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TUMOR-SPECIFIC EXON CREATION OF THE *HELLS/SMARCA6* GENE IN NON-SMALL CELL LUNG CANCERS

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ABSTRACT

In an attempt to identify tumor suppressor genes on chromosome 10 in non-small cell lung cancers, we isolated 10 types of splicing variants of the *HELLS/ SMARCA6* gene transcripts. HELLS/SMARCA6 is a novel member of SNF2 family, which is implicated in cellular function like chromatin remodeling. Variant 1 was an alternatively spliced isoform containing an insertion of a 44-ntd intronic sequence between exons 3 and 4, giving rise to a premature termination of translation. The expression of the variant 1 was detected exclusively in the lung cancer specimens (11 of 43 cases, 26%), but was not detected in corresponding normal tissues. D10S520 marker in the proximity of the *HELLS/SMARCA6* gene showed prevalent allelic loss (41%) as compared with flanking markers (25-31%). These results suggest that loss of function of HELLS/SMARCA6 by allelic loss and aberrant proteins by tumor-specific exon creation may result in epigenetic deregulation, leading the lung cells to malignancy or its progression.

Key words: alternative splicing; HELLS; LOH; lung cancer; SMARCA6

INTRODUCTION

Lung cancer is one of the leading causes of cancer-related deaths in the world. Several areas of frequent allelic loss have been found in lung cancers (1-4). Recently, by a high-resolution, genome-wide search of loss of heterozygosity (LOH), new regions of allelic loss have been identified in lung cancers (5, 6). Allelic loss at human chromosome 10q23-24 has been frequently observed in a variety of solid tumors containing lung cancer (7-17). The region is also known as a breakpoint region frequently detected in human leukemia cells (18). Recently, the *HELLS*/*SMARCA6* gene was identified on chromosome 10q24.2. *SMARCA* genes (SWI/SNF2-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A genes) are members of the *SNF2* gene family. The SNF2 family members have helicase and ATPase activities, and are implicated in many features of cellular function, including chromatin remodeling, recombination, methylation and transcription (19-22). The multiple functions of SNF2 family members suggest that these genes may play important roles as tumor suppressor genes, similar to such genes as *BRM/SMARCA2* (23), *BRG1/SMARCA4* (24, 25), and *HLTF/SMARCA3* (26). BRM/SMARCA2 protein inhibited proliferation by binding with RB to induce cell cycle arrest (23). Mutations of the *BRG1/SMARCA4* gene were detected in prostate, breast, pancreas and lung cancer cell lines (24), and it was shown that

loss of BRG1/BRM was correlated with poor prognosis of lung cancer (25). The *HLTF/SMARCA3* gene has been reported to be a tumor suppressor gene candidate and a common target for gene silencing in colon cancers (26). HELLS/SMARCA6 was first reported as a putative helicase in murine lymphocytes (27). The helicase domains of HELLS/SMARCA6 closely resemble those of mammalian RAD54, another member of the SNF2 family, whose mutations are associated with tumorigenesis (28, 29). Recently, it has been reported that HELLS/SMARCA6 plays an important role in DNA metabolism, cellular proliferation, CpG methylation and histone methylation (30-32), and an alternatively spliced isoform of this gene has been implicated in leukemogenesis (33). We analyzed expression of the *HELLS*/*SMARCA6* gene in primary non-small cell lung cancers (NSCLCs) and found tumor-specific exon creation of the *HELLS*/*SMARCA6* gene. Furthermore, we found prevalent loss of the *HELLS/SMARCA6* locus in NSCLCs.

MATERIAL AND METHODS

Clinical samples

Surgically resected tissues from primary lung tumors and corresponding normal tissues were obtained from patients with lung tumors at Department of Surgical Oncology and Thoracic

Surgery, Okayama University Hospital, after acquisition of written informed consent from all patients.

DNA and RNA extraction

Genomic DNAs were isolated from tumor and normal tissues by SDS/proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation. Total RNAs were isolated from rapidly frozen samples by using ISOGEN reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions.

Reverse transcription-PCR (RT-PCR) and cDNA sequencing

Oligo(dT)-primed cDNA was synthesized using the ReverTra Ace kit (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. The whole coding region of the *HELLS/SMARCA6* cDNA was amplified into 4 overlapping fragments by RT-PCR with 4 pairs of the following primers: sense 5'-CCG GGT GAG TGT CCA GGC A and antisense 5'-CCG TCG CAT CAC TCC TCC AG for fragment A (693-ntd, exons 1-8); sense 5'-CCG GAA GTG TAA TGG TCA GC and antisense 5'-TCA AGA GCA ACA TCA GAC TTC A for fragment B (771-ntd, exons 8-13); sense 5'-GCC AGA TGT ATT TGA TGA CTT GA and antisense 5'-CCG TGT TGA AGC TGT GCA TGT for fragment C (788-ntd, exons 12-17); and sense 5'-TGG ACA TTT TGA TGG ATT ACT GC and antisense 5'-AAA CCC AAT

ACA GGG AAA TCA C for fragment D (701-ntd, exons 17-22). Fragment D was used for comparing the expression level of the gene. The gene expression in tumor and paired normal samples was compared with expression of the glyceraldehyde phosphate dehydrogenase (*GAPDH*) gene as an internal control. For mutation analysis, PCR products were processed with the Big Dye terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3100 sequencer (Applied Biosystems).

Nested PCR analysis for alternative splicing variants

Each PCR product was cloned into pBluescript and sequenced. In order to define and screen the alternative splicing isoforms, nested RT-PCR was performed using the following primers: sense primer designed in exon 3 (5'-GAG TCG ACA GAA ATT CGG TAC C) and antisense primer in exon 5 (5'-CTG GCT TCT CTT CAC TTG CAT C) for a 44-ntd insertion between exons 3 and 4 (variant 1). Primers used for specific detection of other variants are available on request. The amplified products were separated by 2% agarose gel electrophoresis to identify each variant.

Loss of heterozygosity (LOH) analysis

To evaluate allelic loss at chromosome 10q23-24, highly polymorphic microsatellite markers D10S520 (about 60 kbp telomeric to the *Hells*), D10S1680 (about 700 kbp centromeric) and

10q23MS3 (about 600 kbp telomeric) were used. PCR primers for D10S520 and D10S1680 are available from the internet genome database (http://gdbwww.gdb.org). For the 10q23MS3 marker (GenBank accession number AB118108), forward primer 5'-CAA GGC ACT AAG CTC TGT ATT C and reverse primer 5'-TAT CCT GAC TGC CTG TGG TAG were used. The forward primers were labeled with iodoacetamidofluorescein at the 5'-end and used for PCR. The PCR products were analyzed on a PRISM 3100 Analyzer with GeneScan Analysis software version 3.7 (Applied Biosystems). LOH was scored if one of heterozygous alleles showed at least 70% reduced intensity in tumor DNA as compared with the corresponding normal DNA.

Immunohistochemistory

Primary lung tumors fixed in 4% paraformaldehyde were embedded in paraffin and cut 5 μm increments. They were stained with hematoxylin and eosin, and immunostained with goat polyclonal antibody against carboxyl terminus of Lymphoid-Specific Helicase, Lsh/Hells (M-15, x100; Santa Cruz Biotechnology, Santa Cruz, CA) using avidin-biotin-horseradish peroxidase complex method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine.

Statistical analysis

For analysis of relationship between clinical data and *SMARCA6* variants, Fisher's exact test and Spearman's correlation coefficient test were used.

RESULTS

Expression of the Hells/SMARCA6 gene in NSCLCs

In order to identify the tumor suppressor genes for NSCLCs on chromosome 10q23-24, the expression level of the *HELLS/SMARCA6* gene was analyzed by semi-quantitative RT-PCR with mRNA from the 34 tumor tissue samples and corresponding normal tissue samples. Decreased expression of the *HELLS/SMARCA6* gene was detected in 9 of the 34 tumor tissues (26%) as compared with those of the matched normal tissues (data not shown). In 18 of the 34 tumor tissues (53%), a similar level of the gene expression was observed in tumors and matched normal tissues. Increased expression of the *HELLS/SMARCA6* gene was found in 7 of 34 (21%) tumor tissues.

Mutation analysis of the HELLS/SMARCA6 gene

We amplified the *HELLS/SMARCA6* transcripts by RT-PCR with four pairs of primers to produce four fragments A, B, C and D, and sequenced these to find mutations in NSCLCs. No mutations were detected, but we found a single nucleotide polymorphism (SNP) at codon 77

(ATA or ATC) that yielded no amino acid change (data not shown).

Identification of 10 splicing variants of the HELLS/SMARCA6 gene

As RT-PCR and sequencing analyses of the *HELLS/SMARCA6* gene suggested the existence of some splice variants, each fragment of the RT-PCR products from some of the tumor samples was cloned into plasmids and sequenced. We found ten alternatively spliced variants (GenBank accession numbers AB102716-AB102723, AB113248 and AB113249). Variant 1 showed a 44-ntd insertion between exons 3 and 4, which was detected in 11 of 42 plasmid clones of fragment A (26.2%). Sequencing analysis revealed that this 44-ntd insertion was derived from intron 3 (Figure 1). No mutation was detected in the exon-intron boundaries and intron 3 (data not shown). Variant 2, which shows a splicing out of exon 6, was detected in 9 of 42 clones (21.4%) (Figure 2). A partial deletion of exon 10 (variant 3) was detected in 3 of 38 clones of fragment B (7.8%). Variant 4, which shows a splicing out of exons 10 (3'-half), 11 and 12, was detected in 4 of 38 clones (10.5%). A skipping of exon 11 (variant 5) was detected in 1 of 38 clones (2.6%). A deletion of both exons 11 and 12 (variant 6) was detected in 8 of 38 clones (15.8%). Variant 7, which shows a deletion of the 3'-half of exon 10 and a 15-ntd insertion between exons 10 and 11, was detected in 1 of 38 clones (2.6%). A 138-ntd insertion between exons 12 and 13 (variant 8) was detected in 1 of 38 clones (2.6%). Variant

9, which has a 75-ntd deletion in exon 18, was detected in 8 of 42 clones (19%) of fragment D. Variant 10, which does not splice intron 20 out, was detected in 3 of 42 clones (7.1%). Variants 1, 2, 5, 7, 9 and 10 cause frameshifts, and result in premature termination of translation, while variants 3, 4, 6 and 8 retain the open reading frame (Figures 1 and 2).

Detection of splicing variants by nested PCR analysis

In order to analyze the expression of these splicing variants in NSCLCs and corresponding normal tissues, we performed RT-PCR analysis specific for each variant. Variant 1 was detected in 11 of 43 (26%) NSCLC tissues, however, interestingly, we could not find this variant in any of the 43 normal lung tissues we examined. Representative cases are shown in Figure 1D. We found a cryptic blanching point which has a conserved flanking sequence (ccctgac) identical to the consensus sequence, and a polypyrimidine tract located in the intron just before the 44-ntd sequence. No mutation was detected in the exon-intron boundary and intron 3. Furthermore, we analyzed expression level of the *hnRNP A2* and *B1* genes in NSCLC tissues by RT-PCR, as it has been reported on association of expression ratio of the *hnRNP A2/B1* genes with RNA splicing error (34, 35) and association of the elevated *hnRNP B1* mRNA level with human lung cancers (36). However, the expression of *hnRNP A2* and *B1* was detected with similar level in tumor samples with and without variant 1, and no

remarkable differences of expression level were detected between tumor and normal tissues (data not shown). We examined the relationship between the variant 1 expression and clinical data (Table I). However, there was no significant correlation between the expression and the clinical data. The variant 1 was also found in 6 of 8 lung cancer cell lines (data not shown). Other variants (2 to 10) were detected in both tumor and normal tissues. Detection frequency of the alternative variants and representative cases are shown in Figures 2 and 3, respectively.

Loss of heterozygosity (LOH) around the HELLS/SMARCA6 gene

We analyzed allelic loss with 3 microsatellite markers at chromosome 10q23-24 in 37 primary NSCLCs along with corresponding normal lung tissues (Figure 4). Microsatellite marker D10S520, which is located near the *HELLS/SMARCA6* gene, showed highly-frequent LOH in 11 of 27 (41%) informative cases. However, LOH with the flanking markers at a distance of about 700 kbp from D10S520 was shown in only 6 of 24 (25%) with centromeric marker D10S1680, and in only 9 of 29 (31%) with telomeric marker 10q23MS3. In particular, cases 117 and 119 showed LOH only at D10S520, and cases 195, 106, 134 and 140 exhibited common LOH at D10S520, suggesting the existence of tumor suppressor genes in this region. Of 11 tumors that expressed the variant 1 of the *HELLS/SMARCA6*, 7 showed LOH (64%), 3 were non-informative, and only 1 sample showed retention of the D10S520 locus. There was

no significant correlation between the expression level of the *HELLS/SMARCA6* gene and allelic loss at D10S520.

Immunohistochemical analysis of HELLS/SMARCA6 expression

We examined the expression of HELLS/SMARCA6 protein in some specimens of NSCLCs with commercial antibody against the carboxyl terminal region (Figure 5). In 5 cases of the tumor in which the variant 1 transcript was detected, the protein expression was almost same level as that of 5 tumor cases in which the variant 1 transcript was not detected. We found no difference of the immunostaining signal in the tumor and corresponding normal lung tissues.

DISCUSSION

In this study, we identified 10 splicing variants of the *HELLS/SMARCA6* gene in NSCLCs. It is remarkable that variant 1, with a 44-ntd insertion between exons 3 and 4, was detected exclusively in tumor samples (26%, 11/43 cases) but not in corresponding normal tissues. There were no registered expressed sequence tags (EST) harboring the 44-ntd sequence in the NCBI hEST database, indicating that the 44-ntd is a truly intronic sequence. A cryptic blanching point and polypyrimidine tract, which we detected in intron upstream of the 44-ntd insertion, might be used for formation of splicing lariat structure upon the alternative splicing. Previous results, that this protein is required for normal CpG methylation and is crucial for

formation of normal histone methylation (31, 32), and our results, that showed the frequent loss of this locus, suggest that this gene product may normally function as a tumor suppressor. As the variant 1 mRNA is expected to produce a prematurely-terminated polypeptide of 97 amino acid residues, the peptide may function in a dominant-negative manner against the normal HELLS/SMARCA6 protein (838 aa). It may be supported by the recent report that the nuclear localization domain of this protein is located between at least amino acids 31 and 176 (37). It will be desired to perform transfection experiments with the variant 1 and/or full-length *HELLS/SMARCA6* cDNA expression vectors, and the protein expression analysis with customized HELLS/SMARCA6 antibodies, in order to clarify these unsolved issues. Confirmation of the variant 1 protein by western blotting was not performed, because the commercial antibody was made against the carboxyl terminal region of HELLS/SMARCA6.

The *hnRNP A2* and *B1* genes have been reported to be involved in RNA splicing, transport (34, 35), and are associated with human lung cancers (36). Therefore, we analyzed the ratio of *hnRNP A2/B1* gene expression in NSCLCs. However, the expression was at almost the same level in most samples, suggesting that the expression of the *hnRNP A2* and *B1* genes is not correlated with the production of variant 1. However, we can not exclude the possibility that the up-regulation of hnRNPA2/B1 occurs at the post-transcriptional level (38).

It has been reported that the alternative isoform with 75-ntd deletion in exon18 (variant 9) was detected in 57% of acute myelogenous leukemias and 37% of acute lymphoblastic leukemias, but was not in ovarian caricinomas and normal tissues containing thymus, bone marrow, peripheral blood mononuclear cells, testis, ovary and lymph node (33). In the present study, the variant 9 was detected in 7 of 37 (19%) primary lung tumors and in 5 of 37 (14%) normal lung tissues. These results suggest that the normal lung tissues in our cases may be already affected by initiation of tumorigenesis like field cancerization, or that the variant 9 may be expressed in some normal tissues containing lung. Defining the expression pattern in other tissues will be also required for further analyses.

Our results reveal an association of the splicing variant 1 of the *HELLS/SMARCA6* gene with a considerable subset of NSCLC by a novel “exon-creation” mechanism for gene inactivation. To our knowledge, there are few descriptions of such a mechanism relevant to tumorigenesis with high frequency, though it has been reported that cryptic exons are involved in the activation of the EWS-FLI1 chimeric gene in Ewing sarcomas (39). Although it is possible that progression of lung cancer leads to a disturbance of splicing machinery and produces alternatively spliced variants in a tumor-specific manner, the tumor-specific variant 1 could be useful as a tumor marker for NSCLCs. Further study, including a functional

analysis, will be required to uncover the role of this variant in the tumorigenesis of the lung epithelium.

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TABLE I - RELATIONSHIP BETWEEN VARIANT 1 AND CLINICAL DATA

	No. of cases	No. of variant 1 (+) (%)	No. of variant 1(-) (%)	*p* - value
Age				
< 65 year	22	3 (14)	19 (86)	
65 year<	28	10 (36)	18 (64)	$p = 0.11$
Gender				
Male	41	10 (24)	31 (76)	
Female	9	3 (33)	6 (67)	$p = 0.68$
Smoking history				
Non-smokers	9	3 (33)	6 (67)	
< 400	1	0 (0)	1 (100)	
400<	37	9 (24)	32 (76)	$p = 0.56$
TNM classification				
I	29	8 (28)	21 (72)	
II	10	3 (30)	7 (70)	
III	10	1 (10)	9 (90)	
IV	1	1 (100)	0 (0)	$p = 0.74$
Tumor size				
T1	21	5 (24)	16 (76)	
T2	17	5 (29)	12 (71)	
T3	10	2 (20)	8 (80)	
T4	2	1 (50)	1 (50)	$p = 0.81$
Lymph node metastasis				
Positive	16	3 (19)	13 (81)	
Negative	34	10 (29)	24 (71)	$p = 0.51$
Histology				
AD	30	9 (30)	21 (70)	
SQ	20	4 (20)	16 (80)	$p = 0.52$
Differentiation				
Well	13	2 (15)	11 (85)	
Moderate	20	7 (35)	14 (65)	
Poor	17	4 (24)	13 (76)	$p = 0.71$

Tumor staging was determined according to International Union Against Cancer (UICC) TNM classification of malignant tumors. AD, adeno carcinoma; SQ, squamous cell carcinoma.

FIGURE LEGENDS

Figure 1. Splicing variant of the *HELLS/SMARCA6* gene with a 44-ntd insertion (variant 1). (A) Sequencing result of variant 1. (B) Schematic illustration of variant 1. Arrows show the alternative splicing pattern. An asterisk shows a premature termination site. (C) Nucleotide and amino acid sequences of variant 1. Upper case letters show exons, and lower case letters show splicing junctions in introns. Amino acids are shown according to triplet nucleotides with underlining. (D) Nested PCR products for variant 1. Numbers at *top* show patient number. M, molecular weight marker; T, tumor sample; N, normal sample. Only tumor samples exhibit PCR products of variant 1 with a 44-ntd insertion (Var 1, 238-ntd) in addition to the normal transcript (Norm, 194-ntd).

Figure 2. Schematic illustration of 9 splicing variants of the *HELLS/SMARCA6* gene. Arrows show the alternative splicing pattern. Shaded boxes show the region which was spliced into the mRNAs, and open boxes show the exon which was spliced out. Asterisks show the premature termination site. PMT stands for premature termination. Frequency of each splicing variant detected in tumor (T) and normal (N) samples is shown at *right*. E, exon; Int, intron; ins, insertion.

Figure 3. Representative cases of splicing variants of the *HELLS/SMARCA6* gene. (A) Nested PCR products for variant 2. Numbers at *top* show patient number. M, molecular weight marker; T, tumor sample; N, normal sample. (B) Nested PCR products for variants 3-6. PCR products with 105T or 53T sample show variants 3-6 and normal transcript while the variant 5 is faint in 105T. PCR products with the 105N sample shows variants 3, 4 and normal transcript. 107T shows variants 4, 5, 6 and a trace amount of normal transcript, while 107N shows variants 3, 6 and normal transcript. (C) Nested PCR for variant 9. PCR products with 113N or 125T samples show normal transcript (285-ntd) and variant 9 (210-ntd), while those of 113T, 125N, 136T and 136N samples show normal transcript. (D) Nested PCR for variant 10. PCR products with primers designed on exons 20 and 22 show normal transcript (199-ntd) and variant 10 (277-ntd) which has intron 20 (78-ntd) but not intron 21.

Figure 4. LOH analysis around the *HELLS/SMARCA6* gene on chromosome 10q. Microsatellite marker D10S520 which is located near the *HELLS/SMARCA6* gene, the centromeric marker D10S1680, and the telomeric marker 10q23MS3 were used. Black box, LOH; open box, retention; shaded box, not informative.

Figure 5. HELLS/SMARCA6 expression in lung cancer and normal tissues.

The specimens of the patients with lung tumor in which the variant 1 was detected (A, B an C) and with lung tumor in which the variant 1 was not detected (D, E and F) were immunostained using the antibody against HELLS/SMARCA6 (B, C, E and F) and counterstained with hematoxylin and eosin (A and D). A, B, D and E, lung tumor; C and F, corresponding normal lung tissue.

Fig 1

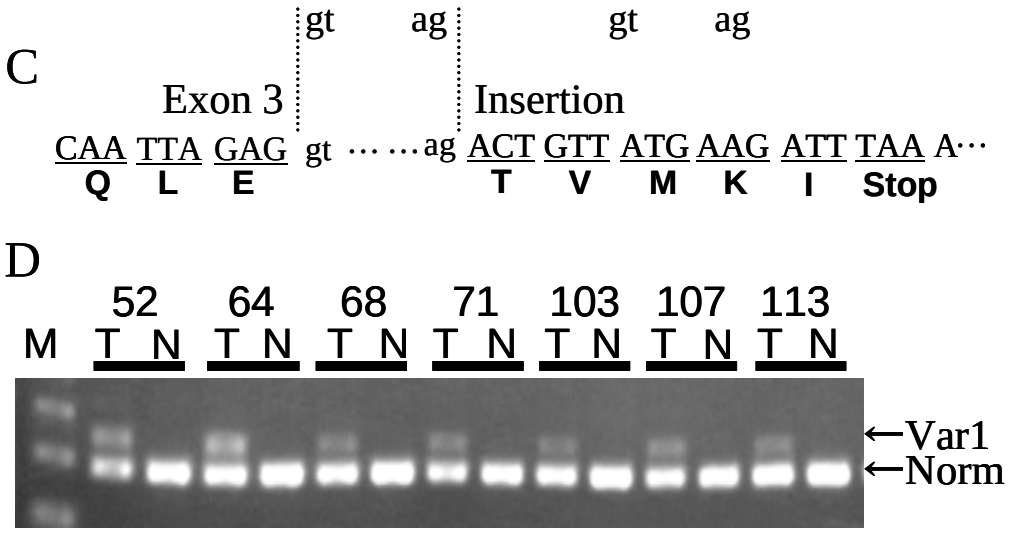

C
gt ag gt ag
Exon 3
Insertion
CAA TTA GAG gt … … ag ACT GTT ATG AAG ATT TAA A…
Q L E T V M K I Stop
D
52 64 68 71 103 107 113
M T N T N T N T N T N T N T N
←Var1
←Norm

Fig 2

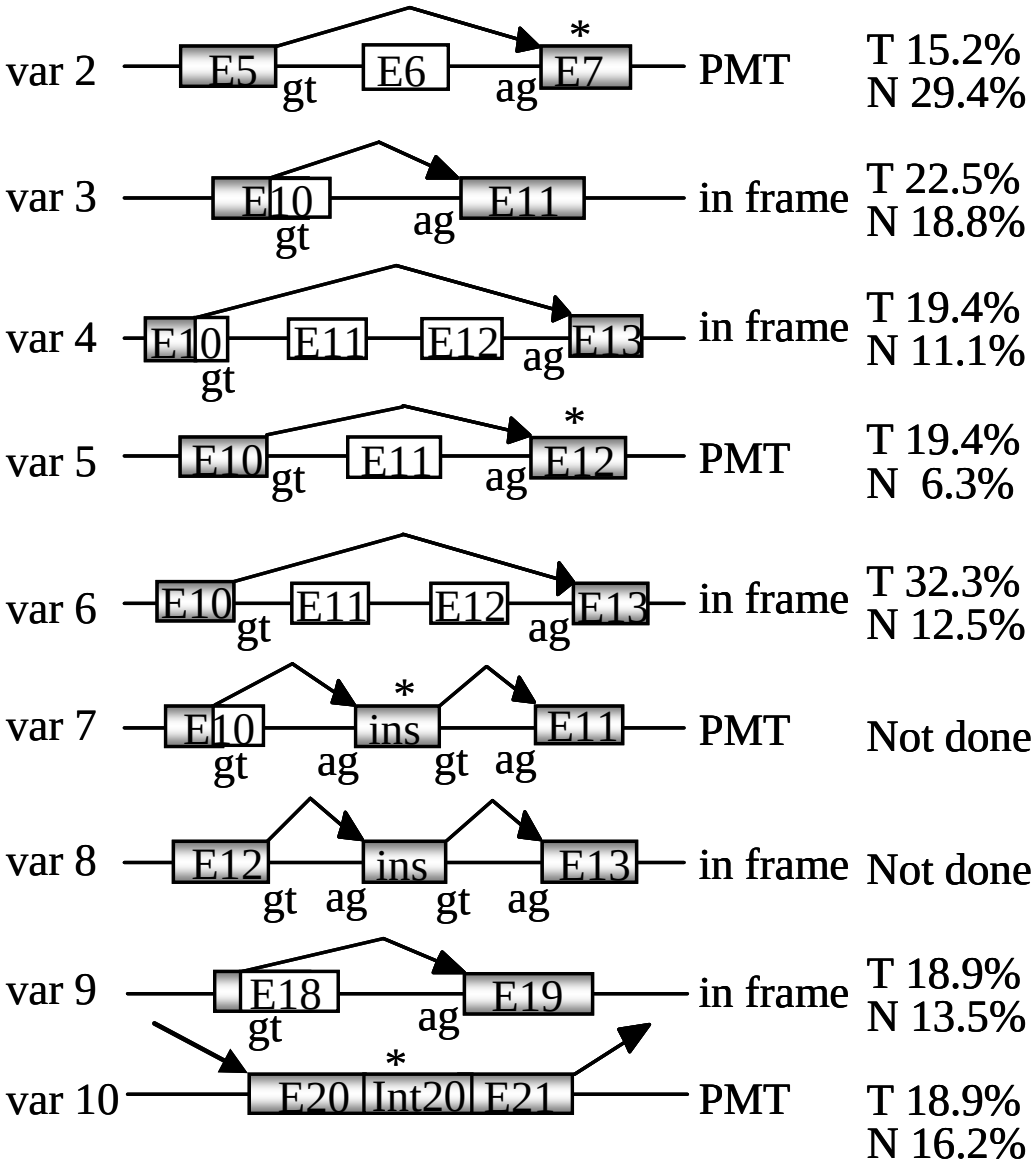

var 2
E5
gt
E6
ag
E7
*
PMT
T 15.2%
N 29.4%
var 3
E10
gt
ag
E11
in frame
T 22.5%
N 18.8%
var 4
E10
gt
E11
E12
ag
E13
in frame
T 19.4%
N 11.1%
var 5
E10
gt
E11
ag
E12
*
PMT
T 19.4%
N 6.3%
var 6
E10
gt
E11
E12
ag
E13
in frame
T 32.3%
N 12.5%
var 7
E10
gt
ag
ins
*
gt
ag
E11
PMT
Not done
var 8
E12
gt
ag
ins
gt
ag
E13
in frame
Not done
var 9
E18
gt
ag
E19
in frame
T 18.9%
N 13.5%
var 10
E20
Int20
E21
*
PMT
T 18.9%
N 16.2%

Fig 3

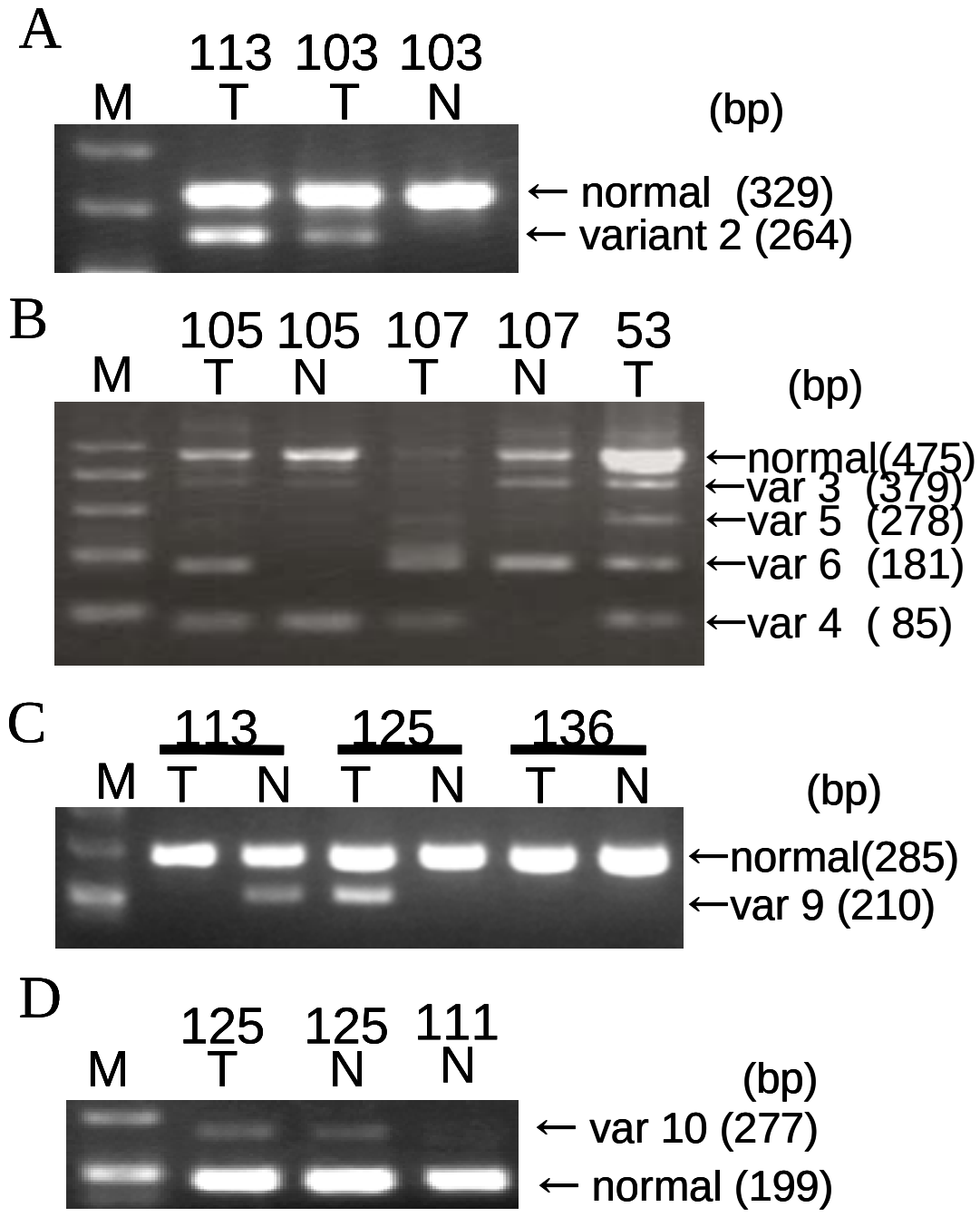
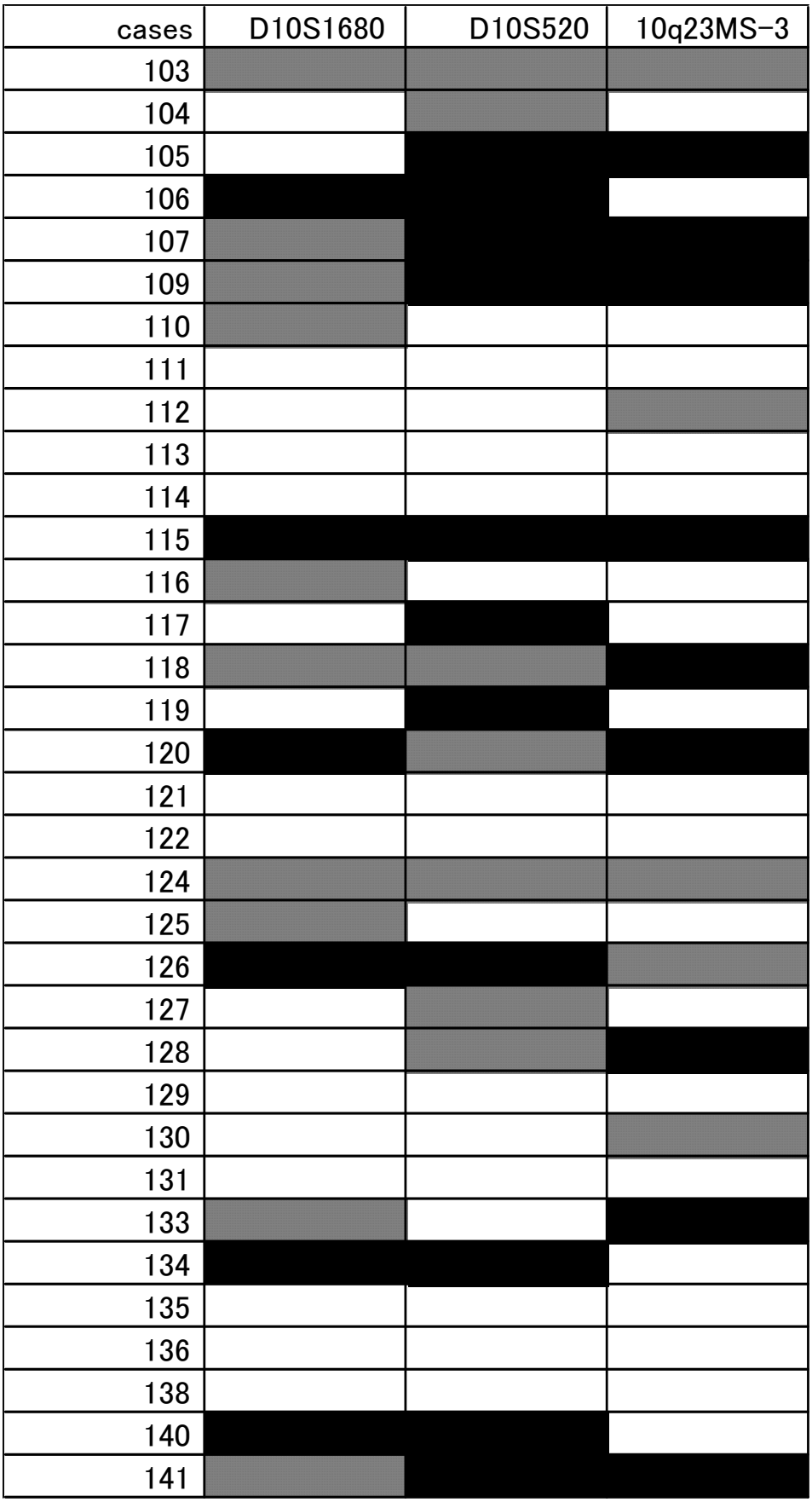

A
113 103 103
M T T N
(bp)
← normal (329)
← variant 2 (264)
B
105 105 107 107 53
M T N T N T
(bp)
←normal(475)
←var 3 (379)
←var 5 (278)
←var 6 (181)
←var 4 (85)
C
113 125 136
M T N T N T N
(bp)
←normal(285)
←var 9 (210)
D
125 125 111
M T N N
(bp)
← var 10 (277)
← normal (199)

Fig 4


cases
D10S1680
D10S520
10q23MS-3
103
104
105
106
107
109
110
111
112
113
114
115
116
117
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141

Fig 5