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Characterization of aberrant *FHIT* transcripts in gastric adenocarcinomas

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Abbreviations: *FHIT*, fragile histidine triad; RT-PCR, reverse transcription-polymerase chain reaction; nt, nucleotide(s)

Abstract

Aberrant transcripts of *FHIT* (fragile histidine triad) have been reported in several types of primary tumors and cell lines, including gastric carcinoma. The role of these aberrant transcripts in tumorigenesis is not clear yet. Forty-eight aberrant-sized *FHIT* transcripts with various lengths and number in 35 cases of gastric adenocarcinomas were further characterized. Aberrant transcripts, with deletions and/or insertions, were frequently observed in 20 cases of tumors. Sequence analysis demonstrated that different types of aberrant transcripts used normal splice sites but skipped exons, contained the inserts with the part of intron 5 sequences, or used the *FHIT* cDNA sequence 179-180 as a cryptic splice acceptor site. Most of aberrant transcripts lacked exon 5 and were presumably non-functional as the translation initiation codon is located in exon 5. Additionally, other transcripts, indicative of additional splice processing, either deletions or insertions, were expressed in several tumors. Taken together, our data indicate that the *FHIT* gene expression is frequently altered in gastric adenocarcinomas by aberrant splicing, and suggest that different types of aberrant transcripts may result during the multi-step splice processing.

Keywords: *FHIT,* gastric adenocarcinoma, aberrant transcript, carcinogenesis, splicing fidelity

Introduction

Fragile sites are specific chromosomal regions prone to genetic instability, chromosomal breakage and rearrangements, and thus their disruption may play a mechanistic role in carcinogenesis. In gastric cancer, chromosomal regions 3p (Gemma *et al*., 1997), 5q (Tamura *et al*., 1996), 7q (Kuniyasu *et al*., 1994), 11q (Baffa *et al*., 1996), 17p (Sano *et al*., 1991), and 18q (Uchino *et al*., 1992) have been identified as fragile sites. The *FHIT* (fragile histidine triad) gene, a putative tumor suppressor gene, is located at human chromosome 3p14.2, a region of high fragility (*FRA3B*) and recombination, as well as of specific deletions and translocation in several cancers (Ohta *et al*., 1996; Croce *et al*., 1999). Homozygous deletions within the *FHIT* locus, genomic DNA rearrangements, and aberrant *FHIT* transcripts have been described in a variety of human cancer cell lines and tumor tissues, including lung cancer (Sozzi *et al*., 1996b), gastrointestinal tumors (Ohta *et al*., 1996), breast cancers (Ahmadian *et al*., 1997), head and neck squamous cell carcinomas (Virgilio *et al*., 1996), and Merkel cell carcinomas (Sozzi *et al.,* 1996a). A good correlation between alterations of the *FHIT* gene and its reduced protein expression has been observed (Yoshino *et al*., 2000). In addition, suppression of tumorigenicity and tumor cell growth in nude mice by replacement of exogenous wild-type *FHIT* gene point to the role of *FHIT* as a tumor suppressor gene (Siprashvili *et al*., 1997; Ji *et al*., 1999). Despite numerous reports on the status of the *FHIT* gene, the question of whether the *FHIT* gene acts as a classical tumor suppressor is still controversial.

High frequency of aberrant *FHIT* transcripts in various cancers, especially in epithelial type, is the most prominent finding. Large deletions and insertions within coding region have been identified in several types of cell lines and primary tumors, even matched normal tissues, by sequence analysis of RT-PCR-rescued transcripts of the *FHIT* gene. Such abnormalities usually occurred at exon-intron junction, mostly accompanied by a normal-sized transcript. Exon 5 and exon 8 were targets for more frequent deletions (Ohta *et al*., 1996). Thus, most of truncated transcripts are missing the start codon of the *FHIT* gene and cannot encode functional protein. Actually, few studies analyzed truncated *FHIT* protein expression. Furthermore, there has been conflicting data concerning occurrence of aberrant *FHIT* transcripts in various cancers, including gastric cancer.

Chromosomal instability including genomic rearrangement and breakage (Ohta *et al*., 1996; Croce *et al*., 1999), disruption of RNA splicing mechanism (=mis-splicing) (Gayther *et al*., 1997), or hypermethylation of the *FHIT* promoter region (Tanaka *et al*., 1998; Laux *et al*., 1999), as yet unidentified, can be considered possible mechanisms involved for aberrant transcription of the *FHIT* pre-mRNA.

We previously investigated altered expression of *FHIT* gene in primary gastric adenocarcinomas. Using 35 tumors and their matched normal tissues, we identified significant rate of LOH at D3S1300, high frequency of aberrant *FHIT* transcripts, and reduced *FHIT* protein expression (Lee *et al*., 2001). Focusing on the *FHIT* transcripts, 48 aberrant-sized *FHIT* transcripts with various lengths and number have been further characterized in this study. The results provide the evidence that *FHIT* is frequently altered in gastric adenocarcinomas by aberrant splicing and activation of cryptic splice acceptor sites within intron 5 and/or exon 6, and suggest that different types of aberrant transcripts may result during the multi-step splice processing.

Materials and Methods

RNA isolation and reverse transcription

Total RNA was extracted from frozen tissues by the total RNA isolation kit (Totally RNA, Ambion Inc., Austin, Texas, USA) according to manufacturer's instruction. Reverse transcription was performed in 20 μl final volume containing 1 μg RNA, 5 mM $MgCl_2$, 1 mM of each dNTP, and 2.5 U MuLV reverse transcriptase (Perkin Elmer, Norwalk, CT, USA). The samples were first denatured for 5 min at 70°C and annealed with oligo $d(T)_{16}$ primers at room temperature for 3 min, and then 2.5 U MuLV reverse transcriptase were added. Samples were incubated at 37°C for 60 min. Inactivating the enzyme at 95°C for 5 min then stopped the reaction.

RT-PCR and cDNA sequencing

Twenty microliter of cDNA reaction was used for the first PCR amplification in a volume of 100 μl containing 0.5 μM of primers 5U2 (5'-CATCCTGGAAGCTTTGAAGC-TCA-3') and 3D2 (5'-TCACTGGTTGAAGAATACAGG-3'), as published by Ohta *et al*. (1996), 1× PCR buffer, 2 mM $MgCl_2$, and 1.25 U AmpliTaq DNA polymerase (Perkin Elmer). The PCR consisted of an initial denaturation at 94°C for 3 min and 25 cycles at 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min, followed by 7 min post-extension at 72°C. Amplified products were diluted 20-fold with TE buffer (10 mM Tris-HCl, 1 mM EDTA), and 1 μl was used for the second-round PCR by the nested primers 5U1 (5'-TCCGTAGTGCTATCTACA-TC-3') and 3U1 (5'-CATGCTGATTCAGTTCCTCTTGG-3') in the same conditions as the first-round PCR, except for 30 cycles of reaction instead of 25 cycles. The nested PCR products were resolved in 10% polyacrylamide gels. Selected aberrant transcripts were recovered from ethidium bromide-stained gels and were cloned into a pGEM-T vector system according to manufacturer's instruction (Promega, Madison, WI, USA). DNA inserts from positive colonies were amplified with T7 and SP6 universal primers and sequenced using an ABI Prism BigDye Terminator Cycle Sequencing kit and an ABI 310 sequencer (Perkin Elmer). DNA sequences were compared to the GenBank databases utilizing the Blast program available at the web site, "http://www.ncbi.nlm.nih.gov".

Results

To study abnormal *FHIT* transcription in gastric carcinogenesis, 35 gastric adenocarcinomas were analyzed for a 707-bp cDNA fragment, which encompass exon 3 to 10 of the *FHIT* gene, using nested RT-PCR and sequencing. In addition to the wild-type *FHIT* transcript, small-sized transcripts with various number and lengths were observed in 20 of 35 (57.1%) tumors, with two

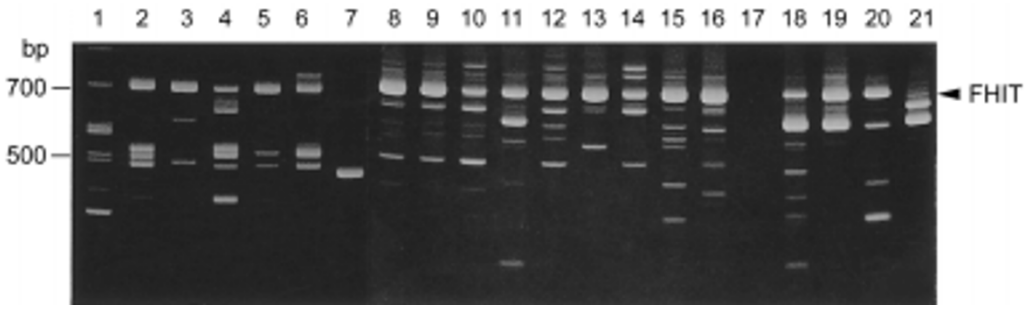

Figure 1. Aberrant transcripts of the *FHIT* gene detected in gastric adenicarcinomas by reverse transcription-polymerase chain reaction (RT-PCR). Additional aberrant-size bands were detected in 20 tumor tissues. Lane 17 shows no *FHIT* transcription. The normal-sized 707-bp transcript is indicated by arrowhead. Band patterns for lanes 1-7 were reported earlier (Lee *et al*., 2001).

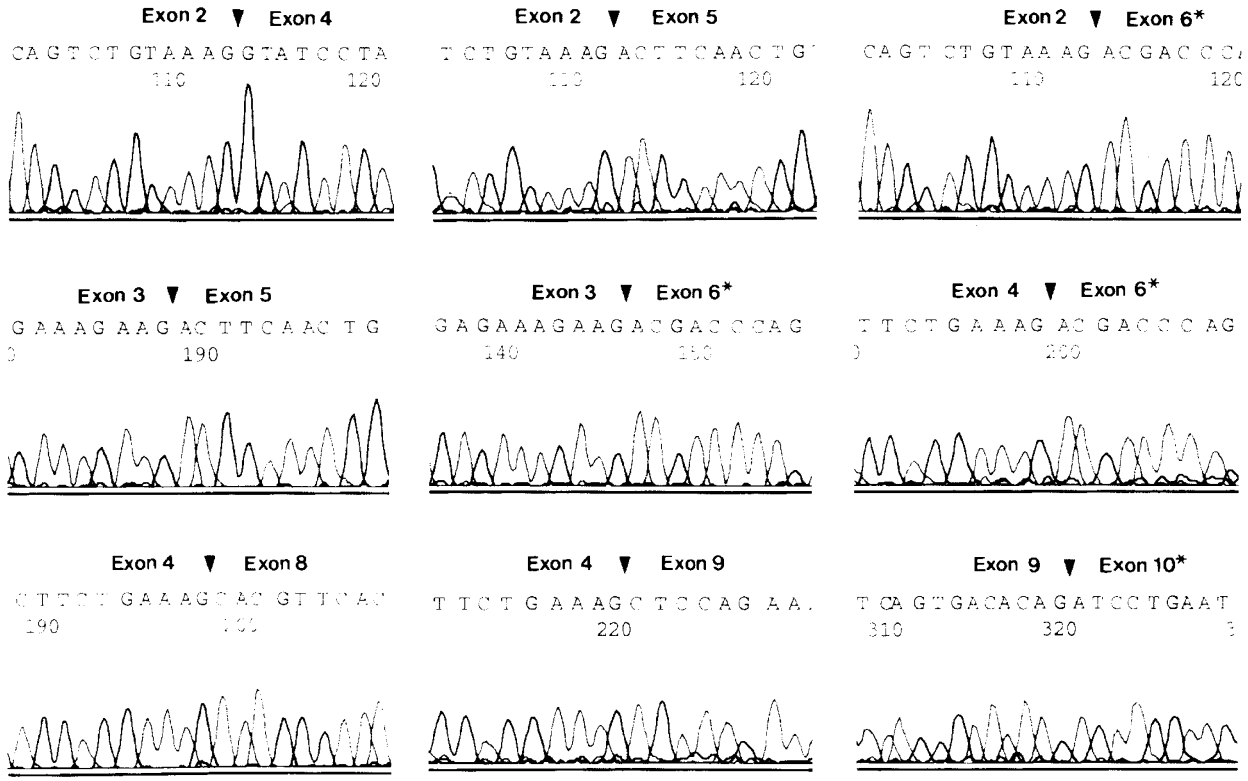

Figure 2. Nucleotide sequence of different aberrant splice junctions within abnormal-sized transcripts observed in gastric adenocarcinomas. Aberrant splice junctions are indicated by arrowhead. Exon 6* represents the last 69 nucleotides of exon 6.

tumors expressing only abnormal *FHIT* transcripts. A total of 48 aberrant transcripts were characterized from the 20 tumors. Sixty-two point five percent (30/48) of these transcripts contained only deletions, the others (37.5%) had deletion with insertion of intron sequence. Aberrant band sizes were diverse (~360 – ~830 bp), with a range of 1 to 7 bands. No *FHIT* transcript was expressed in one case (Figure 1).

All the PCR products were excised from the polyacrylamide gel and subsequently cloned into a pGEM-T vector system. Sequence analysis of normal-sized transcripts showed no deletion or insertion in any case. Figure 2 shows a typical sequence of 9 types of aberrant transcripts with losses of various exons from 3 to 8, resulting in fusion of exons 2 and 4, 2 and 5, 3 and 5, 4 and 8, or 4 and 9. Transcripts with fusion of exons 2, 3, or 4 and the last 69 nucleotides (nt) of exon 6 (nt 181 to 249 of the *FHIT* cDNA sequence; Ohta *et al.*, 1996) were frequently observed. The first 11-bp deletion (nt 450 to 460) at the 5' end of exon 10, ATGTTTTTCAG, was detected in 3 cases (cases 3B, 5B, and 13A) with losses of other exons.

Sequence analysis of 48 aberrant-sized transcripts revealed that aberrant *FHIT* transcripts lacked one or more exons of the *FHIT* or lacked exons with the insertion of intron sequence as previously reported (Lee *et al.*, 2001). Loss of the sequence from exon 5 to the first 77 nt of exon 6 (nt 17 to 180) was the most common abnormality (Table 1). The use of a cryptic splice acceptor sequence (-AG-) at position 179-180 resulted in fusion of exon 4 and the last 69 nt of exon 6. The deletion of this type was detected in 17 aberrant transcripts including 9 cases with deletion only and 8 cases with both deletion and insertion. Of the inserts with various lengths (59-, 81-, 112-, or 138-bp) detected in the

Table 1. Position and number of deletions and/or insertions in aberrant *FHIT* transcripts in gastric adenocarcinomas

cDNA sequence (Position)	Deletion only[1]	Deletion and Insertion
-163 to -111	3	0
-163 to -18	3	0
-163 to 180	1	2
-110 to -18	7	0
-110 to 103	2	1
-110 to 180	3	3
-17 to 180	9	8
-17 to 279	1	0
-17 to 348	1	0
-17 to 103	0	3
104 to 348	0	1
450 to 460	3	0

[1]Data for deletion only were reported earlier (Lee *et al.*, 2001).

Figure 3. Nucleotide sequence of the inserts within aberrant transcripts observed in gastric adenocarcinomas. Bold letters represent the parts of intron 5 sequence flanking on the 5'-side of the coding exon 6 (b, d, and f) or on the 3'-side of the coding exon 5 (g).

above 8 cases, a 138-bp insertion is most frequently observed in 10 of total 20 tumor tissues displaying aberrant transcripts. This insert of identical size was previously reported in a variety of human cancer cell lines and tumor tissues, including lung cancer cell lines and primary lung tumors (Fong *et al.*, 1997), nasopharyngeal cancers (Sung *et al.*, 2000) as well as squamous cell cancers of cervix (Yoshino *et al.*, 2000). Other aberrant transcripts involving loss of exon 4 or loss of exon 4 through the first 77 nt of exon 6 (nt -110 to 180) were detected in 7 or 6 cases, respectively. Three of the latter 6 cases also contained a 59-bp or 138-bp insertion. The exon 4 was frequently spliced to several coding regions, including exon 6, 8, 9, and the last 69 nt of exon 6, which frequently act as an acceptor for exon 2, 3, and 4.

A Blast search in GenBank database identified that the origin of the inserts with various sizes is the part of intron 5 sequences in the *FHIT* gene. Figure 3 shows the nucleotide sequence of the inserts identified in 18 aberrant transcripts from 12 tumors. All of the insertions occurred in combination with in-frame deletions. The sizes of the inserts were diverse (59-, 81-, 103-, 112-, 138-, 158-, and 250-bp), but some of them were overlapped. Some aberrant *FHIT* transcripts with 138-bp, 81-bp, or 59-bp insertions were found between various exons (E3, E4, or E5) and the last 69 nt of exon 6. Some insertions were the parts of intron sequence flanking on the coding exon, indicative of aberrant splicing through the selection of cryptic splice site in intron sequence. An 112-bp or 103-bp insertion was identical to the intron 5 sequence flanking on the 5'-side of the coding exon 6 or on the 3'-side of the coding exon 5, respectively. Some insertions were overlapping; a 250-bp insertion replacing exon 5 comprised this 138-bp as well as 112-bp sequence identical to the insert in aberrant transcripts 6A and 26A. Another 158-bp insertion comprised this 112-bp and the other 46-bp insertion. The deletions or insertions within cDNA sequence occurred at exon-intron junction of genuine splice site, or at cryptic splice site located within exon or intron. Analysis of the sequence flanking the deletion or insertion revealed that two distinctive types of conserved sequences -the GU-AG and AC-AG motifs- were placed in the beginning and the end of the deleted portions of aberrant *FHIT* transcripts. Proportion of two motifs in splice sites is 58.5% for GU-AG and 33.8% for AC-AG.

Multi-step splice patterns were observed in several

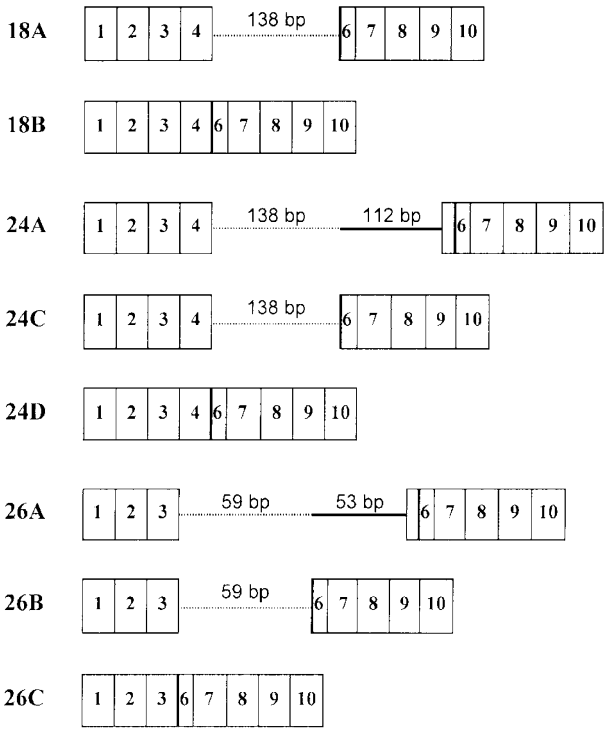

Figure 4. Schematic diagram of representative transcripts suggesting the multi-step splicing in gastric adenocarcinomas. Exon numbers are indicated within the boxes. The intron 5 sequence (112-bp and 53-bp) flanking on the 5'-side of exon 6 is shown by the thick line, whereas the dotted line represents the part of intron 5 sequence. Vertical thick line represents a cryptic splice site within exon 6.

tumor specimens, in which intron sequences remained were finally spliced out (Figure 4). In cases 18, 24, and 26, transcript "A" lacking exon 4 and/or 5 with 138-bp, 250-bp (138-bp and 112-bp), or 112-bp (59-bp and 53-bp) inserts, respectively, appeared to be a precursor of transcripts B, C, or D that underwent additional downstream processing.

Discussion

Characterization of aberrant transcripts in tumor tissues may be a basic step toward understanding the possible role of the *FHIT* gene in carcinogenesis. Early studies of a number of human tumor types showed that *FHIT* transcription was frequently altered, and a significant proportion of such alterations were shown to correlate with deletions within the *FHIT* gene (Ohta *et al.*, 1996; Druck *et al.*, 1997).

In this study, investigation of primary gastric cancers demonstrated 48 aberrant transcripts in 57.1% (20/35) of the tumors. The frequency is significantly higher than 10 in 46.7% (7/15) of Chen *et al.* (1997), and 11 aberrant transcripts in 35% (11/32) of Baffa *et al.* (1998). To extract the abnormal bands, we used 10% polyacrylamide gel instead of agarose gel because low resolution of agarose gel results in co-migration of cDNA fragments and makes the interpretation of results harder. Here, many more aberrant transcripts were observed, although there is a background of nonspecific bands. Most of tumor specimens had multiple bands. Sequence analysis confirmed that faint bands of smaller size, which were not seen in agarose gels, were low-abundance transcripts that were alternatively spliced.

Aberrant transcripts of different size were the results of exon skipping and/or insertion of *FHIT* intron sequence, or selection of cryptic splice sites. Excepting the regions of deletions or insertions, the exons retained in all cases of aberrant transcripts were correctly spliced, indicating the correct use of their splicing signals. An 11-bp deletion from nt 450 to 460 was also identified as described previously (Muller *et al.*, 1998; Nakagawa *et al.*, 1999). The exons 5-6 deleted splice variants, with or without loss of exon 4 and/or 7, were the prominent transcripts. These results were consistent with those found in other various carcinomas (Gemma *et al.*, 1997; Hendricks *et al.*, 1997). Considering the facts that the translation initiation codon is located in exon 5, most of aberrant transcripts may not generate any protein products.

A recent Blast search in GenBank database identifies the origin of the inserts whose sequences remained unknown in most of previous studies. From the findings that all of the inserts seen in our study were the part of intron 5 sequence and usually were found between various exons (E2, E3, or E4) and the last 69 nt within exon 6, it is possible that the inserts, replacing of the missing exons, occurred by aberrant splicing and/or activation of the cryptic splice site within intron 5 and exon 6 of the *FHIT* gene. It is noteworthy that all of the insertions, except for case 30B, occurred in aberrant transcripts with loss of exon 5, although these transcripts lacked variable number of exons. In previous study, loss or reduced expression of *FHIT* protein and its strong association with occurrence of aberrant *FHIT* transcript in cervical carcinomas suggested that *FHIT* gene inactivation might contribute to clonal outgrowth during gastric carcinogenesis and that aberrant expression might be a common mechanism impairing the *FHIT* gene (Yoshino *et al.*, 2000). Although some type of silencing mechanism might be involved, the reason why the aberrant transcripts have anything to do with the absence of *FHIT* expression requires further study. In this study, high rate of insertion and frequent activation of cryptic splice sites within intron 5 and/or exon 6 are striking, suggesting that splicing patterns of aberrant transcripts might correlate with the type of tumor or tissue. In addition, there appeared to be an order by which the additional splice processing occurs (Figure 4). In cases 18, 24, and 26, their transcripts (A, B, C, or D) appeared to represent sequential processing for splicing

out the intron sequence already inserted as a consequence of aberrant splicing, suggesting that sequential reactions of alternative splicing within a given tumor might be derived from a transcriptional unit in a single allele. Similar report has been described in Burkitt's lymphoma (Ferrer *et al*., 1999).

These aberrations may be possibly due to the characteristics of the *FHIT* gene locus as fragile site. The structural alterations of *FRA3B* might interfere with the normal splicing and thus result in the generation of numerous aberrant transcripts. Indeed, homozygous deletions in the *FHIT* gene mostly involve intronic sequences within the *FHIT* gene locus (Boldog *et al*., 1997), suggesting that partial deletions of the *FHIT* gene might affect transcription fidelity. Previous reports indicated that breakage and rearrangements of DNA within the *FHIT* gene resulted in occurrence of aberrant transcripts and subsequently reduced *FHIT* protein expression (Fong *et al*., 1997; Baffa *et al*., 1998). However, genomic deletions did not always affect exons of the gene. Indeed, aberrant *FHIT* transcripts were also found in tumors and in cell lines in which homozygous deletions of *FHIT* exons were not detected (Mao *et al*., 1996; Ozaki *et al*., 2000), indicating genetic and/or epigenetic alterations in other regions, such as promoter regions. Although there is still controversy as to whether the aberrant *FHIT* transcripts reflect a chromosomal instability, these results provide evidence that splicing of the *FHIT* pre-mRNA is frequently altered and processed in gastric carcinomas, and suggest that the disruption of regulated pre-mRNA splicing may be occurred as a consequence of disturbed cellular function during malignant progression. The possible role of these mechanisms, however, remains to be determined in further studies of the genome structure.

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