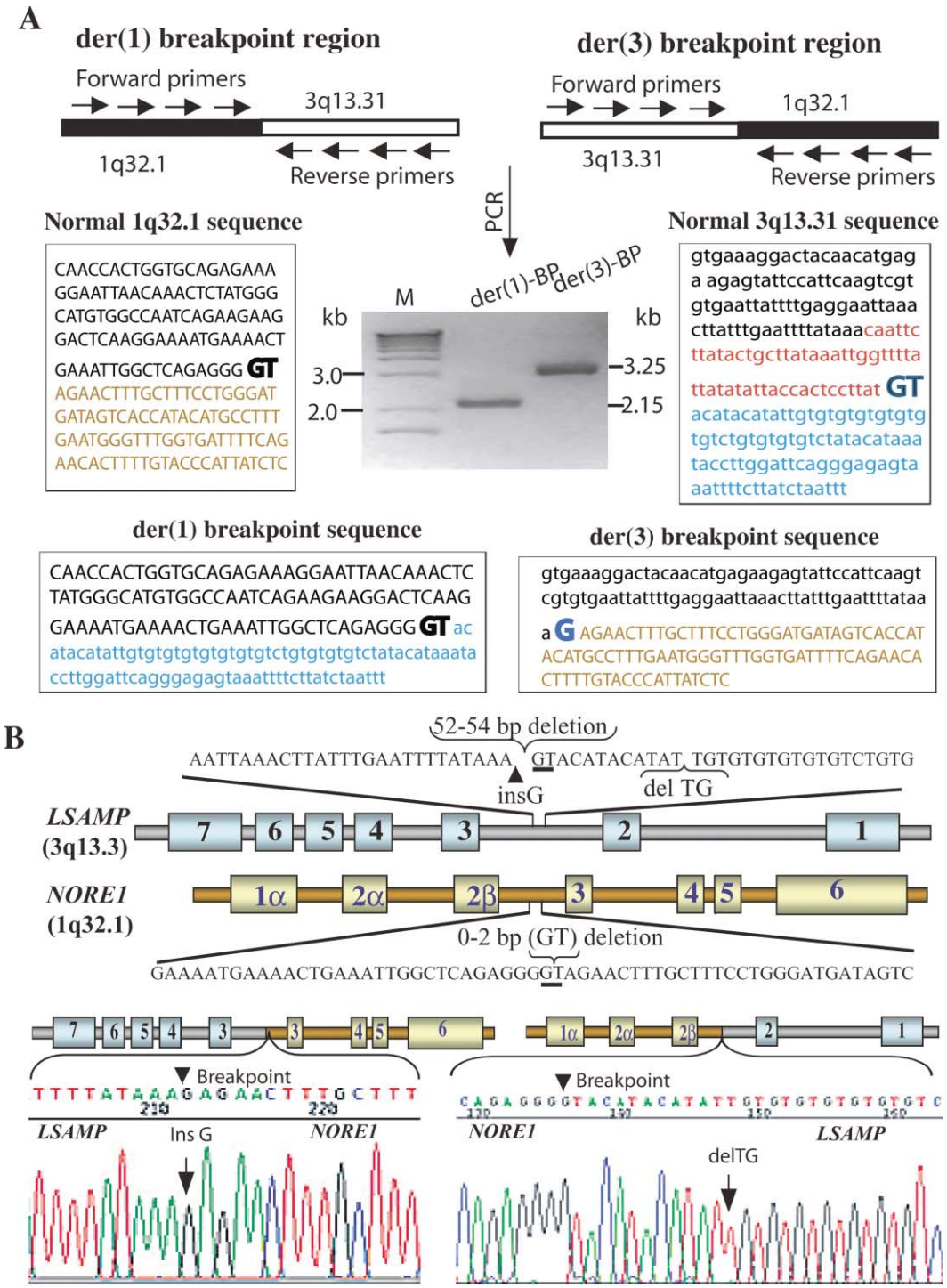


Figure 2. Cloning of both der(1) (1q32.1) and der(3) (3q13.31) breakpoints through long-range PCR and DNA sequencing

A: Amplification of der(1) and der(3) breakpoints via long-range PCR. A 2.15 kb der(1) breakpoint fragment (der(1)-BP) and a 3.25 kb der(3) breakpoint fragment (der(3)-BP) were amplified. The breakpoint fragments were sequenced and are shown in the lowest boxes. The normal sequences around the breakpoints on 1q32.1 and 3q13.31 are also shown for comparison. The uppercase sequences are from 1q32.1 and the lowercase sequences are from 3q13.31. The sequences in red on 3q13.31 are deleted from the breakpoints.

B: Schematic illustration of the identification of breakpoint-spanning genes. The translocation breakpoints occur within intron 2 of both breakpoint-spanning genes *LSAMP* at 1q32.1 and *NORE1* at 3q13.31; this is accompanied by loss of 52 or 54 bp (red sequences in panel A) from *LSAMP* and of 2 or 0 bp from *NORE1*. An insertion of nucleotide G (ins G) in the breakpoint junction and a loss of 2 bp (delTG) in *LSAMP* in the distal part of breakpoint were also observed. *NORE1* has two isoforms, *NORE1A* and *NORE1B*. *LSAMP* contains seven exons and sits in the reverse strand of chromosome 3.

and *NORE1B* (Figure 2B). NORE1 is homologous to a family of RAS binding proteins, including RASSF1, rat Maxp1, and murine Nore1 (Vavvas et al., 1998; Dammann et al., 2000; Vos et al., 2000; Ortiz-Vega et al., 2002; Tommasi et al., 2002) that have been proposed to be effectors for the small GTPase or Ras. Nore1 and RASSF1 have been shown to induce apoptosis (Vos 2000; Khokhlatchev et al., 2002). Other studies, however, have shown that Nore1 family members are cytostatic and modulate cyclin D1 levels, thereby influencing the activity of cell cycle-dependent kinases (Khokhlatchev et al., 2002). *RASSF1* maps to 3p21, a region of frequent loss of heterozygosity (LOH) in CCRCC (van den Berg and Buys, 1997; Dammann et al., 2000), and this gene has recently been shown to be epigenetically inactivated in kidney cancer (Dreijerink et al., 2001; Morrissey et al., 2001; Yoon et al., 2001). Thus, we proceeded to investigate *NORE1* as a candidate RCC suppressor.

Similarly, we performed mutation screening and methylation analysis on *NORE1* in all the RCC cell lines and tumors. Two alterations, GTG(Val189)>ATG(Met189) and CGG(Arg248)>CAG(Gln248), were identified (see Supplemental Figure S2 on *Cancer Cell* website). The former was present in 5% of the 100 tested normal subjects, whereas the latter was not found in any of them. As both were also present in the matched normal kidney tissues, it is likely that they represent polymorphisms. We then perceived that *NORE1A* expression was also downregulated in the 9 RCC cell lines, and the *NORE1A* promoter was methylated in 6/9 RCC cell lines and 17/53 (32%) sporadic RCC tumors (Figures 3A and 3C), whereas methylation in the *NORE1B* promoter was detected only in RCC cell lines A-498 and A-704. *NORE1A* expression in examined 10 of the 17 affected tumors was also downregulated (Figure 3A). Two normal kidney control samples (N3 and N44) also showed *NORE1A* promoter methylation at lower extents compared with their matched tumors (3T and 44T), probably due to contamination from the tumor tissues. Interestingly, *NORE1A*-promoter methylation does not overlap with *LSAMP*-promoter methylation except in four tumors. These results suggest that *NORE1A* may be also associated with sporadic CCRCC. Yet, unlike the methylation situation in *LSAMP*, only 1/4 hereditary tumors showed even slight *NORE1A* promoter methylation, indicating that one wild-type allele of *NORE1A* still exists in these hereditary tumors. Whether *NORE1A* undergoes haploinsufficiency in tumorigenesis remains indeterminate.

In addition, 7/14 tumors (50%) with *LSAMP*-promoter methylation showed LOH of the *LSAMP* locus. However, LOH was also observed in 17/39 tumors (44%) without *LSAMP*-promoter methylation. Similar LOH results were obtained on *NORE1A* (methylated, 5/17 [29%]; unmethylated, 8/36 [22%]), indicating that the LOH may be correlated with CCRCC tumorigenesis but is not methylation dependent.

Promoter methylation in both *LSAMP* and *NORE1A* may also be linked to other types of cancers. *NORE1A*-promoter methylation has recently been detected in cancer cell lines and in 24% NSCLC (Hesson et al., 2003). Here we found that the *LSAMP* promoter was methylated in 5/19 (26%) colorectal cancers.

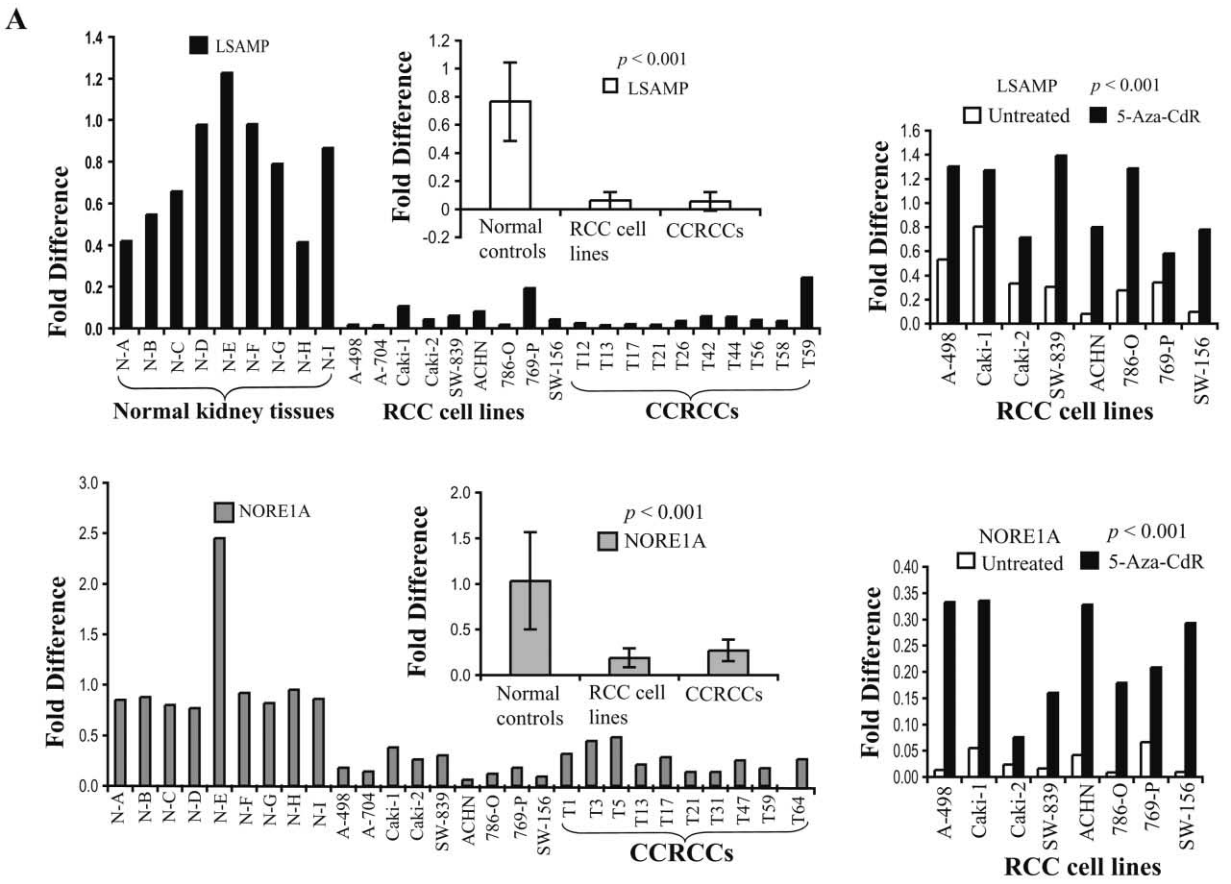
The exact role of these genes in tumorigenesis is unclear. In the familial cases, however, the underlying mechanism is still the three-step model of chromosome 3 translocation-related hereditary CCRCC tumorigenesis (Bodmer et al., 1998, 2002c; Kanayama et al., 2001). Considering the complexity of the multistep process in tumorigenesis, the possibility exists that the breakpoint-disrupted genes, especially *LSAMP*, may contribute to the occurrence of familial tumors by acting as components in the three-step model of tumorigenesis of hereditary CCRCC. We have previously demonstrated that four examined familial CCRCC tumors lost the der(3) chromosome and two of them carry *VHL* mutations, supporting the three-step model of tumorigenesis (Kanayama et al., 2001). Here, we supplement this model with our *LSAMP* and *NORE1A* data. The constitutional translocation t(1q;3q) and disruption of a copy each of *LSAMP* and *NORE1*, as the first set of steps of tumorigenesis, act as the predisposing factors in development of CCRCC. The translocation also results in the increased susceptibility to somatic loss of the chromosome der(3). The following nondisjunctional loss of der(3) deletes a copy each of the RCC-related genes in chromosome 3 (e.g., *VHL*, *RASSF1A*), which further increases the predisposition to CCRCC. This second set of steps will accelerate the transformation process and cellular growth, leading to the third set of steps involving either the inactivation of the other *VHL* allele (e.g., somatic mutation) or the genetic/epigenetic alterations in other CCRCC-related genes, including *LSAMP*, in the remaining copy of chromosome 3. These factors may act synergistically and finally lead to the occurrence of CCRCC.

Epigenetic inactivation of these genes can be reversed by demethylation treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-CdR). The demethylation treatment resulted in significantly increased expression of LSAMP and NORE1 in eight cell lines (Figure 3A), indicating that repression is at least in part mediated by methylation.

Finally, to further evaluate the role of *LSAMP* and *NORE1* as tumor suppressor candidates in cancer, enhanced green fluorescent protein *EGFP-LSAMP*, *-NORE1A*, and *-Nore1* expression plasmids were microinjected or transfected into two RCC cell lines, A-498 and/or Caki-1, in which the *LSAMP* and *NORE1A* promoters were methylated. Cells were then counted at indicated times and were monitored for cell number and/or proliferation. Alternatively, cells were monitored by epi-fluorescence/phase-contrast microscopy to evaluate proliferation, fluroescent protein expression, or apoptosis. While cells expressing EGFP continued to proliferate at rates similar to those of uninjected neighbors, cells expressing EGFP-LSAMP, -NORE1A, and -Nore1 failed to grow (Figure 4B). There was no evidence of apoptosis in any of the experiments. This growth inhibition role was also demonstrated in 293-T cells stably transformed with an inducible *Nore1* gene by lipid-mediated transfection (see Supplemental Figure S3c on the *Cancer Cell* website).

We also observed that EGFP-LSAMP seemed to be cytoplasmic, and EGFP-NORE1A appeared in both cytosol and nucleus. EGFP-Nore1 was predominantly nuclear and tended to occupy discrete puncta within the nucleus (Figure 4A). This was observed in both formaldehyde-fixed and living cells; thus, the localization was unlikely to be due to a fixation artifact. Furthermore, the nuclear localization of EGFP-Nore1 was also confirmed in the transfected Caki-1 RCC cell line and in the 293-T cells by nuclear fractionation (see Supplemental Figures S3a and S3b online).

Our observations are consistent with a growth suppression role for LSAMP, NORE1A, and Nore1. Also, despite the presence of a putative Ras-association region, our results suggest



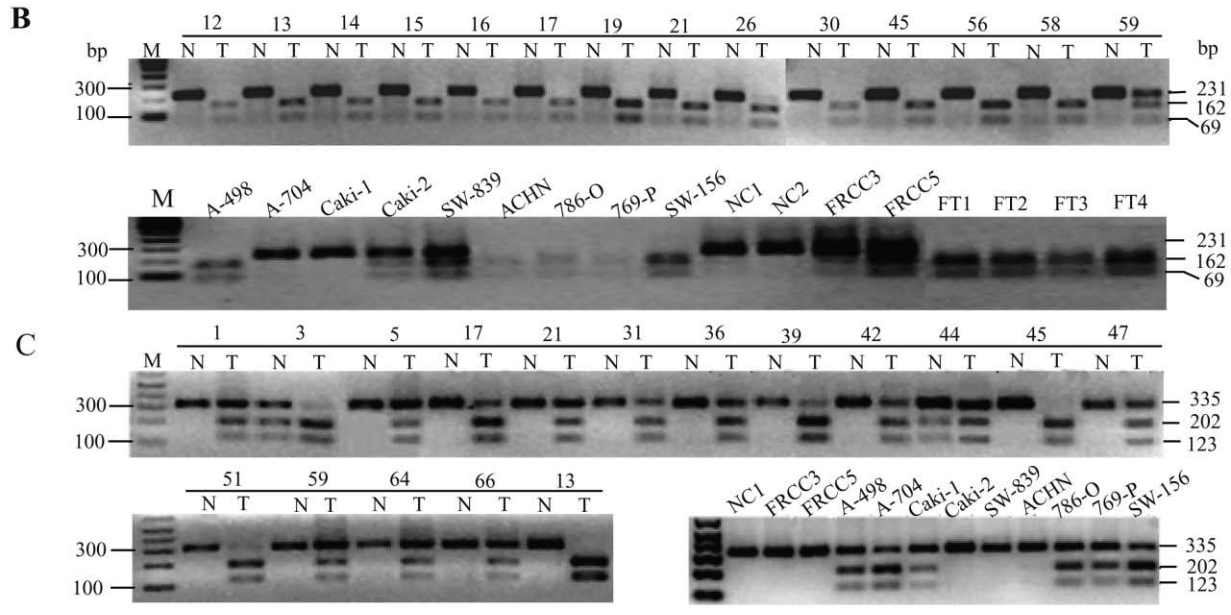


Figure 3. Lower expression and promoter methylation of *LSAMP* and *NORE1A* in RCC cell lines and sporadic RCC tumors

A: The expression of LSAMP and NORE1A in nine RCC cell lines and sporadic tumors (LSAMP: 0.06 ± 0.06 for cell lines and 0.05 ± 0.07 for tumors; NORE1A: 0.19 ± 0.10 for cell lines and 0.27 ± 0.12 for tumors) is significantly lower than that in nine normal kidney tissues (LSAMP: 0.77 ± 0.28; NORE1A: 1.03 ± 0.53) using real-time PCR assay (t test of SSPS, $p < 0.001$). Right panel, eight RCC cell lines were demethylated using 5-aza-CdR and the expression of both *LSAMP* and *NORE1A* was significantly increased in each line (LSAMP: untreated, 0.35 ± 0.23; 5-aza-CdR, 1.02 ± 0.33; NORE1: untreated, 0.03 ± 0.02; 5-aza-CdR, 0.24 ± 0.10) (t test of SSPS, $p < 0.001$).

B: Methylation analysis of the *LSAMP* promoter. Bisulfite-treated DNA from 53 matched pairs of human CCRCC tumors and normal DNA samples, 9 RCC cell lines, 2 t(1;3)-positive lymphoblastoid cell lines, and 2 control lymphoblastoid cell lines (NC1 and NC2) were amplified and digested with HhaI. The *LSAMP* promoter (540 bp) contains 28 CpG islands. The analyzed 231 bp fragment of the *LSAMP* promoter contains one HhaI site, and digestion leads to

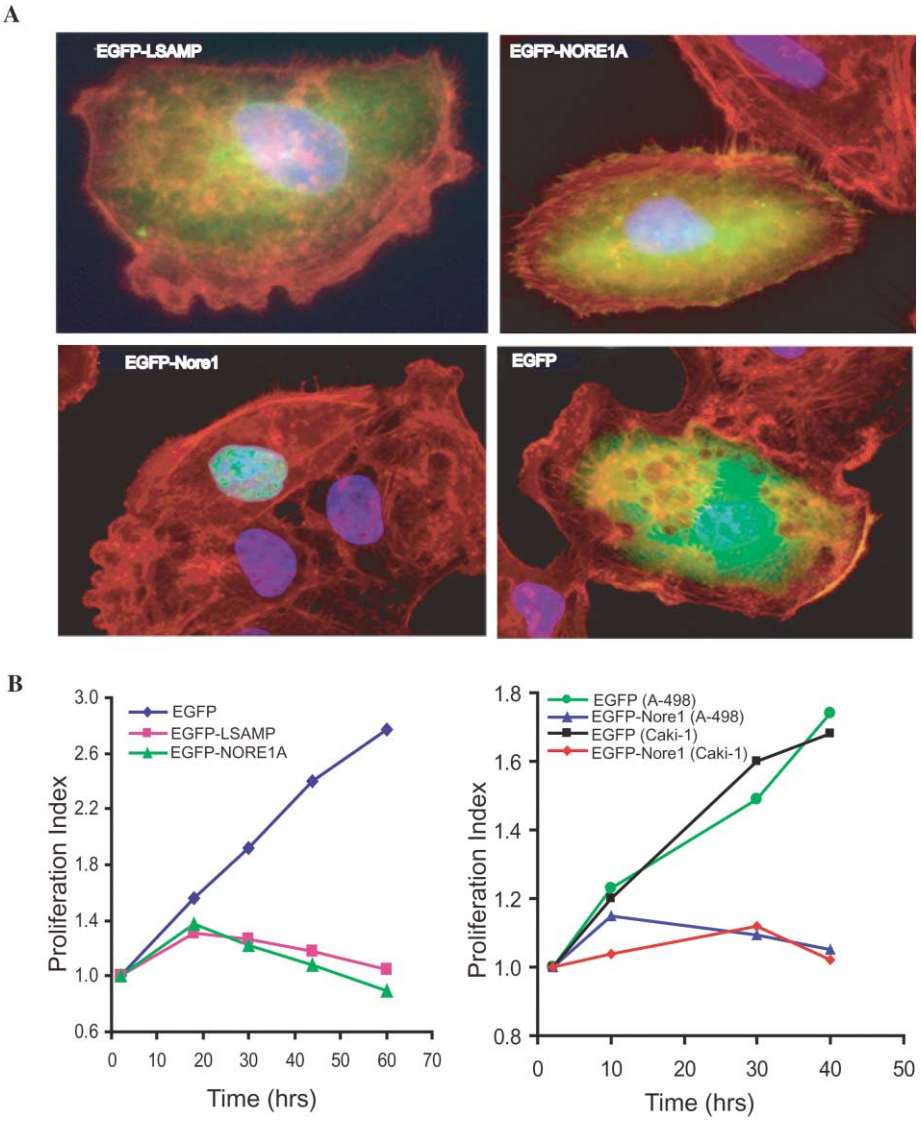


Figure 4. Suppression of LSAMP, NORE1A, and Nore1 re-expression on cell proliferation characteristics

A: Re-expression and localization of EGFP-LSAMP, -NORE1A, and -Nore1 fusion protein 2 hr after microinjection or 24 hr after lipid-mediated transfection of *pEGFP-LSAMP, -NORE1A,* and *-Nore1* plasmids.

B: Growth inhibition assay. A-498/Caki-1 cells were microinjected with *pEGFP-LSAMP, -NORE1A, -Nore1,* or *pEGFP-C1/-N1* vector (negative control). Cell proliferation analysis was performed 2 hr after microinjection. Cells were counted at the indicated times. The "proliferation index" on the *y* axis represents the number of cells counted at those times divided by the number of cells counted 2 hr after injection.

that this nuclear Nore1 protein may not be a bona fide Ras effector, whose family members tend to be lipid modified, membrane bound positive regulators of cell proliferation. Further investigation into its role in growth regulation (potentially through the regulation of cyclin D1 and G1/S progression) and its role in the nucleus are needed.

Based on these data, LSAMP and NORE1A (a homolog of 3p21 tumor suppressor RASSF1A) may represent new tumor suppressor candidates and may act as components in the multistep process of CCRCC tumorigenesis. Inactivation or reduced expression of both LSAMP and NORE1A may be also involved in the occurrence of other types of tumors. Further studies of these genes may lead to the elucidation of novel mechanisms of tumorigenesis.

Experimental procedures

Family with CCRCC and t(1;3)(q32.1;q13.3), paired CCRCC tumors/normal kidney tissues, and cell lines

The clinical and genetic details of the Japanese kindred with familial CCRCC have been previously published (Kanayama et al., 2001). The EBV-transformed lymphoblastoid cell lines FRCC3 and FRCC5 used in this study were established from two affected translocation carriers.

Four tumors were from three members of the t(1;3) family, and 53 matched pairs of CCRCC were collected from the University of Tokushima in Japan.

fragments of 162 bp and 69 bp. Representative aberrant methylation of the *LSAMP* promoter in sporadic and familial CCRCC samples and in RCC cell lines are shown.

C: Methylation analysis of the *NORE1A* promoter by restriction digestion with TaqI in the same cohort of samples. The examined 335 bp of the promoter contains 35 CpG sequences. The methylated fragment contains two TaqI sites and digestion results in bands of 202, 123, and 10 bp. The sizes of molecular weight markers (M) are shown on the left. N, normal kidney sample; T, RCC.

Nine established RCC cell lines were purchased from ATCC: A-498, A-704, Caki-1, Caki-2, SW-839, ACHN, 786-O, 769-P, and SW-156.

Construction of BAC contigs and FISH analyses

Fourty-four 1q32.1 and 3q13.3 BAC clones were obtained from the BACPAC Resource Center (Children's Hospital, Oakland Research Institute) or ResGen Invitrogen Corporation. The clones were selected based on information in the BAC clone mapping databases and Human Genome Sequence Draft database. The details of the BACs are listed in the Supplemental Experimental Procedures available on the *Cancer Cell* website.

Standard dual-color FISH was performed by hybridizing each of the 44 BAC clones to metaphase slides prepared from FRCC3 or FRCC5. In all hybridizations, the PAC clone 160H23 from the 1q subtelomere (Cytocell Ltd, United Kingdom) was included as a marker of the normal chromosome 1 and the der(3) chromosome.

Long-range PCR, Southern blot analysis, and Northern blot analysis

Long-range PCR was used for the amplification of the breakpoints and the generation of DNA probes for Southern blot analysis with an Advantage Genomic PCR kit (Clontech). PCR was carried out following the manufacturer's user manual. Southern blot and Northern blot analyses were performed following the standard protocol. Human multiple tissue Northern blots were purchased from Clontech (Cat. #7780-1). Details of these analyses can be found in the Supplemental Experimental Procedures online.

Mutation analysis

Mutation analysis of *LSAMP, NORE1A*, and *NORE1B* was performed in the 53 sporadic CCRCCs and 9 RCC cell lines. Each exon of *LSAMP*, *NORE1A*, and *NORE1B* was amplified by PCR using primers derived from the flanking intronic or UTR sequences (see Supplemental Table S1 on the *Cancer Cell* website). The PCR products were then purified and subjected to direct DNA sequencing using PE Applied Biosystems.

Real-time quantitative RT-PCR

Total RNA from normal kidney tissues, RCC cell lines, and sporadic tumors was subjected to real-time quantitative PCR using an ABI PRISM 7700 Sequence Detection System. Specific primer and probe were designed for *LSAMP* and *NORE1A* using Primer Express v1.5a (Applied Biosystems). The primer sequences and the details of the real-time RT-PCR analysis are described in the Supplemental Experimental Procedures online.

DNA methylation analysis and demethylation treatment by 5-aza-2′-deoxycytidine (5-aza-CdR)

Methylation analysis was performed for the promoter CpG islands of *LSAMP* and *NORE1A*. Bisulfite-PCR followed by restriction enzyme digestion analysis was used. Eight RCC cell lines were demethylated by 5-aza-CdR (Sigma) treatment. The primers and the details of the analyses are given in the Supplemental Experimental Procedures.

LOH analysis

LOH detection for *LSAMP* and *NORE1* was performed by genotyping the 53 paired normal/tumor DNA samples. The microsatellite markers flanking the *LSAMP* locus are *D3S3681*, *D3S1271*, *D3S1267*, and *D3S1292*. *NORE1* locus markers include *D1S413* and *D1S249*. All the markers were obtained from ABI Prism Linkage Mapping Set version 2, panels 1 and 2 (Applied Biosystems). The details of LOH analysis are described in the Supplemental Experimental Procedures available online.

Cell growth assay

Expression plasmids *pEGFP-LSAMP, -NORE1A*, and *-Nore1* were generated by ligating cDNAs of *LSAMP, NORE1A*, and murine *Nore1* to N- or C-terminal enhanced green fluorescent protein vectors (*pEGFP-N1/-C1*) (Clontech). Expression plasmids were microinjected and transfected into two RCC cell lines, A-498 and/or Caki-1, for cell growth-suppression assay. Inducible experiments and nuclear fractionation assays were also performed for the nuclear location of Nore1. Detailed methods are provided in the online Supplemental Data.

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Accession numbers

The *NORE1A*, *NORE1B*, and *LSAMP* cDNA sequences have been deposited in GenBank with accession numbers NM_182663, AF445801, and NM_002338, respectively; *RASSF1A*, AF132675; *Nore1*, AF053959; VHL, NM_000551.