Virus Genes (2017) 53:477–482
DOI 10.1007/s11262-017-1434-2

Tanapoxvirus lacking the 15L gene inhibits melanoma cell growth in vitro by inducing interferon-λ1 release

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Received: 31 October 2016 / Accepted: 6 February 2017 / Published online: 10 February 2017

Abstract Oncolytic viruses (OVs) have emerged as a promising approach for melanoma treatment by causing tumor lysis and inducing immuno-modulatory activities. Tanapoxvirus (TPV), which causes a mild self-limiting disease in humans and contains a large DNA genome, appears as a promising OV candidate. TPV recombinants were generated with the thymidine kinase/66R gene deletion (TPVΔ66R), the 15L gene deletion (TPVΔ15L), or with both the 15L and 66R gene ablation (TPVΔ15LΔ66R). Our previous studies have shown that treatment of TPVΔ15L resulted in significant tumor regression in xenotransplanted human melanoma in nude mice. Here, we demonstrate that an anti-viral activity identified as interferon-λ1 (IFN-λ1) was secreted in a remarkably higher quantity from human lung fibroblast WI-38 and melanoma SK-MEL-3 cells infected with TPVΔ15L. Furthermore, we show that IFN-λ1 exhibits a more pronounced anti-proliferative effect in melanoma cells than IFN-α and IFN-β in vitro. Additional experiments strongly suggest that TPVΔ15L kills melanoma cells partially through inducing IFN-λ1. Taken together, our results demonstrate the immuno-modulatory activities associated with TPVΔ15L and suggest further exploration of TPVΔ15L as a melanoma virotherapy.

Keywords Oncolytic virotherapy · Tanapox virus · Melanoma · Interferon · Neuregulin · Viral immunomodulation

Edited by Zhen F. Fu.

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Oncolytic viruses (OVs) have emerged as a promising approach for cancer therapy by causing tumor lysis and inducing the anti-tumor immune responses [1, 2]. Talimogene laherparepvec (T-VEC) and JX-594, the modified herpes simplex virus (HSV) and vaccinia virus (VV) both expressing granulocyte–monocyte colony-stimulating factor (GM-CSF), have been approved, respectively, for melanoma and head and neck cancer therapies [3]. Tanapoxvirus (TPV), classified in the family *Poxviridae*, is a geographically limited virus containing a double-stranded (ds) DNA genome (approximately 144 kbp) and causes a mild self-limiting disease in humans (reviewed in [4, 5]). Previous studies have characterized the immuno-modulatory mechanisms exerted by TPV. For example, TPV-2L gene product functions as a high-affinity inhibitor of human tumor necrosis factor (TNF) and TPV-15L gene product functionally mimics neuregulin (NRG) which is a ligand of ErbB3/ErbB4 receptors [6, 7]. We have previously generated TPV recombinants by deleting thymidine kinase (TK)/66R gene (TPVΔ66R), 15L gene (TPVΔ15L), or both 15L and 66R genes (TPVΔ15LΔ66R), and replacing them with different reporter genes [7–9]. While TPVΔ66R and TPVΔ15L were engineered to, respectively, express the mCherry and green fluorescence protein (GFP), TPVΔ15LΔ66R replicated with the expression of both mCherry and GFP. In addition, we showed that TPV-15L, a functional mimetic of NRG [7], significantly promotes melanoma proliferation. Recombinant TPVΔ15L caused significant regression of melanoma tumors in the nude mice as compared to wtTPV [9].

Interferons (IFNs) are a family of pleiotropic cytokines that have been shown to possess anti-viral, anti-proliferative, and immuno-regulatory capabilities [10]. IFNs exert anti-tumor activity via mechanisms including a direct anti-proliferative effect, the induction of growth termination,

and the modulation of tumor antigens [11–15]. Additionally, IFNs are able to modulate the function of the host immune cells including lymphocytes and monocytes [16]. Compared with certain types of IFNs such as IFN-α which has been widely studied and approved by FDA for treating melanoma [17–20], IFN-λs, including IFN-λ1, -λ2, and -λ3, are recently discovered cytokines belonging to type III IFN family [21–23]. It has been shown that type III IFNs exhibit overlapping anti-viral activity as IFN-α and that IFN-λ's anti-viral effect is slower but longer than that of IFN-α [22, 24–26]. Moreover, studies have demonstrated that IFN-λ1 and IFN-α exhibit similar anti-tumor effects by activating a similar set of genes and pathways such as ERK-1/2 and JAK-STAT pathways in regressing neuroendocrine tumor and hepatoma [27–29]. In this study, we show that cells infected with TPVΔ15L secrete IFN-λ1 more abundantly than those infected with wtTPV or other recombinant TPVs. Also, we demonstrate that TPVΔ15L kills melanoma cells partially via inducing IFN-λ1 release from the cells.

As a defense mechanism, host cells secrete IFNs for protecting against viral infection. To evaluate the infectivity of TPVs and the defense mechanism of cells against these viruses, different IFNs were tested in the supernatant of human lung fibroblast WI-38 and SK-MEL-3 cells infected with wtTPV and recombinant TPVs. Type I IFNs secretion has been described in the cells infected with viruses such as rhinovirus and retrovirus [30, 31], however, we have been unable to detect measurable type I IFNs secretion from wtTPV- and recombinant TPVs-infected cells (data not shown). By contrast, secretion of IFN-λ1 was detected and remarkably higher amounts of IFN-λ1 were observed from WI-38 and SK-MEL-3 cells infected with TPVΔ15L, compared to that from cells infected with wtTPV and other recombinant TPVs (Fig. 1a).

As functionally similar to the type I IFNs, IFN-λs are also induced by virus infection and they activate the same intracellular signaling pathways as type I IFNs [32]. Therefore, we sought to assess the anti-viral activity of IFN-λ1 from the cells infected with TPVΔ15L. WI-38 cell monolayers were infected with mock, wtTPV, TPVΔ15L, or TPVΔ15LΔ66R at multiplicity of infection (MOI) of 5. The infected cell supernatant was collected at 72 h post-infection (hpi), and the supernatant samples were named, respectively, as S(m), S(wt), S(Δ15L), and S(Δ15LΔ66R). Owl monkey kidney (OMK) and WI-38 cells were infected with TPVΔ66R at 5 MOI, with the addition of supernatant samples including S(m), S(wt), S(Δ15L), and S(Δ15LΔ66R) separately. At 48 and 96 hpi, the virus was harvested and titrated, and the replication of TPVΔ66R was measured. All viruses used were genetically engineered to express different reporter proteins. While wtTPV expresses no reporter protein, TPVΔ66R expresses mCherry protein. TPVΔ15L expresses GFP and TPVΔ15LΔ66R replicates with the expression of both GFP and mCherry. Administering the TPVΔ66R infection, respectively, with addition of the supernatant samples from mock-, wtTPV-, TPVΔ15L-, or TPVΔ15LΔ66R-infected WI-38 cells [S(m), S(wt), S(Δ15L) or S(Δ15LΔ66R)], critically distinguishes the TPVΔ66R plaques from the cytopathic effect (CPE) of other viruses under fluorescence microscopy. As shown in Fig. 1b, the replication of TPVΔ66R in OMK and WI-38 cells with addition of supernatant from TPVΔ15L-infected WI-38 cells [S(Δ15L)] was significantly decreased than those with addition of mock-, wtTPV- or TPVΔ15 LΔ66R-infected WI-38 cells' supernatant samples [S(m), S(wt), or S(Δ15LΔ66R)] ($P < 0.01$, Tukey's HSD). Data were analyzed by 2-way analysis of variance and post hoc comparisons of treatments using Tukey's HSD. All the P values accordingly have also been reported here. $P < 0.05$ was considered statistically significant. Compared to the titers of TPVΔ66R in the presence of S(wt), the titers of TPVΔ66R incubated with S(Δ15L) were approximately twofold less in OMK cells. These results supported the detection of increased IFN-λ1 secretion in the supernatant of TPVΔ15L-infected WI-38 cells. However, the decrease of virus titers was not proportional to the differences in the amounts of IFN-λ1 as shown in Fig. 1a, which is potentially due to activities and mechanisms hindering the anti-viral activity of the supernatant samples, such as the fast replication of TPVΔ66R at 5 MOI.

The anti-proliferative activity of IFN-α and IFN-β in human melanoma has been well established [27, 33]. Studies have shown that type I IFNs are capable of reducing the proliferation of fresh and long-term cultured melanoma cells in vitro [33–35]. IFN-β has been shown to exert more potent anti-proliferative capability than IFN-α in some melanoma cells such as StML-11, StML-12, and SK-MEL-28 cells in vitro [33]. However, the anti-tumor effect of IFN-λ in melanoma cells has not been well studied. Hence, to determine the anti-proliferative activity of IFN-λ1 compared to IFN-α and IFN-β, SK-MEL-3 cells were incubated with each IFN for 4 days in different concentration levels, and cell proliferation was measured compared to the untreated controls. As shown in Fig. 1c, all three IFNs exhibited dose-dependent anti-proliferative activity. However, the melanoma growth inhibition activity of these three IFNs differed. While melanoma cells treated with 10^3 U/ml IFN-α and IFN-β increased by approximately 89 and 52%, respectively, those treated with 10^3 U/ml IFN-λ1 achieved only 23% growth after 4 days. The data suggest that IFN-λ1 potentially possesses more potent anti-proliferative activity than IFN-α and IFN-β in melanoma cells in vitro.

In light of the secretion of IFN-λ from SK-MEL-3 cells and the remarkable anti-proliferative effect of IFN-λ, we sought to determine if TPVΔ15L kills the melanoma cells

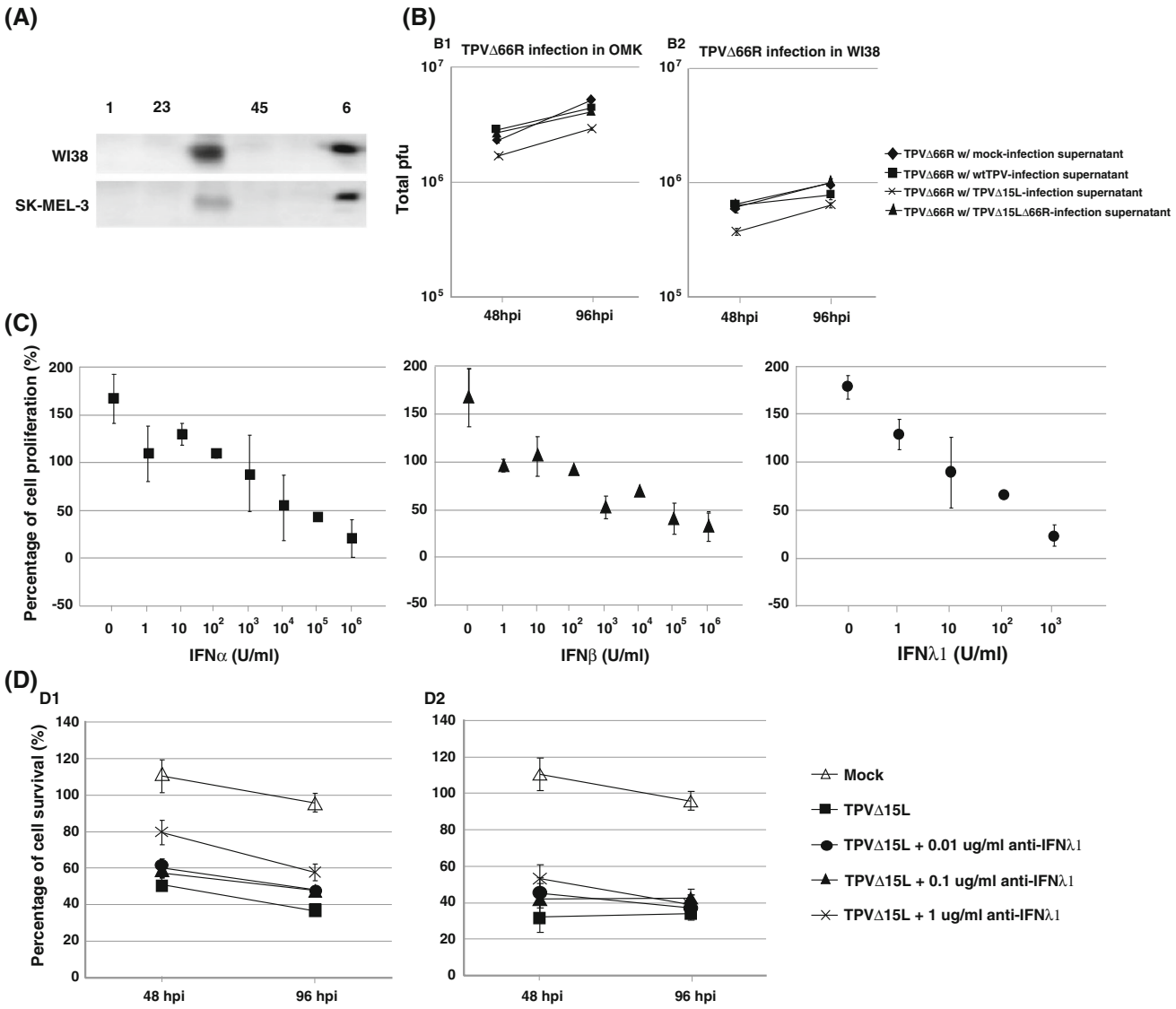

Fig. 1 **a** WI-38 and SK-MEL-3 cells (ATCC product numbers CCL-75, HTB-69) at 70% confluence in 35-mm dishes were infected at 5 MOI with wtTPV, TPVΔ66R, TPVΔ15L, TPVΔ15LΔ66R, and mock separately. The volumes of virus inoculums were adjusted to the same amount (300 µl). After 1-hour virus adsorption at room temperature, the virus inoculums were removed and replaced with the maintenance medium in equal amount (2 ml) to each infection dish. At 72 hpi, the supernatant was collected and 15 µl of each supernatant sample was subjected to 12% SDS-PAGE and western blot analysis using a goat polyclonal antibody against IFN-λ1 and a secondary anti-goat HRP-conjugated antibody. ECL was used for visualization of bands. Lanes represent supernatant samples of cells infected by (1) wtTPV, (2) TPVΔ66R, (3) TPVΔ15L, (4) TPVΔ15LΔ66R, (5) mock (uninfected cells), and (6) positive control, hIFN-λ1, from human cells with a predicted apparent molecular mass of 20.2 kDa. Another independently selected TPVΔ15L mutant also induced IFN-λ1 (results not shown). **b** WI-38 cells were infected with wtTPV, TPVΔ15L, and TPVΔ15LΔ66R at 5 MOI in 12-well plates, and at 72 hpi the supernatant samples were collected and stored at −80 °C. The supernatant of uninfected cells served as control. OMK and WI-38 cells in 48-well plates were infected with TPVΔ66R at 5 MOI, with the addition separately of 50 µl supernatant samples after TPVΔ66R absorption. The virus was harvested at 48 and 96 hpi and titrated. TPVΔ66R plaques were counted. The results represent mean ± SD of triplicate samples. (B1) TPVΔ66R infection in OMK, (B2) TPVΔ66R infection in WI-38. **c** In each well of 96-well plate, 2×10^4 SK-MEL-3 cells were plated in McCoy's 5A (Sigma-Aldrich) with 20% fetal bovine serum (Atlanta Biologicals) and incubated with IFN-α, IFN-β, or IFN-λ1 (Sino biological) in serial dilutions from 1 to 10^6 U/ml, compared with those incubated without IFN (0 U/ml). Total cell numbers were counted after 4 days. Percentage of cell proliferation was calculated as $[(\text{total cell number}—2 \times 10^4)/2 \times 10^4] \times 100$. Shown here represents the average of three independent experiments. **d** In each well of 48-well plates, 9×10^4 SK-MEL-3 cells were plated and cultured in serum-free McCoy's 5A medium. Cells were infected with TPVΔ15L at (D1) 0.1 MOI or (D2) 5 MOI and treated with different concentrations of anti-IFN-λ1 (0.01, 0.1, or 1 µg/ml). At 48 and 96 hpi, total alive cell numbers were counted using trypan blue and the percentage of the dead cells was calculated as [(number of total alive cells)/9×10^4] × 100%. Results represent the average of three experiments.

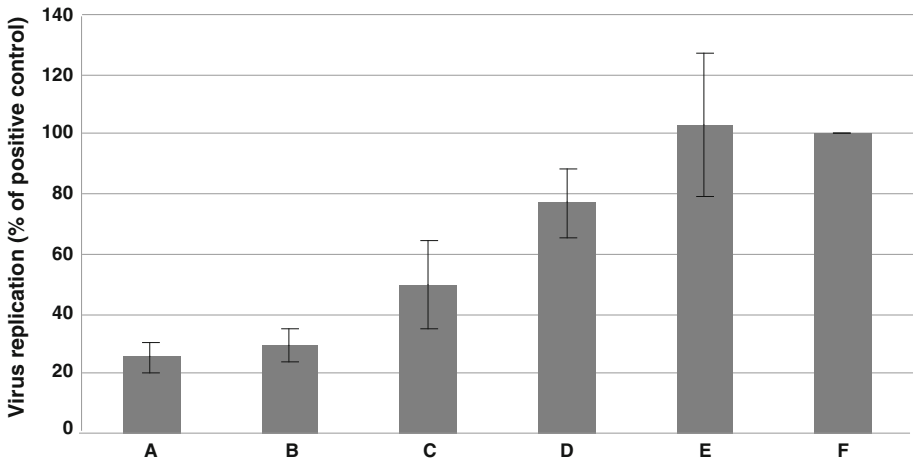

Fig. 2 Neutralization of anti-viral activity exerted by the supernatant of TPVΔ15L-infected cells WI-38 cell monolayers in T75 flasks were infected with TPVΔ15L at 5 MOI. After 1-hour absorption, serum-free EMEM was added to the infected cells which were then incubated in the 37 °C incubator for 4 days. Following incubation, the virus was harvested and ultra-centrifuged at 10,000 rpm for 1 h. The supernatant was collected and filtered through 0.22-μm filter for virus elimination. WI-38 cells were plated in 24-well plates. Adjusted supernatant samples were prepared, respectively: (a) a mixture of 100 μl TPVΔ15L-infected WI-38 cell supernatant sample and 150 μl serum-free medium, (b) a mixture of 100 μl supernatant sample with 150 μl serum-free medium containing 0.0625 μg anti-IFN λ1 (0.25 μg/ml), (c) a mixture of 100 μl supernatant sample with 150 μl serum-free medium containing 0.125 μg anti-IFN λ1 (0.5 μg/ml), (d) a mixture of 100 μl supernatant sample with 150 μl serum-free medium containing 0.25 μg anti-IFN λ1 (1 μg/ml), (e) a mixture of 100 μl supernatant sample with 150 μl serum-free medium containing 0.5 μg anti-IFN λ1 (2 μg/ml), and (f) 250 μl serum-free medium. All the adjusted supernatant samples were incubated for 1 h at 37 °C to neutralize the IFN λ1 in the supernatant samples. WI-38 cells were incubated with each supernatant samples for 2 h at 37 °C before being infected with 50 pfu TPVΔ66R. After 1-hour virus absorption, the virus inoculum was replaced with 500 μl overlay medium (1x EMEM + 2% FBS + 0.5% methylcellulose) in each well. After incubating at 37 °C for 7 days, the virus replication was determined by the number of TPVΔ66R plaques counted under fluorescence microscope. TPVΔ66R replication in WI38 cells pre-treated with adjusted supernatant sample (f) served as the positive control, while that in WI38 cells pre-treated with adjusted supernatant sample (a) as negative control. *Bars* represent the % of positive control (*F*), respectively, as follows: (*A*) TPVΔ66R replication in WI-38 cells pre-treated with adjusted supernatant sample a, (*B*) TPVΔ66R replication in WI-38 cells pre-treated with adjusted supernatant sample b, (*C*) TPVΔ66R replication in WI-38 cells pre-treated with adjusted supernatant sample c, (*D*) TPVΔ66R replication in WI-38 cells pre-treated with adjusted supernatant sample d, (*E*) TPVΔ66R replication in WI-38 cells pre-treated with adjusted supernatant sample e. Shown here represents the average of three independent experiments.

partially through IFN-λ-mediated growth inhibition. SK-MEL-3 cells were infected with TPVΔ15L at 0.1 or 5 MOI, with the addition of anti-IFN-λ1 at different concentrations. Cell survival was determined at 48 and 96 hpi. As shown in Fig. 1d, when melanoma cells were infected at 0.1 MOI, cell survival rate was significantly increased as early as at 48 hpi with addition of anti-IFN-λ1 at 1 μg/ml ($P < 0.01$, Tukey's HSD). At 96 hpi, the number of viable cells was significantly higher with addition of anti-IFN-λ1 in concentration of 0.01, 0.1, or 1 μg/ml ($P < 0.05$). When melanoma cells were infected at 5 MOI, only addition of anti-IFN-λ1 at 1 μg/ml significantly increased the cell survival at 48 hpi ($P < 0.05$). The data clearly show that the addition of anti-IFN-λ1 exhibits a protective effect in the survival and viability of TPVΔ15L-infected cells. Moreover, we show that addition of anti-IFN-λ1 in the TPVΔ15L-infected WI-38 cell supernatant blocked the anti-viral activity, which further confirmed the involvement of IFN-λ1 (Figs. 1b, 2). These results indicate that TPVΔ15L kills melanoma cells partially through inducing IFN-λ1 secretion.

In summary, we show that TPVΔ15L infection induces IFN-λ1 release and TPVΔ15L kills melanoma cells partially through IFN-λ1-mediated cell apoptosis. A variety of immune cells such as natural killer (NK) cells have previously been shown to be responsible for the IFN-λ-mediated anti-tumor effect [36–39]. Based on the significant melanoma reduction of TPVΔ15L that we showed in the previous work [9], further studies are needed to assess the potential activation of the immune cells by IFN-λ1, and to find out the exact mechanisms involving IFN-λ1 for melanoma regression caused by TPVΔ15L in vivo.

Moreover, no detectable IFN-λ1 was shown in the cells infected with TPVΔ15LΔ66R (Fig. 1a). Our results have shown that NRG enhances the melanoma cell proliferation in vitro, and that NRG significantly increases the cell viability of the SK-MEL-3 cells treated with IFN-λ1 (data not shown). However, more studies are needed to further determine if the direct antagonizing relations exist between NRG and IFN. It has been shown that NRG induces cell proliferation through ErbB2/ErbB3 receptor hetero-dimerization and JAK-STAT pathway activation [40]. In

addition, IFN-stimulated growth inhibition is mediated by the activation of JAK-STAT pathway [41]. It has also been demonstrated that TK is required for the cell response to IFN and the induction of anti-viral responses [42]. It would be interesting to speculate that (1) competition exists between NRG and IFN-λ1 and deletion of TPV-15L mimicking NRG induces elevated production of IFN-λ1, and that (2) deletion of 66R/TK gene possibly suppresses the induction of IFN. This could possibly answer why TPVΔ15L infection induced elevated IFN-λ1 expression but TPVΔ15LΔ66R was not able to. However, the exact mechanism requires further exploration.

Acknowledgements We are grateful to Dr. Bruce Bejcek for suggestions and editorial comments.

Author contributions Conception or design of the work—T. Z. and K. E. Data collection—T. Z. Data analysis and interpretation—T. Z. and K. E. Drafting the article—T. Z. and K. E. Critical revision of the article—T. Z. and K. E. Final approval of the version to be published—T. Z. and K. E. Both authors have reviewed the final version of the manuscript and approved for its publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Prior to initiation, all study protocols for treatment and manipulations of mice were approved by the Institutional Animal Care and Use Committee of Western Michigan University (IACUC protocol number 13-07-01).

Informed consent Informed consent was obtained from all individual participants included in the study.

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