Molecular and Cellular Pathobiology

Frequent PVT1 Rearrangement and Novel Chimeric Genes
PVT1-NBEA and PVT1-WWOX Occur in Multiple Myeloma with 8q24 Abnormality

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Abstract

Chromosome 8q24 rearrangements are occasionally found in multiple myeloma and are associated with tumor progression. The 8q24 rearrangements were detected by FISH in 12 of 54 patients with multiple myeloma (22.2%) and in 8 of 11 multiple myeloma cell lines (72.7%). The breakpoints of 8q24 in 10 patients with multiple myeloma and in all multiple myeloma cell lines were assigned to a 360 kb segment, which was divided into 4 regions: approximately 120 kb centromeric to MYC (5’ side of MYC), the region centromerically adjacent to PVT1 (~170 kb region, including MYC, of 5’ side of PVT1), the PVT1 region, and the telomeric region to PVT1. PVT1 rearrangements were most common and found in 7 of 12 patients (58.3%) and 5 of 8 cell lines (62.5%) with 8q24 abnormalities. A combination of spectral karyotyping (SKY), FISH, and oligonucleotide array identified several partner loci of PVT1 rearrangements, such as 4p16, 4q13, 13q13, 14q32, and 16q23-24. Two novel chimeric genes were identified: PVT1-NBEA in the AMU-MM1 cell line harboring t(8;13)(q24;q13) and PVT1-WWOX in RPMI8226 cell line harboring der(16)(16;22)ins(16;8)(q23q24). The PVT1-NBEA chimera in which PVT1 exon 1 was fused to NBEA exon 2 and the PVT1-WWOX in which PVT1 exon 1 was fused to WWOX exon 9 were associated with the expression of abnormal NBEA and WWOX lacking their N-terminus, respectively. These findings suggest that PVT1 rearrangements may represent a novel molecular paradigm underlying the pathology of 8q24 rearrangement–positive multiple myeloma. Cancer Res; 72(19); 4954–62. ©2012 AACR.

Introduction

Genetic abnormalities play a crucial role in the pathogenesis of various malignancies, including multiple myeloma. The primary cytogenetic abnormalities associated with disease development are either nonrandom chromosomal gains known as hyperdiploid, which is characterized by trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, or structural rearrangements involving the immunoglobulin heavy chain gene (IGH) located at 14q32.33 (IGH translocation; refs. 1, 2). Secondary cytogenetic abnormalities implicated in disease progression include 8q24 rearrangements, gain of the long arm of chromosome 1 (1q+), and loss of the short arm of chromosome 17 (17p; refs. 1, 3).

The 8q24 rearrangements have been identified by conventional cytogenetic analysis in 3.5% to 5.0% of patients with multiple myeloma (4, 5), and by FISH and spectral karyotyping (SKY) in 9.5% to 20% (6–9). The 8q24 rearrangements are frequently associated with advanced disease in patients with multiple myeloma and multiple myeloma cell lines (10, 11). Ig chromosomal translocations, such as t(8;14)(q24;q32) and t(8;22)(q24;q11), occur in approximately 25% of multiple myelomas with 8q24 rearrangements, whereas non-Ig chromosomal loci, including 1p13, 1p21-22, 6p21, 6q12-15, 13q14, and 16q22, in which no candidate genes have been delineated so far, have also been identified as translocation partners (7, 8, 12, 13). MYC has long been a possible candidate target gene for 8q24 rearrangements; however, many of the breakpoints within 8q24 have been assigned to various regions that encompassed more than 2 Mb centromeric or telomeric to MYC (9, 11). In contrast to the typical Burkitt lymphoma translocation t(8;14) with breakpoints within the MYC gene (14), rearrangements of plasmacytoma variant translocation 1/Moloney leukemia virus integration-1 locus (PVT1), which is located 57 kb 3’ of MYC, have been identified in variant Burkitt lymphoma translocations t(8;22) and t(28). In the latter...
translocations, fusion of the constant region of the \( I g \) \( \gamma \) or \( \kappa \) chain gene to \( PVT1 \) was detected, resulting in a lack of protein production (15).

In this study, the \( 8q24 \) rearrangements were analyzed in patients with multiple myeloma and cell lines by FISH and SKY combined with oligonucleotide arrays. Results showed frequent \( PVT1 \) rearrangements with several partners and novel \( PVT1-NBEA \) and \( PVT1-WWOX \) chimeric genes.

Materials and Methods

Patients and cell lines

The use of clinical samples was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (Kyoto, Japan). Informed consent was obtained from all patients. Primary samples were obtained from the bone marrow of 53 patients and the lymph node of 1 patient between April 2005 and January 2011. Eleven multiple myeloma cell lines, AMU-MM1, KMS-12-BM, KMS-18, KMS-20, KMS-28-PE, KMS-34, AMO1, IM9, LP-1, NCI-H929, and RPMI8226, were also analyzed. AMU-MM1 was established at Aichi Medical University (Aichi, Japan) from the tumor cells of the cerebrospinal fluid of a 72-year-old Japanese female patient with IgA-\( \kappa \) multiple myeloma (16).

FISH analysis

The FISH was conducted as described previously (17). To assess the \( 8q24 \) rearrangement patterns and identify the genes involved, 3 sets of probes were used. The first set of probes was the \( 8q24 \) probe-LSI \( MYC \) Dual Color, Break Apart Rearrangement Probe. It consists of the SpectrumOrange-labeled 5' LSI \( MYC \) probe, which begins at 119 kb upstream of the 5' end of \( MYC \) and extends 266 kb toward the centromere, and the SpectrumGreen-labeled 3' LSI \( MYC \) probe, which starts approximately at 1.5 Mb 3' of \( MYC \) and extends 407 kb toward the telomere (Abbott Japan). The second set of probes was designed to hybridize to both adjacent sides on the \( PVT1 \) gene, defined as \( PVT1-adjacent \) (\( PVT1-A \)) probe. The \( PVT1-A \) probe set was composed of 2 specific bacterial artificial chromosome clones CTD-3066L1, a fragment approximately 170 kb in length adjacent to the 5' end of \( PVT1 \) and RP11-628C14, a fragment approximately 190 kb in length adjacent to the 3' end of \( PVT1 \). The third set of probes was the \( PVT1 \)-spanning (\( PVT1-S \)) probe composed of 2 bacterial artificial chromosome clones, CTD-2226H2, a fragment approximately 120 kb in length covering 5' regions of \( PVT1 \), and RP11-16I24, a fragment approximately 190 kb in length covering 3' regions of \( PVT1 \) (Fig. 1A). For interphase analysis, signals were evaluated in a minimum of 100 nuclei with hybridization efficiency greater than 90%.

SKY analysis and SKY combined with FISH analysis

The SKY analysis was conducted as described previously (18). For the SKY–FISH analysis, SKY and FISH probe mixtures simultaneously hybridized to chromosomes for 2 days at 37°C. Ten to 20 metaphase spreads were analyzed, and karyotypes were determined according to ISCN 2009 (19). For complex abnormalities with rearranged \( 8q24 \) locus, such as translocations or insertions, which cannot be detected by either FISH or SKY, a SKY–FISH procedure was used to detect chromosomal locations involving \( MYC \) and \( PVT1 \).

Genome copy number analysis

The DNA gain and loss assay on the basis of high-density oligonucleotide microarrays (GeneChip Human Mapping 50K, 250K, or 6.0 single-nucleotide polymorphism (SNP) array, Affymetrix) was conducted with genomic DNA extracted from cell lines and tumor specimens. Breakpoints in chromosomal translocations were identified by the means of genome copy number analysis combined with SKY, and the SNP array data were analyzed to determine total copy numbers using the CNAG3.0 or 3.3 programs (20).

Reverse-transcription PCR and sequencing analysis

Reverse-transcription PCR (RT-PCR) analysis was conducted as described previously (21). The following primers were used: P1S (forward primer in exon 1 of \( PVT1 \), NR_003367.1), \( 5'\)-TTGCGGAAAGTGTGCGC-3', and N3A (reverse primer in exon 3 of \( NBEA \), NM_015678.3). \( 5'\)-GCTCCA-TATTCTGTGACA-3', for the detection of any chimeric gene on der(8)(t(8;13)); N2S (forward primer in exon 2 of \( NBEA \), 5'-CATACAGTCTCGAGAAGTC-3', and P2A (reverse primer in exon 2 of \( PVT1 \), 5'-AGGGCTTCCACCGGCTCAAT-3', or P3A (reverse primer in exon 3 of \( PVT1 \), 5'-GGTTCATCCACTCA-TAGG-3', for the detection of any chimeric gene on der(13)t(8;13); and P1S and W9A (reverse primer in exon 9 of \( WWOX \), NM_016373.2).\( 5'\)-CAGGGACTAGCAAGCTCA-3', for the detection of any chimeric gene on der(16)(t(16;22)ins(16;8)) (q23q24). The nucleotide sequences of PCR products were determined with the fluorometric method (Dye Terminator Cycle Sequencing Kit, Applied Biosystems).

Real-time quantitative RT-PCR

\( NBEA \) and \( WWOX \) mRNA levels were measured with specific primer probe sets from Assays-on-Demand (Applied Biosystems) or SYBR Green method using the ABI Prism 7300 system (Applied Biosystems) according to the manufacturer's instructions. Primers were Assays-on-Demand \( NBEA \) 2-3 (Hs00995629_m1), \( NBEA \) 58-59 (Hs00995655_m1), W8S (forward primer in exon 8 of \( WWOX \)), \( 5'\)-GCAACATCTCTCTT-CCAAAGCA-3', \( W9A \) (forward primer in exon 10 of \( WWOX \)), \( 5'\)-TGGGACACCGACAGATCACA-3', \( W9S \) (forward primer in exon 9 of \( WWOX \)), \( 5'\)-CCGGTT-CTTGGACCTCCTGC-3', and WPA3 (reverse primer in exon 9 of \( WWOX \), \( 5'\)-CCGGTT-CTTGGACCTCCTGC-3'. These primer sets were used to distinguish abnormal chimeric \( NBEA \) or \( WWOX \) transcripts from normal transcripts: \( NBEA \) 2-3, spanning the breakpoint of \( 8q24 \) in (t(8;13)(q24q14)), can detect only the normal \( NBEA \) transcript, whereas \( NBEA \) 58-59 can detect both normal and chimeric transcripts, similarly, combination of \( W8S \) and \( W9A \) (2) can detect only the normal \( WWOX \) transcript and \( W9S \) and \( W9A \) (3) can detect both normal and chimeric \( WWOX \) transcripts. The \( \beta\)-actin mRNA level was measured as an internal control. In addition to the 11 multiple myeloma cell lines, normal peripheral lymphocytes, an Epstein–Barr virus-transformed B-cell line derived from a normal healthy volunteer, the erythroleukemia cell line K562, and the Burkitt...
lymphoma cell line, Daudi, were analyzed. Each assay was done in triplicate.

Results

Frequent involvement of PVT1 locus in 8q24 rearrangements

The 8q24 rearrangements were detected using 3 FISH probe sets in 12 patients (22.2%) and 8 cell lines (72.7%). The break- points of 8q24 were assigned to a 360 kb segment containing the MYC and PVT1 genes in 10 patients with multiple myeloma and cell lines with 8q24 rearrangement. Breakpoint region A is the 120 kb length region of centromeric to MYC, B is the 170 kb length region centromerically adjacent to PVT1 including MYC, C is the PVT1 region, and D is the region of telomeric to PVT1.

Figure 1. Identification of breakpoints region at 8q24 by FISH analysis. A, location of FISH probes and mapping of putative breakpoints at 8q24. FISH probes are depicted as color bars. Vertical arrows indicate breakpoints and horizontal double-headed arrows indicate the possible range of breakpoints on the basis of interphase FISH analysis. The combination of the FISH analysis with 3 sets of probes reveals 4 breakpoint regions and 11 breakpoint types as shown in B and Supplementary Fig. S1. B, mapping of breakpoints in patients with multiple myeloma and cell lines with 8q24 rearrangement. Dark gray boxes, the breakpoint regions; light gray boxes, duplicated regions. Pt, patient number. Breakpoint region A is the 120 kb length region of centromeric to MYC, B is the 170 kb length region centromerically adjacent to PVT1 including MYC, C is the PVT1 region, and D is the region of telomeric to PVT1. Frequency shows the case number for each of the breakpoint regions.
Supplementary Fig. S4). Chromosomal breakpoints, partner genes, and 8q24 rearrangement patterns identified 5 partner loci of PVT1 translocations or insertions, 4p16, 4q13, 13q13, 14q32, and 16q23-24, in 5 of 8 cell lines and 1 of 2 primary multiple myeloma cells (Table 1).

Identification of the PVT1-NBEA and PVT1-WWOX chimeric genes in multiple myeloma cell lines

In AMU-MM1 cell line, the SKY analysis identified the unbalanced chromosomal translocation t(8;13)(q24;q13), which resulted in 2 der(8) and 1 der(13) (Fig. 3A). The SNP array analysis clearly showed that the copy number change at 8q24 occurred within the region including PVT1 exon 1 and intron 1 (between the physical positions of 128871130 and 128909458, Fig. 3B). At 13q13, the copy number change occurred within intron 2 of NBEA (between the physical positions of 34477756 and 34734115, Fig. 3B). On the basis of these results, RT-PCR analysis was conducted to detect chimeric products using primers P1S and N3A and N2S and P3A. Direct sequencing of the PCR products generated using primers P1S and N3A and N2S and P3A revealed the fusion of 50-PVT1 exon 1 with NBEA exon 3-30, and that of 50-NBEA exon 2 with PVT1 exon 3-30 (Fig. 3C–E). The primers N2S and P2A did not yield any PCR product, suggesting the splicing out of PVT1 exon 2 (Fig. 3C–D). In RPMI8226 cell line, the SKY–FISH analysis identified der(16)(t(16;22)ins(16;8)(q23;q24) in RPMI8226, t(8;14)(q24;q32) and t(8;15)(q24;q7) in AMO1, t(8;14)(q24;q32) and t(8;20)(q24;q13) in KMS-28-PE, t(8;20)(q24;q11) in NCI-H929, t(4;8)(q7;q24) in Pt-1, and t(8;8)(q25;p24), and t(8;19)(q24;p13) in Pt-8. *, in KMS-34, interphase FISH signal pattern was YYYRR (Supplementary Table S1), however, one of the red signals could not be detected in SKY–FISH analysis because of few metaphases in KMS-34.

Figure 2. Detection of partner loci of 8q24 rearrangements by metaphase analysis. A, metaphase FISH with PVT1-A probe (left), inverted 4', 6-diamidino-2-phenylindole (DAPI) staining (middle), and SKY–FISH (right) analysis of KMS-18. Red signals from the PVT1-A probe were detected on chromosome 14 by SKY–FISH (red arrows). Yellow arrows indicate fusion signals of red and green signals. Scale bar, 10 μm. B, partial karyotype of 8q24 rearrangement-positive samples using SKY–FISH. Red and green signals derived from the 8q24 (KMS-28-PE) or PVT1-A probes (other cell lines and clinical samples) are shown on each chromosome. Partial karyotype shows t(8;13)(q24;q13) in AMU-MM1, t(8;8)(q16;p24) in LP-1, t(8;16)(q25;q23-24) in KMS-34, t(8;14)(q24;q32) in KMS-18, der(16)(t(16;22)ins(16;8)(q23;q24) in RPMI8226, t(8;14)(q24;q32) and t(8;15)(q24;q7) in AMO1, t(8;14)(q24;q32) in KMS-28-PE, t(8;20)(q24;q13) in NCI-H929, t(4;8)(q7;q24) in Pt-1, and t(8;8)(q25;p24), and t(8;19)(q24;p13) in Pt-8. *, in KMS-34, interphase FISH signal pattern was YYYRR (Supplementary Table S1), however, one of the red signals could not be detected in SKY–FISH analysis because of few metaphases in KMS-34.
Table 1. Partners of 8q24 rearrangements and candidate genes

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<thead>
<tr>
<th>8q24 Rearrangements</th>
<th>Translocation partners</th>
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<tr>
<td><strong>Patients and cell lines</strong></td>
<td><strong>Involved genes</strong></td>
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<tr>
<td>AMU-MM1</td>
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<tr>
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<td>Unknown</td>
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<tr>
<td>NCI-H929</td>
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Abbreviation: NA, not available.

aNo copy number change was found at chromosome 16 in KMS-34. The rearranged gene could not be identified, because there were too many genes within the region in which copy number change occurred in KMS-28-PE (Supplementary Fig. S4).

bNo gene has been reported as affected in multiple myeloma.

cDuplication of both the MYC locus and centromeric part of PVT1 was detected in RPMI8226 and KMS-18 with PVT1 rearrangement.

Discussion

The present study uncovered 2 aspects of molecular genetics in multiple myeloma. The first finding is the frequent rearrangement of PVT1 gene in multiple myeloma harboring 8q24 rearrangements. PVT1 is the human homolog of the mouse Pvt1 oncogene, which was originally identified as a common retroviral integration site in murine leukemia virus (MLV)-induced T lymphomas. PVT1/Pvt1 is also involved in variants t(2;8), t(8;22), or t(8;14) in human Burkitt lymphoma and in variant t(6;15) in mouse plasmacytomas (10, 15, 22–25). Moreover, amplification of the PVT1 gene has been observed in several cancers including lymphomas (26–30), and PVT1 overexpression was found to contribute to the suppression of apoptosis (31). The PVT1 locus is thought to encode several microRNAs important in oncogenesis (32). Hence, the current study suggests that PVT1 is one of the target genes of rearrangement that may be responsible for driving multiple myeloma, whereas other studies have shown breakpoints within the region centromeric to PVT1 (9–11, 33).

The second finding is the novel chimeric genes, PVT1-NBEA and PVT1-WWOX, in the multiple myeloma cell lines, the AMU-MM1 cell line with t(8;13)(q24q13), and RPMI8226 cell line with der(16)t(16;22)ins(16;8)(q23q24), resulting in high expression of the abnormal chimeric transcript. In PVT1-NBEA, the breakpoint of PVT1 was found within intron 1 and PVT1 exon 1 was found to be fused to NBEA exon 3, resulting in the loss of the start codon in NBEA exon 2. In this setting, the second ATG in exon 3 of the NBEA gene may function as a start codon to produce a putative abnormal NBEA protein lacking 107 N-terminal amino acids (Fig. 3E), although the expression of PVT1-NBEA at the protein level remains to be investigated. Although, knockdown of PVT1-NBEA using siRNA could not inhibit proliferation and induces cell death (Supplementary Fig. S5), it would be important to investigate the function of this NBEA chimera in multiple myeloma tumorigenesis, as NBEA modulates signal transduction and vesicular trafficking.
Recurrent PVT1 Rearrangement in Multiple Myeloma

Figure 3. Identification of PVT1-NBEA and PVT1-WWOX chimeric genes in multiple myeloma cell lines. A, SKY analysis of AMU-MM1 reveals a complex karyotype including t(8;13)(q24;q13) as 46,X,X,-der(1;19)(q10;p10),der(2)(p17;q27;q11.2),der(7)(qter->q11.2::p15 -- q11.2::p17),t(8;13)(p24;q13), = der(8)(8;13),del(12)(p11.2),= der(19)(1;19)(q12;p13) = 2,del(20)(p13). Arrow, breakpoint of der(8)(8;13); arrowhead, breakpoint of der(13)(8;13)(q24; q13). B, copy number changes at 8q24 (PVT1) and 13q13 (NBEA) detected by SNP array. Primers used to detect chimeric transcript. The y-axes indicate the linear scale corresponding to genome copy number of each chromosome. However, copy number data have never been corrected for the influence of tumor cell percentage or real copy number of some chromosomes. C, detection of PVT1-NBEA and NBEA-PVT1 chimeric transcripts by RT-PCR. D, sequencing of chimeric junction of PVT1-NBEA and NBEA-PVT1 chimeric transcripts. E, putative structure of abnormal NBEA fusion transcript. PVT1-NBEA lacks a start codon in NBEA exon 2; the second ATG in exon 3 might function as a start codon, resulting in an abnormal NBEA protein lacking its N-terminus. *ATG indicates start codon. F, SKY-FISH analysis of RPMI8226 reveals a complex karyotype including der(16)(16:22)ins(16;8) (q23;p24). Arrows indicate red signals of PVT1-A inserting to t(16;22). It is difficult to detect the 8q24 locus on der(16) using conventional cytogenetic technique. Scale bar, 10 μm. G, copy number changes at 8q24 (PVT1) and 16q23 (WWOX) detected by SNP array. Primers used to detect chimeric transcript. The y-axes indicate genome copy number. H, detection of PVT1-WWOX chimeric transcript by RT-PCR. I, sequencing of chimeric junction of PVT1-WWOX chimeric transcript. J, putative structure of abnormal WWOX fusion transcript.

in neurons and other cells, and as gene abnormality and aberrant expression of NBEA have been associated with plasma cell dyscrasias (34–36). In addition, the association between the PVT1-NBEA fusion gene and the t(8;13) chromosomal abnormality, which has been reported in a small population of multiple myeloma, remains to be verified (7, 11, 37, 38). Chromosome 13 is often deleted in multiple myeloma and this has been linked to poorer prognosis. In such cases with loss of chromosome 13, RB1 is thought to be a major target and driver. However, a group has reported NBEA to also be a target of recurrent interstitial deletions at 13q13 and proposed that NBEA might be a tumor suppressor gene in multiple myeloma (36). WWOX is generally considered to be a candidate tumor suppressor gene, and known to have a proapoptotic effect by participating in the TNF apoptotic pathway and via direct physical interaction with p53 and its homolog p73 (39). However, immunohistochemistry revealed that WWOX protein levels were rather elevated in gastric and breast carcinoma (40). Therefore, WWOX did not seem to act as tumor suppressor gene simply. Interestingly, although both NBEA and WWOX are located at common fragile site, usually contributing to gene inactivation, FRA13A and FRA16D, respectively, these genes highly express via fusion to PVT1 (41, 42). It would be important to further elucidate the function of NBEA and WWOX. Several translocation/insertion partners of 8q24 rearrangements were identified in the remaining samples (Table 1).
breakpoints identified in the present study warrant further molecular analysis of the candidate genes.

The relationship between MYC and PVT1 in terms of multiple myeloma development and progression is difficult to elucidate. RQ-PCR analysis revealed high expression of PVT1 and MYC in most multiple myeloma cell lines regardless of PVT1 or MYC rearrangement status (Supplementary Fig. S6). Results showed that 8q24 rearrangements included various complex translocations with either deletion or insertion of a part of the 8q24 segment including MYC and the centromeric segment of PVT1. In addition, gains of chromosome 8, including PVT1 and MYC, are frequently identified in multiple myeloma cell lines (Fig 1B, Supplementary Fig. S1B). These gains are likely to contribute to the amplification of the PVT1 and MYC genes (28). Beyond chromosomal abnormalities, the molecular mechanisms underlying MYC overexpression in multiple myeloma, such as interaction with PVT1 or deregulation of miRNAs, warrant further research (32, 50).

In conclusion, PVT1 is frequently involved with various partner loci in multiple myeloma with 8q24 abnormalities, and, PVT1-NBEA and PVT1-WWOX were identified as novel, highly expressed chimeric genes in which NBEA and WWOX are fused with PVT1 in multiple myeloma cell lines harboring t(8;13)(q24;q13) and der(16)t(16;22)ins(16;8)(q23;q24), respectively. These findings suggest that PVT1 rearrangements may represent a novel molecular paradigm underlying the pathology of multiple myeloma with 8q24 rearrangements.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Writing, review, and/or revision of the manuscript: H. Nagoshi, T. Taki, I. Hanamura, K. Nishida, S. Horikke, J. Kuroda, M. Taniwaki

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Nagoshi, T. Taki, I. Hanamura, K. Nishida, S. Horikke


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