An Epigenetically Derived Monoclonal Origin for Recurrent Respiratory Papillomatosis

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Objective: To investigate the contribution of promoter methylation-mediated epigenetic events in recurrent respiratory papillomatosis tumorigenesis.

Design: Archival tissue DNA, extracted from microdissected papilloma lesions, was interrogated for methylation status by means of the novel, multigene methylation-specific multiplex ligation-dependent probe amplification assay.

Subjects: Fifteen subjects with recurrent respiratory papillomatosis, 3 females and 12 males, all with adult onset of illness (age range, 23-73 years) except for 1 female patient with juvenile onset (1 year old).

Results: Promoter hypermethylation was recorded in 14 of 15 cases, and 19 of 22 unique methylation-prone cancer genes in the multigene panel had altered DNA methylation in at least 1 laryngeal papilloma biopsy specimen. Identical abnormally methylated genes were found in 5 of 15 recurrent cases, of which the CDKN2B gene was hypermethylated in all 5 cases. Dissimilar epigenetic events were noted in the remaining cases.

Conclusions: A clonal origin was derived for 5 of 15 recurrent respiratory papillomatosis biopsy specimens based on identical epigenetic events. The high frequency of epigenetic events, characterized by consistent promoter hypermethylation of multiple tumor suppressor genes, points to the use of gene silencing mechanisms in the pathogenesis of recurrent respiratory papillomatosis.


RECURRENT RESPIRATORY (laryngeal) papillomatosis (RRP), an extremely rare condition, is characterized by benign neoplasms within the respiratory tract and can be potentially life threatening because of airway obstruction.1 Recurrent respiratory papillomatosis presents primarily as tiny or larger warts on the vocal chords. Prevalence of RRP worldwide is approximately 100,000, with 2300 new cases in the United States each year.2-4 Juvenile-onset disease occurs primarily in patients younger than 1 year to 8 years old; shows no sex difference5,6; has a rapid but often unpredictable pattern of recurrence;5 tends to be a long-term, often lifelong disease; and exhibits a continuum of severity and aggressiveness. The adult form of RRP has a variable age at onset (peak, approximately 20-30 years),6,7 with a higher incidence in males.6 The severity, aggressiveness, and recurrence of the adult form tend to be less than in the juvenile form.6 Human papillomavirus types 6 and 11 account for 80% to 90% of RRP.5 A small percentage of RRP cases progress to malignancy.9

Laryngeal papillomas usually run a benign but recurrent course. Spontaneous transformation of RRP to squamous cell carcinoma is not easily characterized by a histologic progression through dysplasia over time, making these lesions difficult to diagnose histologically and clinically early in the course of the transformation of the disease.

Clonality, the property that the cells within a tumor are derived from a single parent cell, is often indicated by uniformity or relative uniformity of genetic aberrations contained within many or all cells of the tumor. Such aberrations are assumed to confer or reflect biological distinctions relevant to tumor behavior, and thus to be relevant to tumor initiation and clonal expansion.10,13

Epigenetics is the regulation of changes in gene expression by mechanisms that do not involve changes in DNA sequence. Establishment and maintenance of epigenetic control (gene silencing) has several...
aspects, which include promoter region hypermethylation, methyl-binding proteins, DNA methyltransferases, histone deacetylases, and chromatin state. Aberrant methylation of CpG islands is a hallmark of human cancers and is found early during carcinogenesis. Genes in cellular pathways that are inactivated by promoter region hypermethylation include MGMT (DNA repair), p16INK4a, p15INK4b (cell cycle), DAPK (apoptosis), and GSTP1 (detoxification).

We investigated alterations in DNA methylation in biopsy specimens of recurrences from patients with RRP to assess the contribution of promoter methylation-mediated epigenetic events in RRP tumorigenesis. Aberrant promoter methylation of 22 methylation-prone tumor suppressor genes was evaluated by means of a high-throughput multigene probe panel (41 gene probes, 35 unique genes, including control probes) in 15 RRP cases by using the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay.

**METHODS**

**RRP COHORT**

The RRP cohort comprised 15 subjects, 3 females and 12 males, all with adult onset of respiratory papillomatosis (age range, 23–73 years) except for 1 female patient with juvenile onset (1 year old). The number of biopsy specimens from patients with recurrences ranged from 1 to 6. Archival tissue DNA, extracted from microdissected papilloma lesions, was interrogated for methylation status by means of the MS-MLPA assay.

**DNA EXTRACTION**

As a first step, 300 µL of P-buffer (50mM Tris hydrochloride, pH 8.5; 100mM sodium chloride; 1mM EDTA; 0.1% Triton X-100; 20mM dithiothreitol) was added to tubes containing whole 5-µm tissue sections or microdissected tissue. The tube was heated for 15 to 20 minutes at 90°C in a water bath and allowed to cool to 60°C. Next, 6 µL of 20-µg/mL proteinase K was added, mixed, overlaid with 3 drops of mineral oil, and spun for 5 seconds at 13,000 g. This was followed by a 4- to 16-hour (overnight) incubation at 60°C. The tube was heated for 10 minutes at 90°C to denature the proteinase K and to disrupt nucleic acid formaldehyde adducts. On removal of the oil, the tube was centrifuged for 15 minutes (at 13,000g) at room temperature. Next, 250 µL of the supernatant was transferred to a clean 1.5-mL tube. On addition of 10 µL of 5M sodium chloride and 1000 µL of ethanol to the 250-µL supernatant, the tube was incubated at −20°C for least 60 minutes. This was followed by centrifugation for 15 minutes at 13,000g at −4°C. On removal of the supernatant, an additional centrifugation step for 10 seconds ensured removal of the last traces of the supernatant. Finally, the pellet was air dried and dissolved in 100 µL of double-distilled water.

**MS-MLPA ASSAY**

The MS-MLPA assay allows for the relative quantification of approximately 41 different DNA sequences in a single reaction requiring only 20 ng of human DNA. The standard use of the technique to observe quantitative changes in copy number has been outlined in other studies. Adaptation of the MLPA to detect aberrant methylation (MS-MLPA) has been detailed elsewhere.

The probe design is similar to that of ordinary MLPA probes. For 26 of 41 probes, the recognition sequence detected by the MLPA probe is contained within a restriction site for the methyl-sensitive enzyme HhaI (Figure 1). The 41-gene-probe panel (Table 1) interrogates 35 unique genes implicated in cancer, including head and neck squamous cell carcinoma, for losses and gains in a separate reaction in the absence of the methyl-sensitive enzyme HhaI. Because there are 2 probes each for VHL, CDKN2A, BRCA1, and BRCA2, and 3 probes for MLH1, a normal control DNA sample will generate 41 individual peaks in the absence of HhaI (Figure 2). A concurrently run reaction with the 41-gene-probe set in the presence of HhaI is designed to detect aberrant promoter hypermethylation by taking advantage of an HhaI site in the promoter region of 22 of the 35 unique genes (note that 1 of the 2 BRCA1 probes is designed to recognize a region outside the HhaI recognition site; Table 1). Fifteen of the 41 gene probes are designed outside an HhaI site and serve as undigested controls (Figure 2). On digestion of the sample DNA with...
HhaI, probes that recognize the unmethylated regions will not generate a signal because these sequences have become cut by HhaI.

In this study, approximately 20 ng of DNA was used for each of the 2 reactions, one without HhaI and the other with HhaI. Briefly, DNA from normal controls (male and female) and RRP DNA for each of the 2 MLPA reactions was diluted with water to a total volume of 5 µL. The DNA was denatured and fragmented by heating for 5 minutes at 98°C in a thermocycler. Bi- nary MLPA probes were added and allowed to hybridize to their targets during a 16-hour incubation at 60°C. Digestion buffer and a special ligase (MRC Holland, Amsterdam, the Netherlands) were added to the vial. During 15-minute incubation at 60°C, the 2 parts of a probe could be ligated to each other and become an amplifiable molecule provided that the complementary sequence was present in the sample (Figure 1).

This was followed by the addition of polymerase chain reaction primers, deoxynucleotide triphosphates, and Taq polymerase, followed by the following cycles: 1 cycle of 1 minute at 95°C; 10 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 1 minute at 72°C. All (ligated) probes were amplified by the same primer pair, one of which was tagged with a fluorescent dye. The relative amounts of polymerase chain reaction product obtained reflect the relative amounts of ligated probes at the start of the polymerase chain reaction. Amplification products were analyzed on a DNA sequencer (ABI 310/AIBI 3100; Applied Biosystems Inc, Foster City, California), and the products were normalized and quantified.

Aberrant methylation was identified as the appearance of a signal peak that was otherwise absent in normal DNA samples (Figures 2, 3, and 4). To quantify whether 1, 2, or more copies of a specific gene locus became aberrantly hypermethylated, a mathematical algorithm was used.17

**RESULTS**

Promoter hypermethylation was recorded in 14 of 15 cases. Of the 22 unique methylation-prone cancer genes in the multigene panel, 19 had altered DNA methylation in at least 1 laryngeal papilloma biopsy specimen (Table 2).

Of the 15 recurrent cases, 5 cases had at least 1 abnormally methylated gene in a recurrent biopsy, of which the CDKN2B gene showed consistent hypermethylation in all 5 cases (Table 3; Figures 2, 3, and 4). All 3 biopsy specimens in case 4 indicated hypermethylation of CDKN2B, with gain of a TP73 epigenetic event in the subsequent 2 recurrences. APC and GSTP1 were hypermethylated in 1 recurrent biopsy in case 7. In case 13, all 3 specimens showed hypermethylation of the APC and the VHL promoter regions (Table 3).

Dissimilar epigenetic events were noted in the remaining cases (Table 2). Aberrant methylation of CDKN2B and APC genes was most frequent, occurring in 8 of 14 cases, followed by CDKN2A in 6, TIMP3 and VHL in 5, and DAPK1, HIC1, and GSTP1 in 4 of 14 cases (Table 2).

**COMMENT**

The scientific understanding of RRP has been a slow process, and RRP remains an enigmatic disease that contributes a substantial human and financial cost to the public. Originally called juvenile laryngeal papillomatosis, the disease has been increasingly recognized in adults and is now generally referred to as recurrent respiratory papillomatosis. Histologically, RRP is a benign disease characterized by unregulated growth of wartlike neoplasms on the larynx, trachea, and bronchi. Human papillomavirus has been shown to be the cause.8 Even though a small percentage of RRP cases progress to malignancy,9 spontaneous transformation of RRP to squamous cell carcinoma does not appear to be characterized by a histologic progression through dysplasia over time,22 making these cancers difficult to diagnose histologically and clinically early in the course of the transformation of the disease.

The molecular pathogenesis of RRP implicates dysregulation of apoptosis as determined by abnormal expression of antiapoptotic factors like survivin and XIAP as contributing to papilloma growth and survival.23 Human papillomavirus 11 infection is a likely early event associated with progression of RRP to carcinoma, with
increased expression of p53 and topoisomerase α-II and a reduced expression of p21(WAF1) protein gene as markers of transformation to invasive papillomatosis and squamous cell carcinoma.

Additional markers of an aggressive clinical course include high S-phase fraction, proliferative index, and Ki-67 expression.

There are few reported studies examining the molecular genetics of RRP, especially from the genomic and the epigenetics perspectives, and much remains to be learned. This study should serve as a forerunner in the delineation of genes that succumb to promoter hypermethylation silencing as underlying events in the epigenetic pathogenesis of RRP. In addition, it provides evidence of a clonal origin for RRP and permits the tracing of an epigenetic continuum implicating key tumor suppressor genes in RRP.

Epigenetic alterations produce heritable changes in gene expression without a change in the DNA coding sequence itself. Promoter region hypermethylation is known to be an early event in carcinogenesis. The consequence of CpG island hypermethylation, especially for those islands associated with tumor suppressor gene promoters is the loss of tumor suppressor gene function, which contributes to tumorigenesis. The most commonly methylated genes in this RRP cohort were CDKN2B and APC (8 of 14 cases), CDKN2A (6 cases), TIMP3 and VHL (5 cases), and DAPK1, HIC1, and GSTP1 (4 cases; Table 2). In addition, in the 5 RRP with at least 1 commonly methylated gene (Table 3), CDKN2B was identified in all subsequent biopsy specimens, marking this epigenetic event as an initiating clonal alteration in the recurrence continuum in RRP.

The cyclin-dependent kinase 2A (CDKN2A) and CDKN2B genes map to 9p21 and are in tandem, with CDKN2B located 25 kilobases centromeric to CDKN2A. The CDKN2A locus controls the Rb pathway (which regulates G1/S-phase transition) and the p53 pathway (which induces growth arrest or apoptosis in response to either DNA damage or inappropriate mitogenic stimuli) by generating 2 gene products, p16 and p14. Mutations in CDKN2A/p16 inactivate the Rb pathway, whereas deletion of the CDKN2A locus (CDKN2A/p16 and CDKN2A/p14) alter both the Rb and p53 pathways, which are important in many cancers. Inactivation of the CDKN2B/p15, CDKN2A/p14, and CDKN2A/p16 genes is a frequent event in human oral squamous cell carcinomas. The presence of aberrant methylation of p15 and p16 in precancerous oral tissues implicates methylation of p15 and p16 as early events in the pathogenesis of oral lesions.

Hypermethylation of the APC gene was another consistent epigenetic event in RRP, occurring in 8 of 14 cases, including biopsy specimens of recurrences in cases 7 and 13. APC (adenomatosis polyposis coli) is a tumor suppressor gene originally implicated in colon cancer. It has an important role in the Wnt signaling pathway, which is involved in the development of colorectal carcinomas. Genetic and epigenetic alterations in this gene have since been recognized in other malignant neoplasms, including oral squamous cell carcinomas, gastric cancers,
and esophageal adenocarcinomas. Uesugi et al\(^3\) previously reported \(APC\) as being mutated and/or deleted in primary oral squamous cell carcinoma tissues and suggested that loss of \(APC\) function contributes to carcinogenesis in the oral region. Promoter hypermethylation is also an important mechanism of \(APC\) inactivation in oral cancers, occurring in 25% of oral squamous carcinoma cells.\(^3\)

In Barrett metaplasia and dysplasia,\(^3\) hypermethylation of \(APC\), \(CDKN2A\), and \(ESR1\) were usually found in a large contiguous field, suggesting either a concerted methylation change associated with metaplasia or a clonal expansion of cells with abnormal hypermethylation.

Hypermethylation of the \(TIMP3\) and \(VHL\) genes occurred in 5 of 14 RRP cases, and of the \(VHL\) gene in all 3 biopsy specimens of case 13. The \(VHL\) gene is a tumor suppressor gene located at 3p26-p25 and is responsible for Von Hippel–Lindau syndrome, which is an inherited familial cancer syndrome that makes patients susceptible to a variety of neoplasms, malignant and benign. A study of clear-cell renal carcinomas showed that hypermethylation of \(VHL\) promoter region was associated with absence of transcript expression. It was also found that treatment of these methylated \(VHL\) tumors with a demethylating agent resulted in reexpression of the \(VHL\) transcripts.\(^3\)

\(TIMP3\) induces apoptosis,\(^3\) inhibits angiogenesis,\(^3\) impedes cell migration,\(^3\) and is a physiologic regulator of inflammation.\(^3\) Promoter methylation of \(TIMP3\) has been observed in many tumor types\(^3\) and is involved in the genesis of esophageal adenocarcinoma notably during the progression from dysplasia to carcinoma.\(^3\)

Hypermethylation of \(DAPK1\), \(GSTP1\), and \(HIC1\) was less frequent, occurring in 4 of 14 cases as well as in biopsy specimens of recurrence in case 7. Death-associated protein kinase 1, \(DAPK1\), located at 9q34.1, encodes a 160-kDa cytoskeletal-associated calcium/calmodulin-dependent serine/threonine kinase that was initially identified as a positive mediator of interferon.

![Figure 3. Methylation-specific multiplex ligation-dependent probe amplification probe mix without and with \(HhaI\) enzyme (DNA sequencer, ABI 3100) in a control DNA specimen (A) and 3 biopsy specimens from case 4 (B-D). Note methylation of \(CDKN2B\) in all 3 biopsy specimens and methylation of \(TP73\) in specimens 2 and 3. bp indicates base pairs; PCR, polymerase chain reaction.](http://example.com)
γ-induced programmed cell death in HeLa cells. DAPK1 expression is commonly lost in urinary bladder, breast, and B-cell neoplasms and renal cell carcinoma cell lines because of promoter hypermethylation. Aberrant promoter methylation of DAPK1 has been shown to frequently occur in human head and neck cancers, non–small-cell lung carcinomas, gastric and colorectal carcinomas, and uterine cervical carcinomas. In head and neck squamous cell carcinoma, DAPK1 promoter hypermethylation has been associated with metastasis to lymph nodes as well as advanced disease stage.

HIC1 is a tumor suppressor gene that encodes a transcriptional repressor with 5 Kruppel-like C2H2 zinc finger motifs and an N-terminal BTB/POZ domain. Epigenetic silencing of HIC1 has been shown to significantly influence tumorigenesis. Loss or reduced HIC1 messenger RNA in pediatric tumor cell lines with aberrantly methylated HIC1 became reexpressed in all cell lines by treatment with the demethylating agent 5-aza-2'-deoxycytidine.

Glutathione S-transferase π (GSTP1) encodes for the glutathione S-transferase π enzyme, which plays an important role in detoxification. It maps to 11q13 and also has a role in susceptibility to cancer and other diseases. Inactivation of GSTP1 by promoter hypermethylation is characteristic of corticosteroid-related neoplasms such as breast, liver, and prostate cancers. The class of glutathione S-transferase enzymes has been associated with preneoplastic and neoplastic changes. Promoter hypermethylation pattern of the p16, MGMT, GSTP1, and DAPK genes have been used as molecular markers for cancer cell detection in paired serum DNA, and almost half of the patients with head and neck squamous cell carcinoma with methylated tumors were found to display these epigenetic changes in paired serum.

Persistence of the same aberrantly methylated gene in 36% of multiple recurrent biopsy specimens (5 of 14 cases) in this study cohort supports a monoclonal origin for RRP. Neoplasia typically develops as a clonal expansion from a single cell of origin. There is ample experimental and

Figure 4. Methylation-specific multiplex ligation-dependent probe amplification probe mix without and with HhaI enzyme (DNA sequencer, ABI 3100) in a control DNA specimen (A) and 3 biopsy specimens from case 7 (B-D). Note methylation of CDKN2B in all 3 biopsy specimens and methylation of APC and GSTP1 in specimens 1 and 3. bp indicates base pairs; PCR, polymerase chain reaction.
clinical evidence favoring a monoclonal origin of cancer, and some of the strongest arguments are derived from cytogenetic investigations. More recent approaches have used a combination of fluorescence in situ hybridization and karyotyping of tumors with chromosome rearrangements as clonal markers. Loss of heterozygosity patterns at different loci have also been useful as clonal markers, and p53 mutations have been used as clonal markers.

Clonal epigenetic alterations in precancerous lesions may reflect biological peculiarities pertinent to tumor behavior. Knowledge of whether a neoplasm has a single or multiple cell origin may provide important information about its etiology and pathogenesis. The high frequency of epigenetic events characterized by consistent aberrant promoter hypermethylation of multiple tumor suppressor genes points to the use of gene silencing mechanisms as one of the driving forces behind the growth of recurrent laryngeal papillomas. Additional studies to further confirm and validate the results of this study in a larger sample are in progress.

Recurrent genomic aberrations are good indicators of genes that are causally associated with cancer development or progression. Because promoter hypermethylation is potentially reversible, the molecules that regulate the methylation status of DNA are considered promising targets for new cancer therapies. Identifying epigenetic alterations in a precancerous lesion may lead to the discovery of biomarkers that add to the knowledge of risk assessment and early detection, and may provide molecular targets for chemopreventive interventions.

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Author Contributions: Drs Stephen and Worsham had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data.

<table>
<thead>
<tr>
<th>Table 2. Case Summary and Methylation Status</th>
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<tbody>
<tr>
<td>Patient No.</td>
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<tr>
<td>No. of biopsies</td>
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<tr>
<td>TIMP3</td>
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<tr>
<td>CDKN2A</td>
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<tr>
<td>MLH1</td>
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<td>KLLK10</td>
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<td>MEC</td>
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<td>CDKN24B</td>
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<td>VHL</td>
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<td>TP73</td>
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<td>FANCD2</td>
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<td>IGFBP4</td>
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<td>CDKN18</td>
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<tr>
<td>BRCA1</td>
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<tr>
<td>GSTP1</td>
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<tr>
<td>Abbreviation: M, methylated.</td>
</tr>
<tr>
<td>a Cases with dissimilar epigenetic events in multiple biopsy specimens.</td>
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<tr>
<td>b Cases with similar epigenetic events in multiple biopsy specimens.</td>
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<tr>
<td>c Case with absence of M genes.</td>
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<td>d Commonly M genes (present in &gt; 3 cases).</td>
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<tr>
<th>Table 3. Epigenetically Linked Recurrent Laryngeal Papilloma Cases</th>
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<tr>
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<tr>
<td>2 (10 mo)</td>
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<td>3 (30 mo)</td>
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<td>7</td>
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<td>2 (3 mo)</td>
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<td>3 (6 mo)</td>
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<td>11</td>
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<td>12</td>
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<td>2 (14 mo)</td>
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<td>13</td>
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<tr>
<td>2 (1 mo)</td>
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<td>3 (3 mo)</td>
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Abbreviation: M, methylated.

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REFERENCES


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