Microwave ablation combined with anti-PD-1 therapy enhances systemic antitumor immunity in a multitumor murine model of Hepa1-6

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ABSTRACT

Background: To evaluate the changes of immune environment of distant tumors after combined microwave ablation (MWA) and anti- programmed death receptor \textsuperscript{-} 1 \textsuperscript{(anti-PD-1)} therapy, and assess the changes of systemic immune response.

Methods: Bilateral hepatocellular carcinoma model was established in mice, which were then subsequently treated with MWA, or anti-PD-1, or no treatment, or MWA + anti-PD-1. The contralateral tumor volume and mice survival time were recorded. Flow cytometry and immunohistochemistry were used for evaluation of the immune cells subgroup change of contralateral tumor. In addition, tumor rechallenge tests were conducted on unilateral tumor-bearing mice to examine the systemic immune effects of the combination therapy.

Results: We found that MWA treatment alone failed to produce a significant abscopal effect. In contrast, the combination group had longer survival than the MWA or anti-PD-1 group alone, with slower distant tumor growth. Moreover, the tumor-specific immune responses induced by combination therapy are stronger than anti-PD-1 or MWA alone. Combination therapy also elevated the levels of Th1-type cytokines in peripheral blood. In addition, after tumor rechallenge, the combination group showed more rejection to the reimplanted tumors (6 out of 10 mice).

Conclusions: The combination of MWA and anti-PD-1 therapy resulted in the inhibition of distant tumor growth and the construction of a systemic anti-tumor immune environment that can reduce recurrence.

1. Background

Microwave ablation (MWA) is a widely adopted local treatment technique for hepatocellular carcinoma (HCC), which is less invasive, implementation-friendly, and can be performed for multiple times [1]. Through heat damage, MWA causes necrosis and shrinkage of the target lesion, thereby reducing the tumor load [2,3]. However, MWA alone is of limited use and has a high recurrence rate due to underlying cirrhosis and micrometastases, especially for tumor lesions larger than 3 cm, with distant recurrence occurring in more than 60% of patients within 3 years [4].

Previous studies have found that MWA has not only local effects but also enhances systemic anti-tumor immunity [5]. Leuchte et al. demonstrated that elevated specific T cells responsive to tumor-associated antigens (TAA) were detected in 30% of HCC patients undergoing MWA treatment, and that this enhanced tumor antigen-specific immune response was correlated with longer progression-free survival [6]. Regrettably, antigen released by MWA alone may not be enough to overcome the tumor’s immune escape [7]. In addition, although rare, in some cases MWA has shown a post-ablation abscopal effect, manifested as a reduction or even disappearance of distant non-target lesions. Notably this effect tends to be seen in some patients who have received prior immunotherapy [8].

Immunocheckpoint inhibitors play an increasingly significant role in the treatment of renal, colorectal and hepatic carcinoma [9–11]. Programmed death receptor \textsuperscript{-} 1 \textsuperscript{(PD-1)} inhibitors are typical of these. By binding to co-inhibitory molecules of immune cells, they work by relieving the inhibition of immune cells in tumor, thereby enhancing the T-cell response against tumors [12,13]. The CheckMate 459 trial showed improved objective response rates with PD-1/PD-L1 Inhibition alone as first-line treatment for HCC compared to sorafenib, however there was no significant difference in overall survival or progression-free survival rates [14]. More attempts at dual checkpoint inhibitor combinations and combinations of biologic therapies and immunotherapy are being explored. The results of one of these, the IMbrave 150 trial
[15], are promising in terms of the superiority of atezolizumab in combination with bevacizumab over sorafenib in terms of objective response rates and survival. However, the safety and efficacy of combination therapy in subgroups of patients with preexisting autoimmune disease, inflammatory bowel disease or nonalcoholic steatohepatitis remains poorly established [16]. Although PD-1/PD-L1 antibodies have demonstrated promising antitumor activity in clinical trials, it is only effective for a limited number of patients [9,17]. In addition to inter-drug combinations, combinations of locoregional therapies and immunotherapy are increasingly being explored in the quest to achieve synergistic anti-tumor effects, and this includes the use of local therapies in combination with PD-1 inhibitors. Shi et al. constructed the murine tumor model and found that radiofrequency ablation (RFA) combined with anti-PD-1 therapy enhanced tumor specific immune response [18]. Zhao et al. showed that the combination of irreversible electroporation and anti-PD-1 blockade promoted selective infiltration of CD8+ T cells into tumors and significantly prolonged survival in mice. [19]. These preclinical evidences provide preliminary proof of the effectiveness of the conjunction of local therapy and immunotherapy.

However, most of the current research mainly focus on locally treated lesions, there is a lack of attention to the untreated lesions. Based on this, this study aims to assess the alteration of untreated tumor and systemic immunological alterations after MWA and PD-1 blockade by constructing a multitumor murine model of hepatocellular carcinoma. By answering this question, it will provide further evidence for the combination of ablation and immunotherapy for patients with HCC, especially for those with multiple lesions or metastatic tumors.

2. Materials and methods

2.1. Cell line and cultures

Mouse hepatoma cell line Hepa1-6 was from Procell Life & Technology Co., Ltd., Wuhan, China. The cells were cultured in Dulbecco’s Modified Eagle Medium (PM150210, Procell, China) with 10% fetal bovine serum (164210-500, Procell, Technology Co., Ltd., Wuhan, China). The cells were cultured in an atmosphere of 37°C and 5% CO2.

2.2. Animal model and treatments

After the cells have been cultured to sufficient numbers, a total of 1 × 10^6 cells were injected into the bilateral symmetrical back of male C57BL/6 mice. Treatment was performed when the right tumor grew in size to approximately 500 mm^3.

Ablation was conducted on the right side of the tumor using a microwave generator (KY-2000A, Kangyou Medical Technology Co., Nanjing, China). The mice were anesthetized with sodium pentobarbital and then an L-shaped incision was made in the right skin along the long diameter of the tumor. After exposing and the tumor, a 17 G ablation needle was inserted along the long diameter of the tumor for ablation. According to a previous study, the ablation was set to be performed at 5 watts power for 3 min. The wounds of the mice were sutured after the procedure. PD-1 antibody (clone: J43, BioXCell) was intraperitoneally injected into mice from Day3 after ablation for 4 times, each time at an interval of 2 days. The dose is 200 μg.

2.3. Study design

All mice were randomly assigned to four groups: untreated group (no treatment), MWA group (MWA was administered in the right tumor), anti-PD-1 group (anti-PD-1 was administered), MWA + anti-PD-1 group (MWA and anti-PD-1 treatment were combined).

For the rechallenge test, a right lateral tumor model was first established, and then the mice were assigned randomly to untreated group, MWA group, anti-PD-1 group, and MWA + anti-PD-1 group for treatment. The tumor rechallenge attempt was then performed on the left side 15 days after MWA, and the time to tumor formation was recorded (tumor formation was defined as the longest diameter of the rechallenged tumor ≥5 mm).

2.4. Tumor evaluation

The maximum diameter L and the shortest diameter S of the left tumor were recorded by electronic measuring instrument every 2 days. The tumor volume was calculated according to the formula (L × S^2)/2. The curative effect of the tumor was evaluated according to the RECIST1.1 standard [20], and tumor volume on the 0th day was compared with the last measured tumor volume.

2.5. Flow cytometry testing

The left tumor was excised and mechanically dissected by surgical scissors, then digested with RPMI containing collagenase IV (1 mg/ml), hyaluronidase (0.1 mg/ml) at 37°C for 30 min. After termination of digestion, cells were filtered through a filter membrane and resuspended with Hank’s solution containing 1% FCS. Antibodies to CD45, CD4, CD3, CD8, FoxP3, Gr1, CD11b and CD45 were obtained from Biolegend. Cells were stained according to the experimental protocol. Analysis was performed on a BD Bioscience Canto II flow cytometer. FlowJo software (FlowJo, LLC) was used to perform the analysis of the data.

2.6. Quantitative real time-PCR

According to the instructions, the total RNA purification kit (Servicebio Technologies) was used to extract total RNA from the left tumor tissue. Using the first strand cDNA synthesis kit (Servicebio Technologies) to synthesize the first strand cDNA. The mRNA levels of TNF- α, IFN- γ, and reference gene β-actin were measured by real-time PCR machine. Using the ΔΔCt method to calculate the Changes in gene expression.
2.7. Histopathologic examination

Untreated left-sided tumors were fixed in 10% formalin, embedded in paraffin and cut into 6-μm-thick sections, followed by staining with hematoxylin and eosin. Immunohistochemical staining was performed with anti-CD4 (dilution 1:200, GB13064-2; Servicebio) and anti-CD8 (dilution 1:200, GB11068; Servicebio) antibodies, and hematoxylin-stained nuclei were blue and positive staining was brown. Five areas were randomly selected to count positive cells under 100× and 200×magnification. The count results were then averaged and statistically analyzed.

2.8. Enzyme-linked immunospot assay

Mouse interferon-γ (IFN-γ) ELISPOT kit (BD Biosciences) was used according to the instructions for use. The IFN-γ antibody was added to ethanol-treated PVDF membrane plates, followed by mouse spleen cells (5 × 10^5 cells/well) along with mitomycin C-treated Hepa1-6 cells (2.5 × 10^5 cells/well). After 48 h of incubation, cells were removed, plates were washed and biotinylated detection antibodies were added. Finally, the plates were then developed by adding AEC (3-amino-9-ethylcarbazole) substrate. Bioreader 3000 LC (BioSys, Germany) was used to analyze plates.

2.9. Enzyme-linked immunosorbent assay

Plasma was separated from the peripheral blood of mice and stored at low temperature for analysis. Plasma concentrations of IL-2 (ELK1150), IL-4 (ELK1153), IL-18 (ELK2269), IL-10 (ELK1143) and IFN-γ (ELK1132) were determined using mouse ELISA kits (ELK Biotechnology).

2.10. Statistical analysis

SPSS software (version: 25; Chicago, USA) was used to perform statistical analyses. Continuous data were expressed as mean ± SD or medians with interquartile ranges (IQRs), and numbers with percentages was used to represent categorical data. Differences between groups were compared by ANOVA and further compared by t-test with Bonferroni correction or Dunnett’s test if significant. Survival differences were assessed by Kaplan–Meier curves and analyzed by the log-rank test for multiple comparisons. The significance level was set at a p value < .05.

3. Results

3.1. Combination of anti-PD-1 and MWA resulted in the inhibition of distant tumor growth

The left lateral tumor growth curve showed that there was no significant difference in tumor volume between the four groups at Day0. On Day 20 after ablation, no significant difference was shown between the MWA group and the untreated group, while the tumor volume in the combined MWA and PD-1 inhibitor group was significantly smaller than that in the MWA, anti-PD-1 and untreated groups. In addition, there was no significant difference between the anti-PD-1 and MWA groups, and between the untreated and anti-PD-1 groups (Figure 1).

In untreated group and MWA group, all tumors continued to increase in size until the end of the observation period (or death) and no animals were classified as having stable disease (SD), while in the MWA + anti-PD-1 group, 2 mice out of 7 showed SD, and one mouse showed SD in anti-PD-1 group (Table 1).

![Figure 1](image_url). Combined MWA and anti-PD-1 therapy significantly lowered tumor growth rates. (A) Growth of untreated tumors during the observation period. (B) Untreated tumor volume in each group at day 20. Data were obtained from two independent experiments with 7 mice in each group. ** Represents p < .01, *** represents p < .001. MWA: microwave ablation.
3.2. Combined anti-PD-1 and MWA therapy improved survival of the tumor-bearing mice

By the end of the observation period, all mice in the untreated and MWA groups were dead. 1 in anti-PD-1 group, and 3 mice in MWA + anti-PD-1 group survived the entire observation period. Median survival time was 25 days (95%CI 17 – 33 days) and 27 days (95%CI 14 – 40 days) in the untreated and MWA groups, respectively, and 37 days (95%CI 32 – 42 days) and 46 days in anti-PD-1 group and combination group respectively. The survival time was longer in combination group than in untreated group (p=.001), MWA group (p=.001) and anti-PD-1 group (p=.034). There was no significant difference in survival time between the untreated, MWA and anti-PD-1 groups (p=.192 for MWA group vs untreated group; p = .010 for anti-PD-1 group vs. untreated group; and p=.414 for MWA group vs anti-PD-1 group) (Figure 2).

Table 1. Distant tumor responses of 4 groups.

| Groups              | CR | PR | SD | PD | Disease control (CR + PR + SD) % |
|---------------------|----|----|----|----|----------------------------------|
| Untreated           | 0  | 0  | 0  | 7  | 0                               |
| MWA                 | 0  | 0  | 0  | 7  | 0                               |
| Anti-PD-1           | 0  | 0  | 1  | 6  | 14                              |
| MWA + anti-PD-1     | 0  | 0  | 2  | 5  | 29                              |

CR: complete response; PR: partial response; SD: stable disease; PD: disease progression.

3.3. Combined anti-PD-1 and MWA therapy enhanced intratumoral TIL infiltration in distant tumor

Immunohistochemical analysis of the left side tumor showed that the number of CD8+ T cells was significantly higher in the MWA + anti-PD-1 combination group than in anti-PD-1 group (p=.004), MWA group (p=.000) and untreated group (p=.000). In addition, the number of CD8+ T cells in anti-PD-1 group was significantly higher than that in the MWA (p=.000) and untreated groups (p=.000), whereas there was no significant difference between the MWA and untreated groups (p=.095) (Figure 3).

In addition, the number of intratumoral CD4+ T cells was significantly higher in MWA + anti-PD-1 group than those in untreated group, anti-PD-1 group and MWA group (all p<.05). Compared with the untreated group, MWA also increased the number of CD4+ T cells infiltrated into the tumor (p=.000), while there was no significant difference between the MWA group and the anti-PD-1 group (p=.254).

We also performed flow cytometry on the excised contralateral tumors and found that the proportion of both CD45+ and CD8+ cells was significantly higher in anti-PD-1 group than in untreated group (Figure 4). In MWA + anti-PD-1 group, the number of CD8+ and CD45+ cells in tumor tissue was increased compared to MWA or anti-PD-1 alone. The percentage of CD4+ cells in combined group was significantly higher than other three groups. PCR analysis demonstrated higher levels of IFN-γ and TNF-α expression in the distant tumors of the MWA + anti-PD-1 group than in MWA or anti-PD-1 treatment alone (Figure 4). Moreover, the proportion of Treg cells showed a tendency to decrease in combination group compared with untreated or MWA or anti-PD-1 group, although statistical significance has not been reached.

3.4. Combined treatment of anti-PD-1 and MWA protected mice against invasion of reimplanted tumors in the tumor rechallenge test

To validate effectiveness of the combination treatment, we conducted a rechallenge test (Figure 5). During the 45-day observation period, MWA + anti-PD-1 group had 6 (60%) mice rejected the tumor, and the anti-PD-1 treatment group had 2 (20%) mice rejected tumors. In addition, 0 mice in the untreated group and MWA group rejected tumors. The median time of tumor formation was not reached in combination group, and the median time in anti-PD-1, MWA and untreated groups were 31, 18, and 14 days respectively. The results showed that the median tumor formation time in combination group was significantly longer than in MWA group (p=.000), anti-PD-1 group (p=.000) and untreated group (p=.000).

3.5. Anti-PD-1 and MWA therapy synergistically enhanced tumor specific T cell immunity

In order to explore whether combined therapy can stimulate tumor antigen specific T cells, we analyzed the ability of T cells in mouse spleen to secrete IFN-γ after in vitro restimulation using ELISPOT assay (Figure 6). The results showed that both MWA or anti-PD-1 therpay could stimulate specific immune responses to tumor antigen, and the splenic T cells in combined treatment group were more responsive to tumor antigen-specific stimulation, and the number of splenic T cells with the ability to secrete IFN-γ was increased approximately 1-fold compared to MWA or anti-PD-1 group. This result confirmed that combined treatment with MWA
and PD-1 blockade enhanced tumor antigen-specific T cell immune responses.

4. Discussion

This study demonstrated that in a multi-subcutaneous tumor hepatoma mouse model, the combination of MWA and anti-PD-1 therapy inhibited the growth of distant tumors, improved the tumor microenvironment, improved survival, and protected mice from tumor recurrence by systemic anti-tumor immune enhancement.

Immune tolerance and escape are the basis of tumorigenesis, progression and metastasis [21], and co-inhibitory molecules on immune cells play an important role in the process.
of tumor immune escape [22]. Immune checkpoints are inhibitory signaling molecules expressed on the surface of immune cell membranes, which play an important role in autoimmunity protection but can lead to immune escape of tumor cells in the tumor microenvironment [23]. PD-1 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) are essential immune checkpoints on the surface of T cells that mediate negative regulatory signals and can effectively inhibit T cell function and proliferation, and reduce the secretion of cytokines such as IL-2 [24]. PD-1 is mainly expressed on the membranes of activated CD8⁺ T, CD4⁺ T, and NKT cells, and is considered an important marker of T-cell depletion [25,26]. PD-L1 is mainly expressed on the surface of tumor cells and antigen-presenting cells (APCs), and inhibits the downstream transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) genes by binding...
to the activated T-cell ligand PD-1, which ultimately negatively regulates the activation and proliferation of T cells and leads to immune escape of tumor cells [27]. Blocking the PD-1/PD-L1 signaling pathway can restore the activation and proliferation ability of T cells and restore their anti-cancer activity [28]. Targeted therapies that block the PD-1/PD-L1 signaling pathway are currently achieving promising results in a variety of tumors [9,17,29,30].

Microwave ablation activates the patient’s immune system while inactivating the tumor in situ, and induces the generation of specific immune response against the tumor [31]. When the local temperature of the tumor rises above 60°C, it can lead to protein denaturation and coagulative necrosis in the tumor tissue, resulting in the release of a large amount of tumor-specific antigens and activating the adaptive immune response [32–34]. The release of these so-called ‘danger signals’ can activate the antigen-presenting cells in the body to capture the characteristic tumor antigens released by tumor tissue necrosis. The activated cytotoxic T cells are the key effector cells of the whole tumor immunogenicity [35,36].

The abscopal effect was first used to describe the reduction or disappearance of lesions distant from the target lesion observed after radiation therapy [37]. This effect is also seen in some patients with locally treated tumors, where distant untreated lesions appear to slow in growth or shrink or disappear after the target lesion has been ablated. This is thought to be a manifestation of systemic immune modulation induced by local treatment. The microwave ablation not only activated the local specific immune response, but also induced the generation of systemic adaptive immune response [38]. However, a review of preclinical studies showed that abscopal effect of RFA alone were seen in 2 of the 9 studies, while the abscopal effect of MWA alone were not seen in either of the 2 studies [33]. Also, no significant abscopal effect was observed in the MWA alone treatment group in our results. This differentiation seems to suggest a relative disadvantage of MWA in inducing systemic anti-tumor immunomodulatory effects compared to RFA. However, regardless of the ablation modality, once combined with immunotherapy, significant abscopal effect and tumor rejection immunity were demonstrated. Notably, a meta-analysis of randomized controlled trials showed that despite the similarity in efficacy and safety between MWA and RFA, MWA group had lower rates of distant recurrence and long-term recurrence [39]. It is hypothesized that this is related to the ability of MWA to cause more extensive necrosis, particularly of microsatellite lesions, the presence of which may lead to de novo distant recurrence. This suggests that although there may be a disadvantage of MWA in inhibiting the growth of preexisting distant tumors, it has an additional advantage in reducing the number of distant de novo metastases caused by residual primary lesions. More direct comparative studies are needed to further investigate the differences between the two ablation modalities in terms of the induced changes of distant tumor.

At present, the mechanism of synergistic anti-tumor effect of microwave ablation and immunotherapy is not clear. MWA results in the exposure of tumor antigen and damage-related molecular model (DAMPs) to the host immune system, followed by activation of dendritic cells (DCs) [40]. Phagocytosis of DAMPs by DCs can activate the NF-κB pathway, thus promoting the expression of costimulatory molecule CD80. Activated DCs activates naïve T cells through ‘triple signals’ to transform them into effector T cells and exert tumor killing effect. When immune checkpoint inhibitors such as PD-1 inhibitors are combined, the ‘brake’ of effector T cells killing tumor is released, thus enhancing the host’s immunity to the tumor, which can not only make the ablation target focus disappear, but also cause the distant tumor focus or metastatic focus to disappear.

Clinical studies have shown that increased intratumoral cytotoxic T lymphocytes (CTL) infiltration in HCC patients is associated with improved survival [41,42]. In our study, the detection of distant tumors showed significantly higher CD8+ T cells in the MWA plus anti-PD-1 group than in the other three groups, which may explain the suppressed tumor growth. Moreover, an increase in the number of T cells capable of specifically secreting IFN-γ was observed in the spleen of mice in combination group, suggesting that the combination treatment enhanced systemic tumor-specific immunity, which may explain the tumor rejection of combination group shown in the tumor rechallenge test. Furthermore, Treg cells, one of the representatives of immunosuppressive cells, did not show a significant decrease in the distant tumors of the combination group in our experiments. In contrast, in a study of RFA combined with PD-1 inhibitor treating mice with colon cancer [18], the number of Treg cells in the combined group was shown to be reduced compared with the control groups. Such different results may arise from natural differences in the type of cancer or else, may indicate differences in the pathways by which different ablation modalities affect immune cells. Therefore more studies are needed here to explore this in depth.

In the current study, the immune infiltration of ablation target tumor was not evaluated. In another model of radiotherapy combined with immunotherapy (immune checkpoint inhibition) in mice with bilateral melanoma [43], it showed that the change of the number of tumor infiltrating T cells in both of them was the same trend. In other words, the number of T cells infiltrating in both primary and abscopal tumors after combined treatment was higher than that with no-treatment or radiotherapy alone group. In addition to the changes of T cells, the infiltration of CD45+ cells, dendritic cells and monocytes/macrophases in primary tumors and abscopal tumors increased in the combined treatment group compared with no-treatment group. This suggests some similarities in the immune infiltration of primary and abscopal tumors following combination therapy. CD8+ T cells were likely to mediate the abscopal effect. These effector cells were induced by combination therapy and newly infiltrated into distant tumors rather than existing before.

Our study has some limitations that need to be noted. First, the experimental model was not an in situ hepatocellular carcinoma model, this means that more tests are needed...
to generalize the findings to the real immune environment of patients with liver cancer. Second, the target lesion ablated was not evaluated. Although complete ablation was performed to the extent possible, post-ablation tumor residuals are inevitable, and evaluation of residual lesions lacks some technical feasibility. More importantly, this is not the main objective of this study. Third, for the combination of MWA and anti-PD-1 treatment in chronological order, we chose one of them, i.e., postoperative combination with anti-PD-1 treatment. In fact, the difference in the effect of different combination modalities on the outcome may not be negligible. This is to be further confirmed in future preclinical and clinical studies. Finally, antitumor immunity may vary considerably depending on the tumor host system. Therefore, the immune effects of MWA and anti-PD-1 treatment should be verified in other animal models.

5. Conclusions

In conclusion, this study was the first to describe the abscopal effect of MWA in conjunction with PD-1 blockade for treating HCC in a multi-tumor animal model. We demonstrated that MWA in hepatocellular carcinoma can cause T cell infiltration in distant tumors, and this infiltration was enhanced by combined with PD-1 monoclonal antibody treatment, resulting in the delay of tumor growth. In addition, the systemic tumor immune response induced by combination therapy was proved to be specific to the tumor antigen. What's more, in the rechallenge test, the combined treatment showed stronger rejection against reimplanted tumor, indicating that the induced immune enhancement was persistent. Finally, the findings of this study can provide necessary evidence for the combination of local ablation treatment and immunotherapy for patients with HCC, especially those with multiple lesions or metastatic tumors.

Ethical approval

This study was conducted in accordance with international, national and institutional regulations regarding animal experimentation and biodiversity rights. The research protocol was approved by the Animal Ethics Committee of Huazhong University of Science and Technology (IACUC number 2528).

Author contributions

HSJ: Conceptualization, Software, Writing, Original draft preparation. LTQ: Investigation, Methodology, Visualization. CY: Investigation, Data curation, Visualization. LJC: Visualization, Data curation. WYL: Methodology, Formal analysis. YCT: Methodology, Data Curation. WCY: Visualization. JSG: Methodology. BYW: Software, Validation. YW: Software, Validation. XB: Conceptualization, Supervision, Reviewing and Editing, Project administration.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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