

Identification of Somatic Mutations of the *RNF6* Gene in Human Esophageal Squamous Cell Carcinoma

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Abstract

We mapped a tumor suppressor gene locus to an 800-kb interval on human chromosome 13q12.11 for esophageal squamous cell carcinoma (ESCC). Two genes, *ML-1* and *RNF6*, are located within this 800-kb interval. We analyzed both genes for the presence of mutations in 24 ESCC primary tumors and 16 tumor cell lines by directly sequencing the PCR products that were amplified from each exon. No mutation was detected in *ML-1*. In contrast, three somatic mutations in the *RNF6* gene were detected in the ESCC primary tumors, and one mutation was also found in a tumor cell line. Identification of multiple somatic mutations in *RNF6* suggests that *RNF6* is a potential tumor suppressor gene involved in the pathogenesis of ESCC.

Introduction

Human ESCCs² occur frequently worldwide (1). ESCC is an aggressive tumor with a poor prognosis. People in the high-risk regions such as northern China were much more likely to develop this cancer than individuals residing in low-risk areas of the world. Within the high-risk regions, there was a strong tendency toward familial aggregation, suggesting that genetic susceptibility, in conjunction with environmental exposures, might play a role in the etiology of ESCC. Both genetic factors and environmental factors were associated with ESCC. The environmental factors included smoking, alcohol, dietary inadequacies, and consumption of fermented foods. Multiple genetic changes, including mutations in p53, p15, p16, and overexpression of cyclin D1, have been identified (2–4). A genome-wide scan for LOH in ESCC was performed previously, and several regions, including chromosomes 3p, 5q, 9p, 9q, and 13q, showed very frequent LOH (5). The LOH in chromosome 13q was additionally refined to 13q12.11 by using a panel of 56 pairs of ESCC primary tumors and their matched normal DNAs (6). By using the human genome draft sequence, this region was narrowed down to an 800-kb interval.

Two genes, *ML-1* (ATPase, NM_016529) and *RNF6* (Homo sapiens ring finger protein 6, NM_005977), were mapped to this 800-kb interval. *ML-1* encodes an aminophospholipid transporter-like ATPase. It was identified in an experiment by Sun *et al.* (7) who screened for antisense cDNAs that could convert nontumorigenic cells into a tumorigenic phenotype. The *RNF6* gene was identified by cloning of genes that mapped near a chromosome 13 breakpoint at t(4;13)(q26;q12) in a myelofibrosis patient (8). The *RNF6* gene encodes a Zn-finger protein that belongs to the RING-H2 family. Zn-

finger domain is often found in DNA binding proteins. We report here mutational analysis of the two candidate genes, *ML-1* and *RNF6*.

Materials and Methods

Cell Lines. Genomic DNA for eight human cancer cell lines was purchased from American Type Culture Collection (Manassas, VA). These cell lines included: 45508 (lung cancer), 45512 (lung cancer), 45518 (breast cancer), 45522 (breast cancer), 45528 (breast cancer), 45530 (breast cancer), 45534 (breast cancer), and 45520 (breast cancer). We also obtained eight cancer cell lines, which included CCL-185 (lung cancer), CRL-1435 (prostate cancer), CRL-1848 (lung cancer), CRL-2220 (prostate cancer), CRL-2221 (prostate cancer), CRL-5803 (lung cancer), CRL-5809 (lung cancer), and HeLa (cervical cancer). Genomic DNA was extracted from cultured cells by first harvesting confluent cells in TE9 [500 mM Tris-HCl (pH 9.0), 20 mM EDTA, and 10 mM NaCl]. The cells were then digested overnight with proteinase K (final concentration 0.2 mg/ml) in the presence of SDS (final concentration 0.2%) at 50°C. This was followed by phenol-chloroform extraction and then ethanol precipitation.

ESCC Primary Tumor Samples. We used previously a panel of 56 pairs of ESCC primary tumors and their matched normal DNAs to refine a minimal LOH region to chromosome 13q12.11 (6). Twenty-four ESCC primary tumors that showed LOH in the 800-kb critical region were selected from these 56 ESCC patients. Tumor tissues were microdissected using a laser capture microdissection method (9), and matched normal DNAs were isolated from blood. The entire coding region of the *RNF6* gene and 13 of 14 exons of the *ML-1* gene were analyzed by directly sequencing the PCR products amplified from the tumor DNAs.

PCR and Sequencing of Tumor DNA. The exon and flanking intron sequences of the *ML-1* and *RNF6* genes were obtained from the human draft sequence.³ Primer3 software was used to design the primer pairs for each exon (10). Thirteen pairs of primers were used for the mutational analysis of *ML-1* and 7 pairs of primers were used for *RNF6*. The complete list of the primers is described in Table 1. PCR was carried out in a 25- μ l reaction mixture containing 1 \times Buffer (Applied Biosystems, Foster City, CA, USA), 1.5 mM Mg²⁺, 0.2 mM deoxynucleotide triphosphate, 0.5 μ M primers, 5 ng genomic DNA, and 1 unit Taq DNA polymerase (Applied Biosystems). Initial denaturing was at 95°C for 2 min, then 40 cycles of 95°C for 45 s, 60°C for 30 s, and 72°C for 60 s, followed by extension at 72°C for 10 min. After PCR amplification, the DNA fragments were purified using the QIAquick purification kits (Qiagen, Inc., Valencia, CA). When PCR reactions yielded more than one band, the band with the correct size was cut out and purified using the QIAEX II Gel Extraction kit (Qiagen). Two primer sets, ML-1_13A/ML-1_13B and RNF6_4A/RNF6_4B, required purification using QIAEX II. The PCR fragments were subjected to sequencing reactions using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), and the sequencing was conducted with an ABI PRISM 3100 or 3700 Genetic Analyzer (Applied Biosystems). Analysis of the sequencing traces was conducted using the Sequencher software (Gene Code Corporation, Ann Arbor, MI) and Phred/Phrap (11).

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² The abbreviations used are: ESCC, esophageal squamous cell carcinoma; LOH, loss of heterozygosity; SNP, single nucleotide polymorphism.

³ Internet addresses: <http://www.ncbi.nlm.nih.gov/> and <http://genome.ucsc.edu/>.

Table 1 Primers and sequences

| Primers | Direction | Sequence | PCR sizes (bp) | Codons in the amplicon |
|----------|-----------|-------------------------------|----------------|------------------------|
| ML-1_1A | Forward | agatctcactatgaagatggtttgtacc | | |
| ML-1_1B | Reverse | tgtataagcacgggatacagacacagc | 446 | 163..220 |
| ML-1_2A | Forward | cattcccgtactcttctcagtgaaatgg | | |
| ML-1_2B | Reverse | tgaaggttaaacgccaggtctctcatgc | 342 | 221..281 |
| ML-1_3A | Forward | gagattcctcgggaatctgttttctctc | | |
| ML-1_3B | Reverse | tacatgtgaccaccggctgtagaagatc | 388 | 282..318 |
| ML-1_4A | Forward | cattcccgtactcttctcagtgaaatgg | | |
| ML-1_4B | Reverse | aaggaatgtcactgaagttcttaagttc | 220 | 319..343 |
| ML-1_5A | Forward | tcattttggtagaagggcaacaatgaagc | | |
| ML-1_5B | Reverse | ttggcagatgactagaatcaaacagcg | 274 | 344..384 |
| ML-1_6A | Forward | tgttgagagtttccacagaggtcaagcg | | |
| ML-1_6B | Reverse | aggccagaactgatgaaacctaacagc | 345 | 385..411 |
| ML-1_7A | Forward | ctctgagttaaataggtggccatgctc | | |
| ML-1_7B | Reverse | ttagagaagtgcaagccacacataagc | 195 | 412..431 |
| ML-1_8A | Forward | aagctagttctggagtgagagatggg | | |
| ML-1_8B | Reverse | tattacgtgccaagcaaatgaatcacc | 293 | 432..450 |
| ML-1_9A | Forward | ataaggaaggttagcttggatgcaag | | |
| ML-1_9B | Reverse | tctcctgaagctcatcatggctgtaag | 330 | 451..486 |
| ML-1_10A | Forward | atattatccttgattacattatggtgc | | |
| ML-1_10B | Reverse | gaaagacaattccagggacagatgagc | 231 | 487..516 |
| ML-1_11A | Forward | atgcatggagactgtgaaagctccacc | | |
| ML-1_11B | Reverse | agggccgggagcacctgtgttctgtgc | 305 | 517..550 |
| ML-1_12A | Forward | gtgaagcaccacgccttaccgcagctg | | |
| ML-1_12B | Reverse | attgcctacactcaagctgcacgttcc | 415 | 551..582 |
| ML-1_13A | Forward | cagcatcagcagctagaaacctggggc | | |
| ML-1_13B | Reverse | CGAGGGAAATGTAACCTAACAACTGC | 295 | 583..613 |
| RNF6_2A | Forward | atggcttttaggcaagaagtgaatgccc | | |
| RNF6_2B | Reverse | ttctccttatcttggaaacctctccatc | 497 | 1..65 |
| RNF6_3A | Forward | ggttactaaaagattagctcttacccttc | | |
| RNF6_3B | Reverse | tgcaagtgcctccaatcaagcctgttcc | 232 | 66..97 |
| RNF6_4A | Forward | agaatgaggatttctccatagcgttgc | | |
| RNF6_4B | Reverse | TGGAATGCTGTATAAATCTTCTCCATG | 489 | 98..164 |
| RNF6_4C | Forward | GAGAGTTTCGGTTTAGTTTGAAATCC | | |
| RNF6_4D | Reverse | ACGGCTTCGACTATTGGAAGTAGATCG | 503 | 155..304 |
| RNF6_4E | Forward | CAACGGTTTGAGCAGCACATGTTTGG | | |
| RNF6_4F | Reverse | CTAAACGAGAAATGGTTCGGCGAAAGC | 529 | 282..441 |
| RNF6_4G | Forward | GAACTCGATCCAGAGTAGGGCTAGCAG | | |
| RNF6_4H | Reverse | AGCAAGCGAAGAATGGGTAGTGTTC | 509 | 430..580 |
| RNF6_4I | Forward | CTCATACTCGAAAACAGTGACAGTAGGG | | |
| RNF6_4J | Reverse | TGGTTAGTCAATCAAAAATAACTCAGC | 473 | 568..685 |

Results and Discussion

Mapping of Chromosome 13q11-q13 LOH to an 800-kb Interval. A tumor suppressor locus for ESCC was mapped previously to human chromosome 13q11 through LOH studies (5, 6). One LOH region was defined by markers D13S787, D13S1243, D13S283, and D13S221. To define a critical LOH region, we first selected a marker that showed the most frequent LOH. This identified D13S221, which showed LOH in 89% of the tumors. We next selected flanking markers that showed the largest increase in the retention of heterozygosity among these tumors that showed LOH at D13S221. D13S283 was selected on the centromeric side. By mapping these markers onto the human genome draft sequence,³ we were able to refine the critical LOH region to an 800-kb interval between D13S283 and D13S221. Two genes, *ML-1* (NM_016529) and *RNF6* (NM_005977), were mapped to this 800-kb interval.

Mutational Analysis of *ML-1* Gene. *ML-1* was first identified in an experiment that showed its antisense cDNA construct could cause a tumorigenic phenotype after transfection into nontumorigenic cells (7). Thus, *ML-1* represents a candidate tumor suppressor gene. The transcript of *ML-1* contains 2177 bases, and the coding region contains 614 amino acids. The gene spans at least 14 exons (Fig. 1A). We were able to delineate 13 exons using the human genome draft sequences. However, the first 498 bases were in a gap region of the human genome draft. We synthesized 13 pairs of primers that could amplify each of the 13 exons including both the exonic sequences and ~100 bp of the intron sequences that flank the exons (Table 1). We then amplified each of the 13 exons from 24 primary ESCC tumors and 16 tumor cell lines, and directly sequenced the PCR products. No mutation was detected in *ML-1*. We have identified one nonsynonymous SNP, V185M (G565A), and one synonymous SNP, T193T (C591A; Table 2). V185M represents a conservative substitution. Our mutation analysis indicated that *ML-1* is unlikely to be the tumor suppressor gene at this locus.

Mutations of the *RNF6* Gene in Primary ESCC Tumors and Tumor Cell Lines. The *RNF6* gene was cloned by virtue of its proximity to a chromosome 13 breakpoint at t(4;13)(q26;q12) in a myelofibrosis patient (8). It contains 3503 nucleotide bases, whereas its corresponding protein contains 685 amino acid residues. *RNF6* has a RING-H2 finger at the COOH-terminal end, and, thus, it may function as a DNA-binding protein. We were able to delineate all four

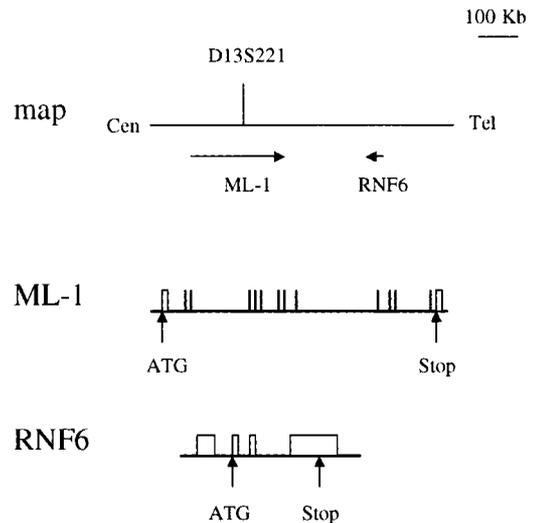


Fig. 1. Map and gene structures of *ML-1* and *RNF6*. Top, D13S221 shows the highest frequency of LOH. The line with an arrow indicates the transcript direction. Cen denotes the centromeric end, whereas Tel denotes the telomeric end. Middle, the vertical lines and open boxes are exons of the *ML-1* gene. The arrows point to start and stop codons. Bottom, *RNF6* gene. The labels are the same as for *ML-1*. The genomic structures of the two genes are displayed from 5' to 3'.

Table 2 Mutations and SNPs in *RNF6* and *ML-1* genes

| Gene | Amino acid change | Nucleotide change | Mutation or SNP | Genotype and tissue | Sequences near the mutation or SNP |
|-------------|-------------------|-------------------|-----------------|--|------------------------------------|
| <i>RNF6</i> | R102K | G665A | Mutation | A in ESCC 034T | gaagtccctaG/Aagaaagttca |
| <i>RNF6</i> | A242T | G1084A | Mutation | A/G in ESCC 308T | aattgggggaG/Acagctggcat |
| <i>RNF6</i> | G244D | G1091A | Mutation | A in ESCC 273T | ggagcagctgG/Acattcctcga |
| <i>RNF6</i> | S623N | G2228A | Mutation | A in HeLa | gagcataacaG/Atattgatagt |
| <i>RNF6</i> | N48S | A503G | ns SNP | G in 5 ESCC | aatgaactcaA/Gtgatgaagat |
| <i>RNF6</i> | I164T | T851C | ns SNP | T/C in ATCC_45522 and ATCC_45528 | ggatttgaaaT/Ctcatggagaa |
| <i>RNF6</i> | R572Q | G2075A | ns SNP | A in 1 ESCC and G/A in its match normal | aggcagttgcG/Aaaatccaaac |
| <i>RNF6</i> | G127G | A741G | SNP | G in 5 ESCC | ctcgaagtggA/Gcaaaatggga |
| <i>RNF6</i> | C635C | T2265C | SNP | C in CRL1848, CRL2221, ATCC_45512 C/T in CCL-185 | gtagtgtttgT/Cattagtgact |
| <i>ML-1</i> | V185M | G565A | ns SNP | G/A in CRL2221 | ggaaaatgacG/Atggccctcat |
| <i>ML-1</i> | T193T | C591A | SNP | A in CRL185, CRL-2200 | atggccacacC/Actgaagtaag |

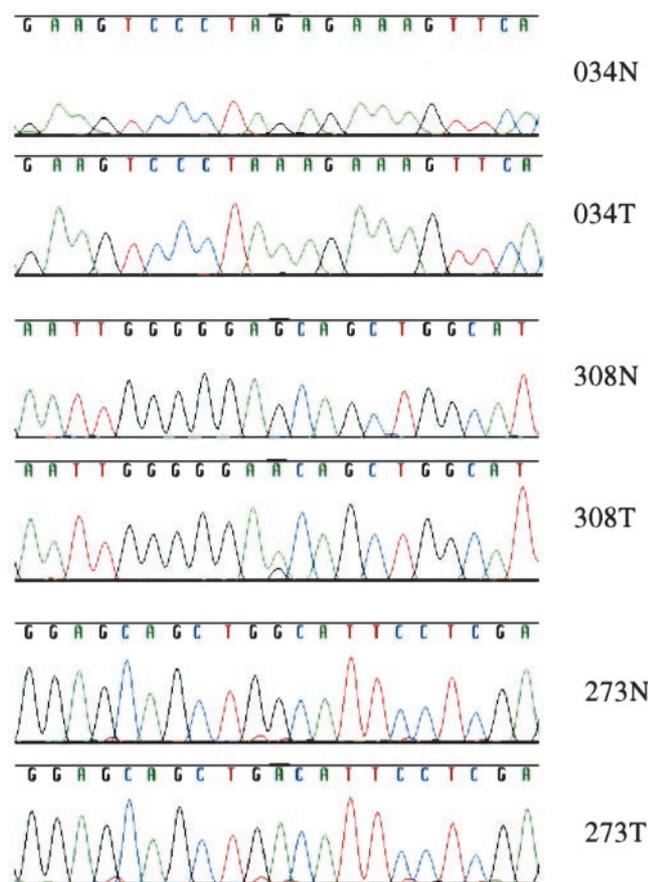


Fig. 2. The sequence chromatographs of somatic mutations. Both normal and tumor sequences are shown here. The mutations are in the center flanked by 10 bases on each side. *N* denotes normal and *T* denotes tumor. The sequences can also be found in Table 2.

of the exons by comparing *RNF6* cDNA sequence with the human genome draft sequence (Fig. 1B). We synthesized seven pairs of primers to span the entire coding region of *RNF6* (Fig. 1B; Table 1). The fourth exon required five overlapping PCR amplicons to cover the coding region. We amplified all of the coding exons from 24 primary ESCC tumors and 16 tumor cell lines. We found three mutations in three ESCC primary tumors (Fig. 2; Table 2) and one mutation in a tumor cell line (Table 2). The amino acid changes in the mutations were R102K, A242T, G244D, and S623N. We also analyzed the blood DNAs from the three patients with mutations in the *RNF6* gene, and all contained the wild-type allele (Fig. 2), thus indicating that the mutations in the ESCC primary tumors were acquired during tumorigenesis in somatic cells. *RNF6* contains three

domains: an arginine domain (amino acids 292–424), a poly-aspartic acid domain (amino acids 598–601), and a Zinc finger domain (amino acids 632–673). The mutation S623N, which is located near the Zinc finger domain, may affect its DNA-binding activity. Both A242T and G244D may perturb the structure and function of the *RNF6* protein. In the first case, the side chain of amino acid Thr is larger than the amino acid Ala. In the second case, the amino acid substitution gains a negatively charged residue. R102K is a conservative substitution as R and K are both positively charged residues. In addition, three nonsynonymous SNPs (N48S, R572Q, and I164T) as well as two synonymous SNPs (G127G and C635C) in the *RNF6* gene were also identified (Table 2). These SNPs were not found in the dbSNP database, thus they represent novel SNPs. The significance of the nonsynonymous SNPs will be analyzed in an epidemiological case-control study. In conclusion, we detected four mutations in the *RNF6* gene from 24 primary tumors and 16 tumor cell lines. Identification of multiple mutations in the *RNF6* gene in ESCC tumors suggests that *RNF6* is a potential tumor suppressor gene involved in the pathogenesis of ESCC.

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