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RAPID COMMUNICATION

A lncRNA MEG3 variant enhances telomerase activity by increasing DNA damage repair ability in human liver cancer stem cells

The maternally expressed gene 3 (*MEG3*) acts as an anti-tumor component in different cancer cells. However, MEG3 variant has been shown to confer cancer susceptibility[1] and certain polymorphisms within MEG3 are implicated in cancer risk (rs7158663, rs4081134, and rs11160608).[2] Moreover, *MEG3*-TTCC and *MEG3*-CCCA promote the differentiation of SCs by activating the *JAK2/STAT3* signaling pathway in different degrees.[3] In particular, the alleles of *MEG3* (AA, GA + AA) and MEG3 (rs7158663) were associated with an increased risk for human liver cancer.[4] Moreover, *Sirt2* is a specific NAD-dependent histone deacetylase for H4–K16 and has a strong preference for histone H4K16Ac in their deacetylation activity.[5] Nevertheless, the functions and mechanism of MEG3 variant on hepatocarcinogenesis have not yet been well-demonstrated.

To investigate the ability of *MEG3* variant to promote the malignant growth of human liver cancer stem cells (hLCSCs), hLCSCs were isolated from liver cancer Huh 7 cells using CD133/CD44/CD24/EpCAM beads (Fig. S1A). In hLCSCs, CD133, CD44, CD24, and EpCAM were positively expressed (Fig. S1B). The MEG3 variant (1169–1174: CTTCC-TCCTT) was cloned into the pLVX-Tet-on-Tight-EF1a-ZsGreen plasmid and the tetracycline (DOX)-inducing lentivirus rLVX-Tet-on-MEG3 variant was prepared rLV-tet on- MEG3 variant. Next, hLCSCs were infected with rLV-tet on-MEG3 variant. In the rLVX-Tet-on-MEG3 variant-hLCSCs treated with DOX (0 μg/mL, 0.5 μg/mL, 1 μg/mL, 1.5 μg/mL, 2 μg/mL), MEG3 variant was significantly increased with the increase in DOX concentration (Fig. 1A; Fig. S2A). Cell proliferation ability (24 h: $P = 0.0396$, 0.0015, 0.014, 0.0117; 48 h: $P = 0.0013$, 0.0056, 0.0086, 0.0377) (Fig. S2B), colony forming ability (38.3% ± 2.86% *vs.* 49.4% ± 5.45%, $P = 0.01$; 49.4% ± 5.45% *vs.* 61.75% ± 2.93%, $P = 0.0068$; 61.75% ± 2.93% *vs.* 72.18% ± 2.8%, $P = 0.0285$; 72.18% ± 2.8% *vs.* 88.19% ± 6.78%, $P = 0.0308$) (Fig. S2C), and tumor formation ability (0.13 ± 0.024 g *vs.* 0.24 ± 0.038 g, $P = 0.0000005$; 0.24 ± 0.038 g *vs.* 0.55 ± 0.076 g, $P = 0.0000017$; 0.55 ± 0.076 g *vs.* 0.669 ± 0.07 g, $P = 0.0002$; 0.669 ± 0.07 g *vs.* 0.906 ± 0.04 g, $P = 0.0000005$, and 10.8 ± 1.93 days *vs* 8.5 ± 2.55 days, $P = 0.0245$; 8.5 ± 2.55 days *vs.* 7 ± 0.81 days, $P = 0.043$; 7 ± 0.81 days *vs.* 6.1 ± 0.875 days, $P = 0.0338$; 6.1 ± 0.875 days *vs.* 5.3 ± 1.06 days, $P = 0.026$) (Fig. S2D, E) significantly increased with the increase in DOX concentration. Moreover, *Sirt2* was decreased with the increase of DOX concentration (Fig. 1B). Therefore, acetylation modification at the 16th lysine of HistoneH4 (H4K16Ac) was significantly increased with increasing DOX concentration (Fig. 1C). Although H4K16Ac was significantly increased in DOX (2 μg/mL) treatment group compared to DOX (0 μg/mL) treatment group, it was not significantly changed in DOX (2 μg/mL) + rLV-Sirt2 group compared to DOX (0 μg/mL) group (Fig. 1D; Fig. S3A, B). Thus, the binding of H4K16Ac and RNApolII to SETD2 promoter was significantly increased with the increase of DOX concentration (Fig. 1E; Fig. S4A, B). Moreover, the SETD2 expression was increased with the increase of DOX concentration (Fig. 1F; Fig. S4C). Although *SETD2* was significantly increased in DOX (2 μg/mL) treatment group compared to DOX (0 μg/mL) treatment group, it was not significantly changed in DOX (2 μg/mL) + rLV-Sirt2 group compared to DOX (0 μg/mL) group (Fig. 1G; Fig. S4D, E). Therefore, MEG3 variant enhances the expression of SETD2 via the Sirt2-H4K16Ac pathway.

The binding of SETD2 to HistoneH3 was significantly increased with the increase of DOX concentration (Fig. 1H). Therefore, H3K36me3 was significantly increased with the

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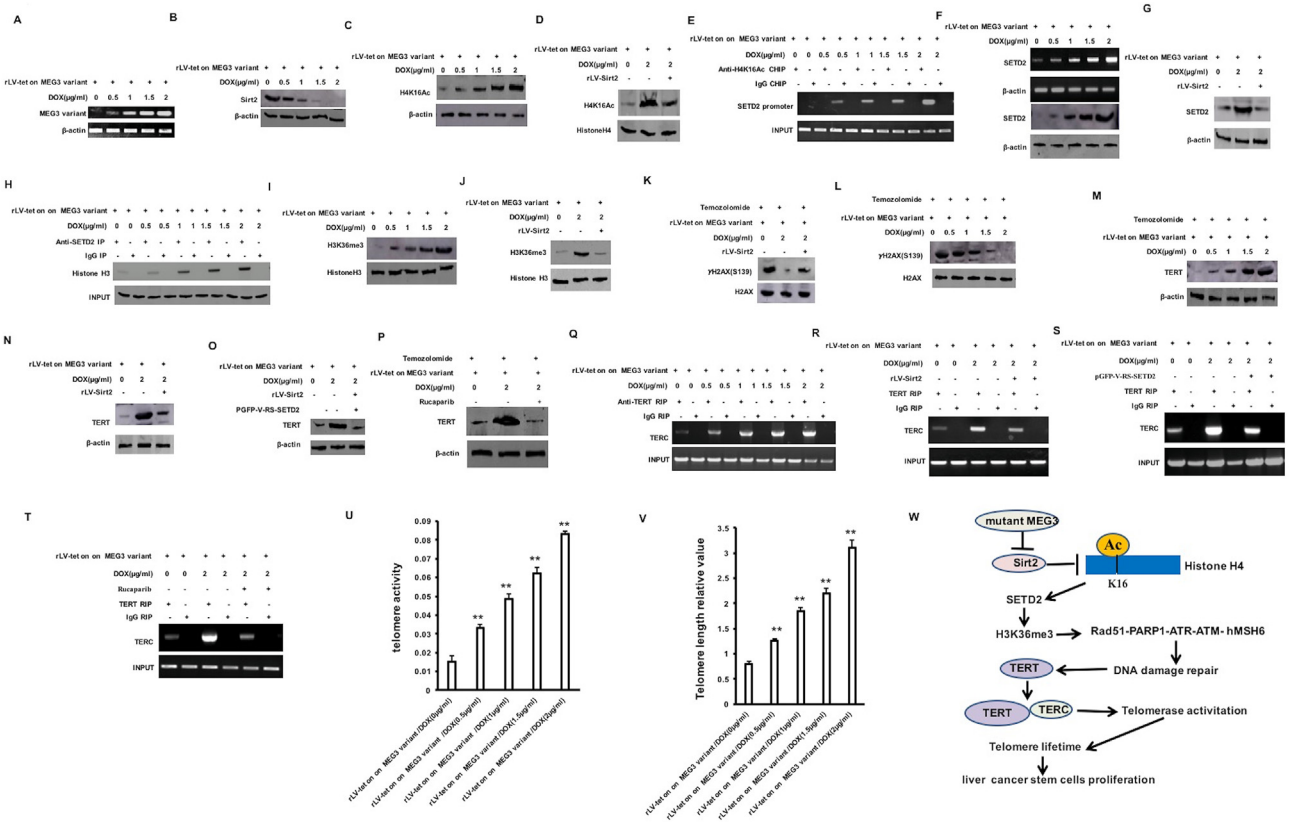

Figure 1 MEG3 variant enhances the activity of telomerase by increasing DNA damage repair ability in human liver cancer stem cells. **(A)** RT-PCR was used to detect MEG3 variant. β-Actin serves as an internal reference. **(B)** Immunoblot analysis with anti-Sirt2. β-Actin serves as an internal reference. **(C)** Immunoblot analysis with anti-H4K16Ac. HistoneH4 serves as an internal reference. **(D)** Immunoblot analysis with anti-H4K16Ac. HistoneH4 serves as an internal reference. **(E)** Chromatin immunoprecipitation analysis with anti-H4K16Ac. **(F)** RT-PCR analysis for SETD2 transcriptional ability and Western blotting analysis with anti-SETD2. Western blotting analysis with anti-SETD2 and anti-Sirt1. β-Actin serves as an internal reference gene. **(G)** Western blotting analysis with anti-SETD2. **(H)** Co-immunoprecipitation analysis with anti-SETD2 and Western blot analysis with anti-HistoneH3. **(I)** Western blot analysis with anti-H3K36me3. HistoneH3 serves as an internal reference. **(J)** Western blot analysis with anti-H3K36me3. HistoneH3 serves as an internal reference. **(K)** Temozolomide (DNA damage inducer) was used to treat these cells and the γH2AX (S139) (a marker of DNA damage) was detected by Western blotting with anti-γH2AX (S139). H2AX serves as an internal reference. **(L)** Temozolomide (DNA damage inducer) was used to treat these cells and the γH2AX (S139) (a marker of DNA damage) was detected by Western blotting with anti-γH2AX (S139). H2AX serves as an internal reference. **(M)** Western blotting with anti-TERT in hLCSCs infected with rLV-tet on-MEG3 variant and treated with DOX plus Temozolomide (DNA damage inducer). β-Actin serves as an internal reference. **(N)** Western blotting with anti-TERT. **(O)** Western blotting with anti-TERT. **(P)** Western blotting with anti-TERT. **(Q)** RNA immunoprecipitation analysis with anti-TER and RT-PCR for TERC. **(R)** RNA immunoprecipitation analysis with anti-TERT and RT-PCR for TERC. **(S)** RNA immunoprecipitation analysis with anti-TERT and RT-PCR for TERC. **(T)** RNA immunoprecipitation analysis with anti-TERT and RT-PCR for TERC. **(U)** Telomerase activity was examined by TRAP. **(V)** Telomere length was measured using the absolute human telomere length quantification qPCR assay kit. The values of each group were expressed as mean ± standard deviation (mean ± standard error of the mean, $n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$. **(W)** Schematic diagram of the mechanism by which MEG3 variant enhances telomerase activity by increasing DNA damage repair ability in human liver cancer stem cells.

increase of DOX concentration (Fig. 1I). Although H3K36me3 was significantly increased in DOX (2 μg/mL) treatment group, it was not significantly changed in DOX (2 μg/mL) treatment + rLV-Sirt2 group and DOX (2 μg/mL) treatment + pGFP-V-RS-SETD2 group compared to DOX (2 μg/mL) treatment group (Fig. 1J; Fig. S5A–C). Thereby, the binding of H3K36me3 to Rad51, PARP1, ATR, ATM, and hMSH6 was significantly increased with the increase of DOX concentration (Fig. S5D). Next, Temozolomide (DNA damage inducer) was treated in these cells and the γH2AX (S139) (a marker of DNA damage and inversely proportional to DNA damage repair ability) was detected. Although DNA damage repair ability was significantly increased in DOX (2 μg/mL) treatment group, it was not significantly changed in DOX (2 μg/mL) treatment + rLV-Sirt2 group (Fig. 1K) and DOX (2 μg/mL) treatment + pGFP-V-RS-SETD2 compared to DOX (2 μg/mL) treatment group (Fig. S5E). The γH2AX (S139) was significantly decreased with the increase of DOX concentration (Fig. 1L; Fig. S5F). Although DNA damage repair ability was significantly increased in DOX (2 μg/mL) treatment group (1.89 ± 0.085 *vs.* 0.513 ± 0.065, $P = 0.0019$), it was not significantly changed in DOX (2 μg/mL) treatment + pGFP-V-RS-SETD2 group compared to DOX (2 μg/mL) treatment group (1.89 ± 0.085 *vs.* 1.98 ± 0.199,

$P = 0.169$) (Fig. S5G). Although DNA damage repair ability was significantly increased in DOX (2 μg/mL) treatment group (2.16 ± 0.19 *vs.* 0.73 ± 0.055, $P = 0.0046$), it was not significantly changed in DOX (2 μg/mL) treatment + rLV-Sirt2 group compared to DOX (2 μg/mL) treatment group (2.16 ± 0.19 *vs.* 2.36 ± 0.47, $P = 0.324$) (Fig. S5H). Therefore, MEG3 variant promotes the DNA damage repair ability through the SETD2-H3K36me3 pathway.

Given that MEG3 variant triggers the DNA damage repair and DNA damage repair to activate telomerase activity, we will further reveal whether the MEG3 variant effects on telomerase activity are dependent on DNA damage repair. In hLCSCs infected with rLV-tet on-MEG3 variant and treated with DOX (0 μg/mL, 0.5 μg/mL, 1 μg/mL, 1.5 μg/mL, 2 μg/mL) plus Temozolomide (DNA damage inducer), the telomerase reverse transcriptase (TERT) expression was significantly increased with the increase of DOX concentration (Fig. 1M, P). Therefore, the interaction between TERT and telomerase RNA component (TERC) was significantly increased with the increase of DOX concentration (Fig. 1Q, T). Furthermore, the telomerase activity and the telomere lengths were significantly increased with the increase of DOX concentration (Fig. 1U, V). Although the telomerase activity was increased in DOX (2 μg/mL) treatment group compared to DOX (0 μg/mL) treatment group, it was not significantly changed in DOX (2 μg/mL) treatment + rLV-Sirt2 group compared to DOX (0 μg/mL) treatment group (0.012 ± 0.0017 *vs.* 0.094 ± 0.006, $P = 0.0009$; 0.012 ± 0.0017*vs.* 0.0156 ± 0.006, $P = 0.163$) (Fig. S6A), DOX (2 μg/mL) treatment + pGFP-V-RS-SETD2 group (0.022 ± 0.003 *vs.* 0.137 ± 0.02, $P = 0.006$; 0.022 ± 0.003 *vs.* 0.026 ± 0.006, $P = 0.169$) (Fig. S6B) and DOX (2 μg/mL) treatment + Rucaparib (DNA damage repair inhibitor) group (0.015 ± 0.0025 *vs* 0.078 ± 0.01, $P = 0.007$; 0.015 ± 0.0025 *vs.* 0.018 ± 0.0015, $P = 0.198$) (Fig. S6C) compared to DOX (0 μg/mL) treatment group. Although the telomere lengths were increased in DOX (2 μg/mL) treatment group compared to DOX (0 μg/mL) treatment group, it was not significantly changed in DOX (2 μg/mL) treatment + rLV-Sirt2 group compared to DOX (0 μg/mL) treatment group (0.723 ± 0.035 *vs.* 1.12 ± 0.066, $P = 0.004$; 0.723 ± 0.035 *vs.* 0.768 ± 0.053, $P = 0.107$) (Fig. S6D), DOX (2 μg/mL) treatment + pGFP-V-RS-SETD2 group (0.917 ± 0.04 *vs.* 1.713 ± 0.075, $P = 0.0023$; 0.917 ± 0.04 *vs.* 0.96 ± 0.026, $P = 0.153$) (Fig. S6E) and DOX (2 μg/mL) treatment + Rucaparib group (0.743 ± 0.064 *vs.* 1.4 ± 0.171, $P = 0.005$; 0.743 ± 0.064 *vs.* 0.77 ± 0.053, $P = 0.358$) (Fig. S6F) compared to DOX (0 μg/mL) treatment group. Therefore, MEG3 variant activates the telomerase activity by increasing DNA damage repair ability. Next, in the DOX (2 μg/mL) group, DOX (2 μg/mL) + pGFP-V-RS-SETD2 group compared with the DOX (0 μg/mL) group, the expression of SETD2 was significantly increased in the DOX (2 μg/mL) group and decreased in DOX (2 μg/mL) + pGFP-V-RS-SETD2 group compared with the DOX (0 μg/mL) group (Fig. S7A). Although the cell proliferation ability (Fig. S7B), colony formation ability (Fig. S7C), and tumor formation ability (Fig. S7D, E) were significantly increased in the DOX (2 μg/mL) group compared with the DOX (0 μg/mL) group, it was not significantly changed in DOX (2 μg/mL) + pGFP-V-RS-SETD2 group compared with the DOX (0 μg/mL) group. In the DOX (2 μg/mL) group, DOX (2 μg/mL) + pGFP-V-RS-TERT group, the expression of TERT was significantly increased in the DOX (2 μg/mL) group and decreased in DOX (2 μg/mL) + pGFP-V-RS-TERT group compared with the DOX (0 μg/mL) group (Fig. S8A). Although the cell proliferation ability (Fig. S8B), the colony formation ability (Fig. S8C), and tumor formation ability (Fig. S8D, E) were significantly increased in the DOX (2 μg/mL) group compared with the DOX (0 μg/mL) group, it was not significantly altered in DOX (2 μg/mL) + pGFP-V-RS-TERT group compared with the DOX (0 μg/mL) group. Thus, the decrease of *SETD2 or TERT* inhibits the cancerous functions of MEG3 variant.

Up to the present, we clearly demonstrate that mutant *MEG3* (31,169—1,174: CTTCC-TCCTT) promotes the growth of human liver cancer stem cells. Mechanistically, our results show that MEG3 variant decreases the expression of Sirt2 and then enhances acetylation modification at the 16th lysine of HistoneH4. Therefore, MEG3 variant enhances the expression of SETD2 through H4K16Ac and then enhances tri-methylation modification at the 36th lysine of HistoneH3. Importantly, MEG3 variant increases the cellular DNA damage repair ability through H3K36me3. Strikingly, MEG3 variant enhances the TERT expression and the telomerase activity by increasing DNA damage repair ability. In particular, TERT and SETD2 play a positive role in the carcinogenic function of MEG3 variant in liver cancer stem cells (Fig. 1W). Thus, we postulate that MEG3 variant may play a vital role in hepatocarcinogenesis through Sirt2-H4K16Ac-SETD2-H3K36me3-DNA damage repair-telomerase-telomere remodeling signaling pathway. Obviously, these observations will play an important role in finding effective liver cancer therapeutic target.

Ethics approval and consent to participate

All methods were carried out in accordance with the approved guidelines. All experimental protocols were approved by a Tongji university institutional committee. Informed consent was obtained from all subjects. The study was reviewed and approved by the China National Institutional Animal Care and Use Committee (Ethics number: TJAB04222101).

Author contributions

Dongdong Lu conceived the study and participated in the study design, performance, coordination, and manuscript writing. Yanan Lu, Shujie Li, Shuting Song, Liyan Wang, Xiaoxue Jiang, Sijie Xie, and Rushi Qin performed the research. All authors read and approved the final manuscripts.

Conflict of interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.11.012.

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