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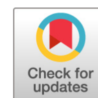
AUTHOR REPLY



Reply to Wang et al., "Assessment of the Abundance and Potential Function of Human Papillomavirus Type 16 Circular E7 RNA"

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In their letter to the editor (1), Richard C. Wang et al. argue mainly three points with our published observation (2): (i) the circular E7 (circE7) abundance relative to the E6*I level and its identity by Northern blotting, (ii) the nuclear versus cytoplasmic distribution of E6*I (linear E7) RNA, and (iii) RNA interference (RNAi) off-target effects on E6*I and translation potentials of circE7. Because the spliced E6*I RNA functions as an E7 mRNA for the translation of the viral oncoprotein E7, our observation concerns the reported biological activity of human papillomavirus type 16 (HPV16) circE7 and the statement in discussion that "other linear isoforms encoding E7 (e.g., E6*I) do not fully support E7 oncoprotein translation, despite the fact that such linear transcripts are present even after circE7 knockdown" (3). We are happy to provide further evidence in this reply.

We have no concern about the presence of circE7 RNA in HPV16 cervical cancer cell lines, but our data do not support the reported biological function in Wang et al.'s previous publication (3). We found by RT-ddPCR (reverse transcription-droplet digital PCR), which has high reproducibility and is more accurate than conventional RT-quantitative PCR (RT-qPCR) for DNA/RNA copy number detection (4–7), the presence of only 0.4 copies of circE7 RNA per CaSki cell, ~1,640-fold lower than the number of E6*I RNA copies (2). TaqMan RT-qPCR in our published observation also showed that circE7 was ~0.3% of E6*I (2). We detected recently by RNA sequencing (RNA-seq) 6 circE7 reads of the total 6206 E6*I reads (0.1% of E6*I) from four CaSki cell samples, of which only two samples displayed detectable circE7 reads (3 reads each) (Table 1). Wang et al.'s letter states that the detected circE7 is about ~0.2 to 1% of E6*I RNA in CaSki cells (1). As shown in Fig. 1b to d in Wang et al.'s letter (1), an oversized, labeled circE7 band (the predicted size should be 472 nucleotides [nt]) is very faint by Northern blotting with an E7 probe compared with the overexposed linear E7 RNA bands detected in CaSki cells (8) and circE7 plasmid-transfected HEK293 cells. If this faint band is truly a circE7 as claimed in Wang et al.'s letter (1), the circE7 should be linearized into a single product of the predicted size after RNase H digestion or after a single nick by hydrolysis, along with a synthetic 472-nt circE7 RNA in both linear and circular forms as size controls in Northern blot assays. We are aware of RNA in a circular form migrating slower than its linear form, but further characterization of this slow-migrating, oversized faint band is necessary for ensuring its identity as an authentic circE7 and not a degraded RNA fragment of E7 transcripts. Since all Northern blot gels in Fig. 1b to d in Wang et al.'s letter were overexposed for linear E7 RNA in order to see a labeled circE7 (1), how could Wang et al. determine ~7 to 12 copies of circE7 RNA per CaSki cell without comparative copy number quantification by the dilution of a synthetic circE7 RNA in parallel for Northern blot analyses? In fact, our observation clearly states that a total of 40 cycles and 500 ng of total cDNA from CaSki cells were needed to detect circE7 RNA by

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TABLE 1 Detection of circE7 RNA reads from CaSki cells by RNA-seq

CaSki RNA-seq sample[a]	Total no. of reads	No. of linear splice junction 226^409 for E6*I	No. of back-splice junction 880^409 for circE7	% circ E7/E6*I
1	133,303,774	1,745	0	0
2	100,417,636	1,441	0	0
3	99,117,888	1,421	3	0.21
4	105,720,990	1,599	3	0.19
Total	438,560,288	6,206	6	0.10

[a]Raw data for the four CaSki RNA-seq samples are available from the NCBI GEO database under accession number GSE158033. RNA-seq on ribo-minus total CaSki cell RNA was performed on a HiSeq 2500 sequencer with a paired-end read length of 150 bp and a depth of ~100 million reads per sample according to the manufacturer's instructions (Illumina). We used the STAR v2.5.2b aligner to identify the linear RNA splice junction reads. We generated alignments for CIRI2 (version. 2.0.6) (https://sourceforge.net/projects/ciri/files/CIRI2/) with BWA (version 0.7.17) to identify the back-splice junction reads.

reverse PCR amplification, but only 25 cycles and 100 ng of total cDNA were enough for E6*I detection by regular RT-PCR (2). Even though cell growth conditions (confluence versus logarithmic) may affect circE7 abundance (Fig. 1a in Wang et al.'s letter [1]), the relative level of circE7 to E6*I RNA under each condition remains to be determined.

Over the years, the HPV field has well recognized that the spliced E6*I RNA isoform from high-risk HPVs is present mainly in the cytoplasm (9) and functions as an E7 mRNA to encode the E7 protein (10, 11). Our published observation demonstrated that both E6*I (linear E7) and circE7 RNAs are mainly cytoplasmic (2), which is in contrast to the data by Wang et al.'s laboratory that the linear E7 (E6*I) RNA was mainly nuclear but that circE7 was cytoplasmic (see Fig. 3b in reference 3).

Regarding RNAi off-target effects and coding potentials of circE7, Wang et al.'s letter provides no convincing data to support the production of E7 protein from circE7 RNA (1). In our observation, two synthetic small interfering circE7s (si-circE7s) at different doses all showed strong off-target effects on E6*I and E6*II by both RT-PCR and RT-qPCR and, more importantly, on E7 protein expression by Western blotting (2). Of the two small interfering RNAs (siRNAs) used in our observation (2), one si-circE7 sequence, si-circE7-2, with a 13-nt sequence overhang to the back-splice junction, was identical (Fig. 1) to the circE7 sh2 sequence in Wang et al.'s early report (3). Despite that, Wang et al.'s letter questions the off-target effect possibly being the result of the two siRNAs used in our observation; however, this contention is not legitimate unless the short hairpin RNA (shRNA) used in Wang et al.'s early report (3) had a different sequence. Other reports have concluded that off-target silencing occurs in a manner reminiscent of target silencing by microRNAs (miRNAs) and that 11 contiguous nucleotides of identity to the siRNA are sufficient to direct the silencing of nontargeted transcripts (12). Moreover, an inducible shRNA is able to silence a subset of transcripts that are silenced by an siRNA of the same sequence (13). Consistently, our observation (2) demonstrated that si-circE7-2 with a 13-nt overhang sequence (Fig. 1) had a greater off-target effect on linear E6*I and E6*II RNAs than si-circE7-1 with an 11-nt overhang sequence.

Wang et al.'s letter also shows circE7 RNA expression from three circE7 expression vectors and E7 protein expression only from circE7 and circResist_WT vectors but not the circResist_noATG vector in Fig. 1e to g in Wang et al.'s letter (1), suggesting E7 translation from circE7 RNA under their experimental conditions. However, we have a different view of these data.

		416 409/880 870	Size
Yu et al.	si-circE7-1	UAAUACAC/CUGCAGGAUCA	19 nt
	si-circE7-2	UAAUACAC/CUGCAGGAUCAGC (868)	21 nt
Zhao et al.	circE7 sh2	UAAUACAC/CUGCAGGAUCAGC	21 nt
	circE7 sh1	AAUACAC/CUGCAGGAUCAGCC (415 ... 867)	21 nt

FIG 1 Nucleotide sequences of the siRNAs used in Yu and Zheng's observation (2) and the shRNAs used in Wang et al.'s previous report (3). Numbers above the nucleotide sequences are the nucleotide positions in the HPV16 genome./, back-splice junction.

Although the E7 RNA transcribed from their three vectors lacks an E6 intron, as designed to prevent RNA splicing, the shortcoming of this design to avoid E6 intron splicing is that all vectors now containing an entire E7 open reading frame (ORF) region under the control of a strong cytomegalovirus (CMV) immediate early (IE) promoter transcribe a large amount of linear E7 mRNA with a size of ~1.5 kb (see Fig. 1d in Wang et al.'s letter [1]) for the translation of E7 protein (see Fig. 1g in Wang et al.'s letter [1]). Thus, Wang et al.'s letter (1) provides no evidence that these vectors did not express linear E7 RNA to encode E7 protein besides quaking protein-binding motif (QKI)-mediated production of circE7. In fact, all three vectors in Wang et al.'s letter expressed only a minimal amount of circE7 by reverse PCR in transfected cells (see Fig. 1f in reference 1) and by Northern blotting (see Fig. 1d for the circE7 wild-type [WT] vector in Wang et al.'s letter [1]). As expected, there was no E7 protein translation from the E7 RNA in cells transfected with the circResist_noATG vector, of which the E7 translation initiation codon ATG was mutated (1). To further prove E7 translation directly from circE7 RNA, a synthetic circE7 RNA would be suggested for *in vitro* and *in vivo* (mammalian cells) translation studies. In conclusion, this negligible amount of circE7 in cervical cancer cells (less than a copy per cell) would be unlikely to play a predominant role biologically important for the cell transformation and carcinogenesis in Wang et al.'s early report (3).

Data Availability. All study data are included in the article and supporting information. The NCBI has assigned the following accession number GSE158033 for deposited CaSki RNA-seq dataset in this report.

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REFERENCES

1. Wang RC, Lee EE, Zhao J, Kim J. 2022. Assessment of the Abundance and Potential Function of Human Papillomavirus Type 16 Circular E7 RNA. mBio 13:e00411-22. https://doi.org/10.1128/mBio.00411-22.
2. Yu L, Zheng ZM. 2022. Human papillomavirus type 16 circular RNA is barely detectable for the claimed biological activity. mBio 13:e03594-21. https://doi.org/10.1128/mbio.03594-21.
3. Zhao J, Lee EE, Kim J, Yang R, Chamseddin B, Ni C, Gusho E, Xie Y, Chiang CM, Buszczak M, Zhan X, Laimins L, Wang RC. 2019. Transforming activity of an oncoprotein-encoding circular RNA from human papillomavirus. Nat Commun 10:2300. https://doi.org/10.1038/s41467-019-10246-5.
4. Taylor SC, Laperriere G, Germain H. 2017. Droplet digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. Sci Rep 7:2409. https://doi.org/10.1038/s41598-017-02217-x.
5. Kojabad AA, Farzanehpour M, Galeh HEG, Dorostkar R, Jafarpour A, Bolandian M, Nodooshan MM. 2021. Droplet digital PCR of viral DNA/RNA, current progress, challenges, and future perspectives. J Med Virol 93:4182–4197. https://doi.org/10.1002/jmv.26846.
6. Park C, Lee J, Hassan ZU, Ku KB, Kim S-J, Kim HG, Park EC, Park G-S, Park D, Baek S-H, Park D, Lee J, Jeon S, Kim S, Lee C-S, Yoo HM, Kim S. 2021. Comparison of digital PCR and quantitative PCR with various SARS-CoV-2 primer-probe sets. J Microbiol Biotechnol 31:358–367. https://doi.org/10.4014/jmb.2009.09006.
7. Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, Emslie KR. 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal Chem 84:1003–1011. https://doi.org/10.1021/ac202578x.
8. Yu L, Lobanov A, Majerciak V, Mirza S, Band V, Liu H, Cam M, Zheng ZM. 2020. Expression of HPV oncogenes from a single integration site in cervical cancer cell lines. bioRxiv https://doi.org/10.1101/2020.06.25.171959.
9. Jia R, Liu X, Tao M, Kruhlak M, Guo M, Meyers C, Baker CC, Zheng ZM. 2009. Control of the papillomavirus early-to-late switch by differentially expressed SRp20. J Virol 83:167–180. https://doi.org/10.1128/JVI.01719-08.
10. Tang S, Tao M, McCoy JP, Jr, Zheng ZM. 2006. The E7 oncoprotein is translated from spliced E6*I transcripts in high-risk human papillomavirus type 16- or type 18-positive cervical cancer cell lines via translation reinitiation. J Virol 80:4249–4263. https://doi.org/10.1128/JVI.80.9.4249-4263.2006.
11. Ajiro M, Jia R, Zhang L, Liu X, Zheng ZM. 2012. Intron definition and a branch site adenosine at nt 385 control RNA splicing of HPV16 E6*I and E7 expression. PLoS One 7:e46412. https://doi.org/10.1371/journal.pone.0046412.
12. Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. 2003. Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol 21:635–637. https://doi.org/10.1038/nbt831.
13. Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS. 2006. Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. RNA 12:1179–1187. https://doi.org/10.1261/rna.25706.