Green tea polyphenols inhibit colorectal aberrant crypt foci (ACF) formation and prevent oncogenic changes in dysplastic ACF in azoxymethane-treated F344 rats

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Green tea and its constituents have shown cancer-preventive activities in many animal models. In order to prepare for a human trial on the inhibition of colon carcinogenesis, we conducted a study with green tea polyphenols as the preventive agent in an azoxymethane (AOM)-induced rat colon cancer model using aberrant crypt foci (ACF) as an end point. F344 rats were given two weekly injections of AOM (15 mg/kg), and then fed a 20% high-fat diet with or without 0.12 or 0.24% Polyphenon E (PPE, a standardized green tea preparation consisting 65% of (–)-epigallocatechin-3-gallate and 22% of other catechins) for 8 weeks. Colorectal ACF were analyzed under a microscope after methylene blue staining. Dietary PPE administration was found to significantly and dose dependently decrease the total number of ACF per rat and the total number of aberrant crypt per rat. Moreover, treatment with 0.24% PPE also significantly decreased the percentage of large ACF (four or more crypts) and the percentage of ACF with high-grade dysplasia in total ACF. The high-grade dysplastic ACF from 0.24% PPE-treated group had increased apoptosis and decreased nuclear expression levels of β-catenin and cyclin D1. Retinoid X receptor (RXR)a expression was reduced in high-grade dysplastic ACF, adenoma and adenocarcinoma during AOM-induced colon carcinogenesis, and the PPE treatment partially prevented the loss of RXRa expression in high-grade dysplastic ACF. Taken together, our results strongly suggest the colon cancer-preventive activity of PPE and identified possible molecular markers for future colon cancer prevention studies.

Introduction

Tea, made from dried leaves of the plant Camellia sinensis, is the second most popular beverage worldwide next to water. In many different animal models, tea and its constituents have been demonstrated to inhibit tumorigenesis in different organ sites including the lung, oral cavity, esophagus, stomach, small intestine, colon, skin, prostate, mammary glands, liver, pancreas and bladder (1–5). Tea polyphenols, which account for about a third of the water extractable biomaterials in dry weight, are the most abundant bioactive components of green tea (summary odds ratio 0.82, 95% confidence interval 0.69–0.98). A prospective study on women in Shanghai indicated a negative association between tea consumption and the risk of CRC (26). We participated in a nested case–control study within the Shanghai Cohort Study, involving 18 244 men, to investigate the association between prediagnostic urinary levels of (–)-epigallocatechin and 4′-methyl (–)-epigallocatechin, markers of tea consumption, were significantly associated with a reduced risk of developing CRC in men. These results provide a strong rationale for conducting human intervention trials on the prevention of human CRC with well-defined tea polyphenol preparations. In such trials, ACF can be utilized as a surrogate end point biomarker, which are detected and characterized by magnifying chromoendoscopy (28,29).

ACF, originally described by Bird (30) in AOM-treated murine colon, have been accepted as pre-neoplastic lesions of the colon. In humans, ACF are commonly observed in familial adenomatous polyposis patients, as well as in sporadic CRC patients (31–34). Human ACF harbor many neoplastic alterations, such as increased proliferation activity, mutations in K-RAS and APC genes, as well as microsatellite instability (33–36). Hao et al. (37) demonstrated that nuclear and cytoplasmic β-catenin expression was increased in human colorectal ACF according to their degree of dysplasia, and moreover, nuclear and cytoplasmic β-catenin expression was increased from dysplastic ACF to adenoma and to adenocarcinoma in the human colon. Consistent with this notion, dysplastic ACF with nuclear β-catenin accumulation recently have been suggested to be more informative pre-neoplastic lesions of chemical carcinogen-induced colon cancer in rats (38).

Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; CRC, colorectal cancer; EGCC, (–)-epigallocatechin-3-gallate; IHC, immunohistochemistry; PPE, Polyphenon E; RXR, retinoid X receptor.

These authors contributed equally to this work.
In preparation for a human intervention trial, we investigated in the current study the effect of a standardized tea polyphenol preparation, Polyphenon E (PPE), on ACF formation in AOM-treated F344 rats. The cellular and molecular changes in the high-grade dysplastic ACF caused by dietary PPE treatment were characterized. Our results provide evidence that PPE is a potential chemopreventive agent for CRC and suggest possible biomarkers for use in future cancer prevention studies in humans.

Materials and methods

Animals
Male F344 rats at 5 weeks of age were purchased from Taconic Farms (Germantown, NY). After 1 week of acclimation, animals were randomly distributed into control and experimental groups. All animals were housed three in a plastic cage with a filter top. The animal room was controlled at 20 ± 2°C, 50 ± 10% humidity and a 12 h light/dark cycle. Animals had free access to food and water at all times. Food cups were replenished with fresh diet twice weekly.

Experimental procedure
At 7 weeks of age, all animals were subjected to two weekly subcutaneous injections of AOM (Midwestern Research Institute, Kansas City, MO) at a dose of 15 mg/kg each. One day after the second injection, animals were fed a modified 20% high-fat AIN-76A diet (39) containing 0, 0.12 or 0.24% PPE (nine animals per group). PPE was a gift from Dr. Yukihiko Hara of the Mitsui Norin Co., Ltd (Tokyo, Japan). This is a standardized green tea polyphenol preparation, containing 65% EGCG, 7% epicatechin-3-gallate, 3% epigallocatechin, 9% epicatechin, 3% gallo1catechin gallate and 0.6% caffeine. Dose selection for PPE was based on our previous study, which showed that oral administration of EGCG at doses of 0.08 or 0.16% in drinking fluid significantly decreased small intestinal tumor formation in Apo^−/− mice (11). To minimize possible degredation of tea polyphenols, all stock diets were stored at 4°C for the entire period of the experiment. Body weight and food consumption were monitored once and twice a week, respectively, until the termination of the experiment at 8 weeks after the second AOM injection. All animals were killed by CO2 asphyxiation. After laparotomy, the spleen and entire large intestine were harvested. Spleen was washed with ice-cold saline, blotted and then weighed. The large intestine from cecum to anus was longitudinally opened, flushed with ice-cold saline and fixed flat between two pieces of filter paper in 10% buffered formalin for 24 h. In another experiment, F344 rats were treated with AOM as described above, and then fed with modified AIN-76A diet containing 20% fat. As shown in Figure 1A, all animals had a steady body weight gain during the treatment, and the administration of AOM and 0.12 or 0.24% PPE did not affect the growth of the rats in all the groups during the treatment, and the administration of AOM and 0.12 or 0.24% PPE did not affect the growth of the rats in all the groups measured at different time points. Food consumption of treatment groups of 0.12 or 0.24% PPE was ~12–15 gram per day per animal, which was not different from that of the AOM-treated positive control group (Figure 1B). During the entire period of experiment, there were no signs of toxicity or conditions, suggesting adverse effects caused by dietary administration of PPE. There was no difference among the mean spleen weights of positive control and treatment animals (average 0.64–0.67 g) at the time of killing.

Identification of ACF
The formalin-fixed colonic tissues were cut into 4 cm segments and stained in 0.2% methylene blue solution for 3–5 min. The total number of ACF and the number of aberrant crypts (ACs) in each focus were counted under a microscope (×40 to ×100). ACF were identified with the following morphological characteristics: (i) the enlarged and elevated crypts than normal mucosa and (ii) increased peri-cryptal space and irregular lumens.

Histological analyses of ACF and tumors
In order to harvest most of the ACF for histological analyses, methylene blue-stained colonic tissues were examined under a microscope, and large ACF containing four or more AC were marked by permanent ink. At least 10–12 pieces of colonic tissues containing the marked ACF were dissected from the mid-distal part of each colon, where most ACF occurred. These tissues were then processed and appropriately oriented in paraffin blocks for longitudinal sectioning serially at 4 μm thickness. Because ACF may develop into corkscrew shape when they progress into high-grade dysplasia, only a few whole crypts were observed in their entirety. Sections 1, 10 and 20 were stained with hematoxylin and eosin for histological evaluation, which allowed ACF visualization at different section levels. Based on nuclear to cytoplasmic ratio, cell polarity, chromatin pattern, mitotic figures and mucin secretion, ACF were classified into three categories: (i) ACF with hyperplasia (no dysplasia); (ii) ACF with low-grade dysplasia (elongated, slightly crowded and pseudosтратified nuclei, but polarity well preserved, normal or slightly reduced numbers of goblet cells); and (iii) ACF with high-grade dysplasia (elongated, crowded and pseudosтратified nuclei; markedly increased nucleus to cytoplasm ratio, significantly reduced number of goblet cells, back-to-back glands and markedly decreased inter glandular stroma) (37). The sections adjacent to hematoxylin and eosin sections that contained histologically confirmed ACF were used for immunohistochemistry (IHC). Fixed colorectal tumors were processed and stained as described above, and adenomas and adenocarcinomas were identified as described previously (37).

IHC
A standard avidin–biotin peroxidase complex method was employed as described previously (37). In brief, after dewaxing and rehydrating, the slides were heated in a pressure cooker in sodium citrate buffer (0.01 M, pH 6.0) for 3 min after reaching full pressure. Endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol. Sections were then blocked for 1 h at room temperature in phosphate-buffered saline containing 3% normal horse or goat serum depending on the origin of the primary antibody. The sections were then immunostained with anti-β-catenin (1:3000, BD Biosciences, Franklin Lakes, NJ), cyclin D1 (1:100, DAKO, Carpenteria, CA), retinoid X receptor (RXR)α (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) or cleaved caspase-3 (1:200, Cell Signaling, Danvers, MA) antibodies overnight at room temperature. The antibodies were diluted in 10% goat or horse serum. The sections were rinsed in phosphate-buffered saline and incubated with a biotinylated secondary antibody and subsequently incubated in VECTORSTAIN ELITE ABC reagent for 30 min, using 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA) as the chromogen. Sections were then counterstained for 2–3 min with hematoxylin (Sigma, St Louis, MO) and mounted with Permount.

Evaluation of the staining
Both positive and negative stained cells in high-grade dysplastic ACF were counted for cleaved caspase-3, nuclear expression of β-catenin, cyclin D1 and RXRα. Apoptotic index, nuclear positivity for β-catenin, cyclin D1 and RXRα were then calculated based on the percentage of positive cells in total ACF cells analyzed. The intensity of membranous staining for β-catenin was scored as follows: (i) 0 = negative, (ii) 1 = weak, (iii) 2 = moderate and (iv) 3 = strong (as intense as normal mucosa). Cytoplasmic staining for β-catenin was scored as follows: (i) 0 = negative (no cytoplasmic staining), (ii) 1 = weak, (iii) 2 = moderate and (iv) 3 = strong (37). Tumor sections were evaluated for β-catenin and RXRα staining as described above.

Statistical analysis
The data on ACF multiplicity, labeling index and intensity of staining were analyzed by Student’s t-test and analysis of variance. Effects of the treatments on the number of ACF in different categories were analyzed by chi-square test.

Results
General observation
As shown in Figure 1A, all animals had a steady body weight gain during the treatment, and the administration of AOM and 0.12 or 0.24% PPE did not affect the growth of the rats in all the groups measured at different time points. Food consumption of treatment groups of 0.12 or 0.24% PPE was ~12–15 gram per day per animal, which was not different from that of the AOM-treated positive control group (Figure 1B). During the entire period of experiment, there were no signs of toxicity or conditions, suggesting adverse effects caused by dietary administration of PPE. There was no difference among the mean spleen weights of positive control and treatment animals (average 0.64–0.67 g) at the time of killing.

Dietary PPE treatment inhibited ACF formation
After 8 weeks of dietary treatment with PPE, animals were killed and the entire colons were harvested. Colonic ACF were identified and analyzed under a light microscope after methylene blue staining. ACF mostly occurred in the distal colon. Table I summarizes the total number of ACF per rat, the total number of AC per rat, the number of AC per focus and the number (percentage) of foci containing one, two, three or four or more AC. All rats developed ACF in the colon 8 weeks after AOM treatment (100% incidence). Compared with the positive control group, dietary PPE treatment at levels of 0.12 and 0.24% dose dependently decreased the total number of ACF per rat by 16 and 37% (P < 0.01), respectively, and decreased the total number of AC per rat by 17 and 43% (P < 0.01), respectively. The rats treated with 0.24% PPE also showed a significantly lower number of AC per focus compared with the positive control animals (P < 0.05). We subsequently categorized ACF based on the number of AC in each
focus. The results showed that PPE treatments generally decreased the number of foci in all four categories compared with the positive control group, especially by 0.24% PPE treatment. Furthermore, the percentage of large ACF (containing four or more AC) in total ACF of 0.24% PPE-treated rats was significantly lower than that of the positive control animals (27% reduction, \( P < 0.05 \)). As a consequence, the percentage of the smallest ACF (containing one AC) in total ACF of 0.24% PPE-treated rats was significantly higher than that of the positive control animals (21% increase, \( P < 0.05 \)).

Dietary PPE treatment inhibited formation of dysplastic ACF

ACF from different groups were subjected to histological analyses. A total of 63 ACF from the positive control rats, 76 ACF from 0.12% PPE-treated rats and 42 ACF from 0.24% PPE-treated rats were examined histologically and classified according to the criteria described in Materials and Methods and shown in Figure 1C–E. The percentages of ACF with dysplasia were significantly lower in both the 0.12 and 0.24% PPE treatment groups (Table II). The percentages of high-grade dysplastic ACF among total ACF were reduced by 50% by PPE treatment at both dose levels. As a consequence, the percentages of hyperplastic ACF were higher in PPE treatment groups.

Induction of apoptosis in high-grade dysplastic ACF by PPE

To shed light on the mechanism of action of PPE, ACF with high-grade dysplasia from positive control and 0.24% PPE-treated rats were analyzed for apoptosis by IHC with anti-cleaved caspase-3 antibody (Figure 2H and I). Cleaved caspase-3-positive cells displaying nuclear staining were observed in ACF, but rarely in the normal mucosa. Apoptotic index was defined as the percentage of cleaved caspase-3 positively stained cells among total cells in high-grade dysplastic ACF observed in each tissue section. About 1.3% cells from the positive control group were positively stained by anti-cleaved caspase-3 antibody, i.e. with an apoptosis index of 1.3%. The group that received dietary treatment with 0.24% PPE had a significantly higher apoptotic index of 3.0% in high-grade dysplastic ACF (2.3-fold higher, \( P = 0.05 \), Table III). PPE treatment did not cause any appreciable increase of cleaved caspase-3-positive cells in normal mucosa.

Modulation of β-catenin expression in high-grade dysplastic ACF by PPE

Aberrant β-catenin expression is a common feature of AOM-induced rat colon tumor and human CRC (40,41). We analyzed β-catenin expression in normal mucosa, high-grade dysplastic ACF, adenoma and adenocarcinoma from positive control rats. The staining patterns with anti-β-catenin antibody are shown in Figure 2A–E. Normal mucosa showed a distinct membranous staining of β-catenin. High-grade dysplastic ACF, adenoma and adenocarcinoma displayed reduced membranous staining, accompanied by increased nuclear and cytoplasmic staining of β-catenin, as compared with the normal mucosa. Moreover, adenocarcinoma showed weaker membranous staining of β-catenin than high-grade dysplastic ACF and adenoma. We then analyzed β-catenin expression in high-grade dysplastic ACF of 0.24% PPE-treated animals and compared it to that of the positive control group (Figure 2C and Table III). In the positive control group, 26.2% of high-grade dysplastic ACF cells showed positive nuclear β-catenin staining. In the 0.24% PPE treatment group, only 7.2% ACF cells showed positive nuclear β-catenin staining (73% reduction, \( P < 0.01 \)). Cytoplasmic β-catenin expression in high-grade dysplastic ACF was significantly lower in 0.24% PPE-treated group by 73%.
Inhibition of cyclin D1 expression in high-grade dysplastic ACF by PPE

Cyclin D1 is an important downstream protein of β-catenin signaling and is involved in cell growth and proliferation. We analyzed cyclin D1 expression in high-grade dysplastic ACF to determine whether cyclin D1 expression would be reduced concomitantly to the prevention of aberrant β-catenin expression by PPE treatment observed above. ACF from both positive control and PPE-treated groups showed nuclear staining patterns for cyclin D1 (Figure 2F and G). In the positive control group, 59.2% of high-grade dysplastic ACF cells showed positive nuclear cyclin D1 staining, while only 31.2% high-grade dysplastic ACF cells showed positive nuclear cyclin D1 staining from 0.24% PPE-treated group (47% reduction, *P* < 0.01, Table III). These results further support the observation that PPE treatment prevented aberrant β-catenin function in ACF with high-grade dysplasia.

Loss of RXRα expression and its prevention by PPE in high-grade dysplastic ACF

Studies have shown that RXRs were associated with carcinogenesis in multiple organ sites (42–46). To shed light on the role of RXRα in colorectal carcinogenesis, RXRα expression was analyzed in AOM-induced colorectal ACF with high-grade dysplasia, adenomas and adenocarcinomas (Figure 3). In positive control rats, normal epithelial cells showed strong nuclear staining of RXRα; whereas in adenomas, the nuclear RXRα staining was much weaker; and in adenocarcinomas, the nuclear RXRα expression was almost lost. It is interesting that a decreased nuclear level was even observed in high-grade dysplastic ACF. Using staining intensity of RXRα in normal epithelial cells as the internal standard, IHC evaluation showed that 34.2% high-grade dysplastic ACF cells showed positive nuclear cyclin D1 staining from 0.24% PPE-treated group (47% reduction, *P* < 0.01, Table III). These results further support the observation that PPE treatment prevented aberrant β-catenin function in ACF with high-grade dysplasia.

Discussion

In the present study, we investigated the effect of PPE, a standardized tea polyphenol preparation, on colorectal ACF formation in AOM-treated F344 rats. To mimic typical American dietary composition, the animals were fed a modified AIN-76A diet that contained 20% fat during the entire period of study. The composition of the 20% fat was a slight modification of the American blend fat developed by the Institute of Shortening and Edible Oils (39). PPE was fed to the animals through the diet. Dietary administration is a more convenient way of delivering PPE to the rat as compared with administration...
through drinking fluid because fluid consumption may be affected by the bitterness of the PPE solution. Our results demonstrated that dietary PPE treatment inhibited the formation of ACF in AOM-treated rats, and this inhibition is characterized by (i) decrease in total number of ACF per rat, total number of AC per rat and number of AC per focus; (ii) reduction in the percentage of large ACF (with four or more AC) among total ACF; and (iii) reduction in the percentage of high-grade dysplastic ACF among total ACF.

In the colon mucosa, mucin-depleted foci and \( \beta \)-catenin-accumulated crypts, all with dysplasia in nature, have been suggested to be more relevant pre-neoplastic lesions for colon cancer, whereas hyperplastic ACF often lack the potential to develop into neoplasia (47–49). Herein, we categorized ACF into three types, i.e. hyperplastic, low-grade dysplastic and high-grade dysplastic. We prefer to use the term ‘dysplastic ACF’ following that by Hao et al (37) and Siu et al (50), even this type of lesions are also known as microadenomas (51,52). Our results showed that high-grade dysplastic ACF, like adenomas and adenocarcinomas, displayed reduced membranous expression, but increased nuclear and cytoplasmic expression, of \( \beta \)-catenin. We demonstrated, for the first time, the inhibitory effect of dietary tea polyphenols on the formation of high-grade dysplastic ACF, and this effect was associated with increased apoptosis, decreased nuclear expression levels of \( \beta \)-catenin and cyclin D1 and increased expression level of nuclear RXR\( \alpha \). Aberrant expression of these molecules was not frequently observed in low-grade dysplastic or hyperplastic ACF, whereas high-grade dysplastic ACF showed relatively uniform aberrant expression of these molecules. Therefore, their responses to PPE treatment were not systematically analyzed in low-grade dysplastic or hyperplastic ACF in the current study.

The biological activities of retinoids are mediated by two families of nuclear receptors: RXRs and retinoic acid receptors. Dysfunctions of RXRs have been associated with carcinogenesis in multiple organ

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**Table III.** Effects of dietary PPE treatment on \( \beta \)-catenin, cyclin D1 and RXR\( \alpha \) expression and apoptosis in high-grade dysplastic ACF in AOM-treated F344 rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of ACF analyzed</th>
<th>( \beta )-catenin</th>
<th>Cyclin D1 (%)</th>
<th>Apoptotic index (%)</th>
<th>RXR( \alpha ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nuclear staining (%)</td>
<td>Membranous staining (intensity)</td>
<td>Cytoplasmic staining (intensity)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>26.2 ± 3.2</td>
<td>1.9 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>59.2 ± 4.4</td>
</tr>
<tr>
<td>0.24% PPE</td>
<td>6</td>
<td>7.2 ± 3.8</td>
<td>2.5 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>31.2 ± 7.8</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0.01</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\( \beta \)-Catenin was analyzed by IHC using antibody against \( \beta \)-catenin for nuclear (positivity), membranous and cytoplasmic (both for intensity). Cyclin D1 was analyzed by IHC using antibody against cyclin D1 and apoptotic index was determined by IHC using antibody against cleaved caspase-3 (Asp175). RXR\( \alpha \) was analyzed by IHC using antibody against RXR\( \alpha \). The positivity was expressed as percentage of positive cells in all ACF cells analyzed. Two-tailed Student’s t-test was used for statistical analysis. Data represent mean ± SD.
sites, such as the breast, lung, thyroid, prostate and pituitary (42–46). In this work, we demonstrated that the expression of RXRα was decreased in ACF with high-grade dysplasia, and it was further decreased in adenomas and adenocarcinomas. These results suggest that decreased nuclear expression of RXRα occurs before the development of the adenoma and may be used as a biomarker for monitoring colon cancer chemopreventive activity.

Recently, Xiao et al (53) demonstrated an APC-independent regulation of β-catenin degradation via RXRα-mediated pathway, in which RXRα repressed β-catenin-mediated transcription by inducing the proteasomal degradation of β-catenin. Consistent with this finding, our results showed that the decreased expression of RXRα occurred in parallel with the increased nuclear accumulation of β-catenin in colorectal lesions during AOM-induced rat colorectal carcinogenesis. More interestingly, we demonstrated that dietary PPE treatment prevented the loss of RXRα expression as well as the aberrant nuclear expression of β-catenin in high-grade dysplastic ACF. Nuclear expression of cyclin D1, an important downstream protein of β-catenin pathway, was also decreased in high-grade dysplastic ACF by dietary PPE treatment. Moreover, dietary PPE treatment significantly induced apoptosis in high-grade dysplastic ACF. These findings suggested that, besides dysfunctions of APC signaling pathways, the decrease in RXRα expression might represent a mechanism by which pre-neoplastic lesions, such as high-grade dysplastic ACF, escape the control of normal growth and differentiation and progress into colorectal tumors. Maintaining RXRα expression level in dysplastic ACF by PPE treatment may contribute to the lower nuclear β-catenin level through proteasomal degradation of β-catenin. It is possible that high-grade dysplastic ACF with high nuclear β-catenin level and low RXRα level may have more potential to develop into adenomas or adenocarcinomas than high-grade dysplastic ACF with lower nuclear β-catenin level and higher RXRα level. Further investigation is needed to establish a relationship between maintaining RXRα level and the prevention of aberrant nuclear β-catenin expression by PPE in this animal model. It is also possible that the decreased nuclear level of β-catenin is due to a decreased nuclear translocation of β-catenin into the nuclear as we reported previously (11).

Taken together, dietary PPE treatment inhibited ACF formation, especially high-grade dysplastic ACF, in AOM-treated F344 rats. We propose that this inhibition was associated with promoting apoptosis, preventing aberrant nuclear β-catenin accumulation, decreasing cyclin D1 level and maintaining RXRα expression. These may be useful biomarkers in colon cancer prevention studies in humans.

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References


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