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MCV Truncated Large T antigen interacts with BRD4 in tumors

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Abstract

Among *Polyomaviridae* family of viruses, Merkel Cell Polyomavirus (MCV) is the only human polyomavirus with convincing data supporting its classification as a direct causative agent of a human skin malignancy, Merkel Cell Carcinoma. Oncogenic transformation by MCV requires the integration of the viral genome into the human genome, truncation of the large T antigen (LT) to render the viral genome replication deficient and expression of small T antigen oncoprotein. The chromatin binding protein BRD4, was recently shown to transcriptionally regulate the expression of virus oncoproteins, thereby enhancing the tumorigenesis of virus-associated cancers, such as HPV associated cervical cancer. Previous work by Wang *et al.* revealed that BRD4 interacts with MCV full length LT during viral replication. In this study, we demonstrated that MCV truncated tumor LT antigen also interacts with BRD4 protein. We showed that the MCV tumor LT antigen and BRD4 protein complex co-localizes within the nucleus. Furthermore, we tested whether BRD4 protein transcriptionally regulates MCV Non Coding Control Region (NCCR), where we found that though full length LT and sT together, along with the BRD4 protein showed enhanced transcriptional activity whereas tumor truncated LT did not. These findings on the interactions of the MCV tumor truncated LT antigen with the BRD4 protein add to existing knowledge about interactions with LT and its role in tumorigenesis, and assist in efforts to more precisely define new therapy targets for this disease.

Introduction

Merkel cell polyomavirus (MCV), the cause of a lethal skin cancer called Merkel cell carcinoma, is a unique member in the list of known human tumor viruses [1] [2]. MCV is a small, non-enveloped virus with a circular double stranded 5 kb genome, divided into early and late regions by a noncoding control region (NCCR) [1]. The early region expresses a large T antigen (LT) and small T antigen protein (sT), which drive tumorigenesis in Merkel cells. MCV LT antigen is found in its full-length form in wild-type episomes of the virus, however in tumor cells, a truncated replication-incompetent form of the LT protein is expressed. Truncated LT antigen always, in every patient tumor thus far, conserves the N-terminal tumor-suppressor targeting domains, but loses the expression of the C-terminal ends responsible for viral replication functions. In 2012, Wang *et al.* uncovered the interaction between an epigenetic reader, the bromodomain protein 4 (BRD4) and the N-terminal end of the full-length LT oncoprotein [3]. Their studies showed that this interaction facilitates the localization of the complex to the MCV replication origin region where it regulates MCV replication, as tested using *in vitro* replication assays in HEK293T and C33A cells [3]. BRD4 is a BET family member that harbors two bromodomains and an ET (extra-terminal) domain [4]. It is a chromatin regulator involved in transcription programs in the development of several aggressive cancers and associates with a number of oncogenic viruses, including Human Papillomavirus (HPV) [4]. Recently McKinney *et al.* showed that BRD4 activates early HPV transcription in primary keratinocytes [5]. Furthermore, Dooley *et al.*found specific nuclear foci of BRD4, MED1 and H3K27ac at the sites of tandem HPV integration in cancer cells [6]. Evidence from their studies supports a BRD4 dependent super-enhancer like element in the viral genome regulating viral transcription [6].

Objective

In this study, we asked whether the truncated LT antigen, that retains the N-terminal

but lacks the replication-important C-terminal, continues to interact with BRD4 protein. If yes, then what role would this interaction play in viral-mediated tumorigenesis? We further tested how this interaction may affect transcription through the MCV NCCR region, which houses the viral promoter/enhancer. The objective of our study was to validate this interaction (BRD4 and LT) and then investigate its implication in Merkel tumors.

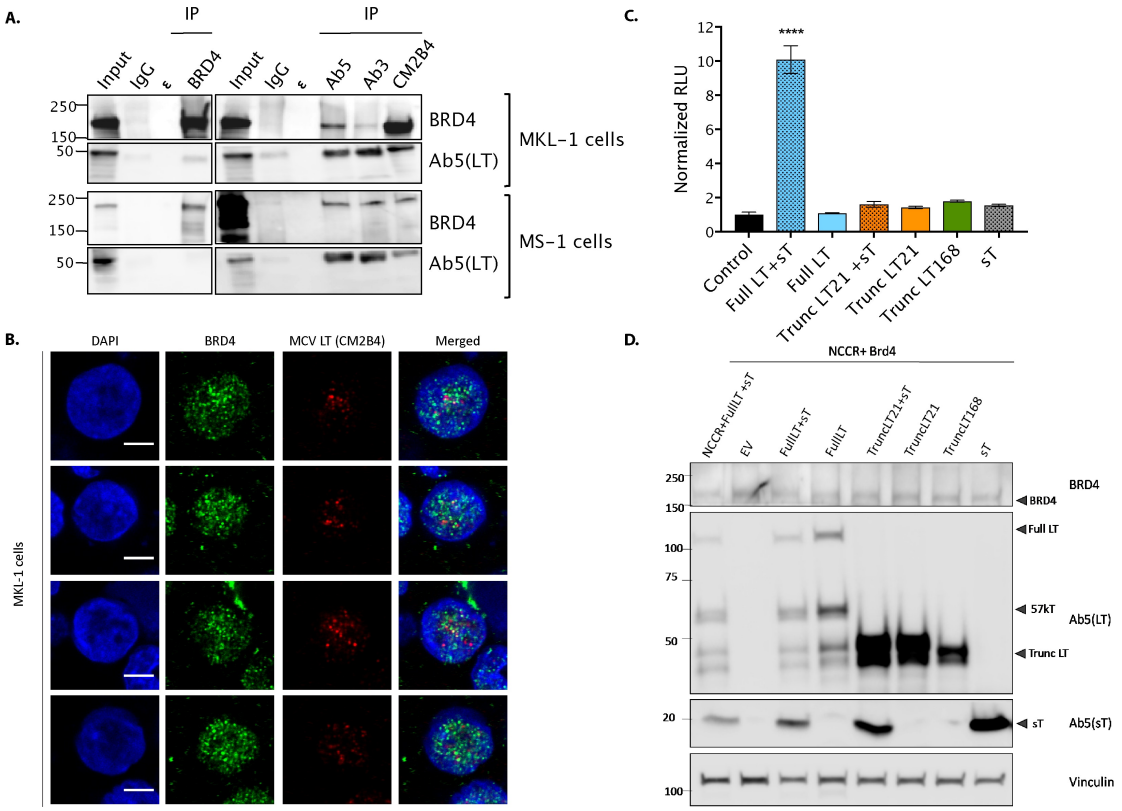

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Figure Legend

Figure 1. Truncated MCV LT antigen interacts with endogenous BRD4 protein in Merkel cell carcinoma cells.

(A) Nuclear proteins were isolated from MKL-1 and MS-1 cell lines and immunoprecipitated with polyclonal BRD4 antibody and 3 different antibodies against LT antigen i.e. Ab5, Ab3 and CM2B4. BRD4 protein was observed to be co-immunoprecipitated with LT targeting antibodies; however vice-versa was not seen. ε represents the empty lanes between the samples. Input is 2.7% (MKL-1) and 0.8% (MS-1) of total lysate.

(B) MKL-1 were immunostained for BRD4 and LT antigen (using CM2B4) and imaged using FV1000 at 60X magnification. The scale bar represents 5 microns. 4 cells imaged are shown here (of a total of 28 cells, in 3 experiments).

(C) Represents the Relative Luciferase activity in U2OS cells transfected with MCV NCCR region and BRD4 expressing plasmid along with different combinations of MCV T antigen. Two different truncated LT antigens (LT21 and LT168) were used to test increase in luciferase activity. Each column represents the mean value obtained from 3 independent experiments. Error bars represent SD. (2 technical replicates each time). One-way ANOVA with post-hoc Tukey's test showed Full LT+sT to be statistically significant in comparison to control and other conditions ($p<0.0001$).

(D) Corresponding western blot for the luciferase analysis confirms the expression of the different T antigen combinations.

Results & Discussion

To test the interaction of truncated tumor LT antigen, we performed an immunoprecipitation (IP) assay in both MKL-1 and MS-1 cell lines [1] [7] [8]. These are both Merkel cell carcinoma cell lines that express truncated-LT and sT. The truncated-LTs from both MKL-1 and MS-1 are of different lengths corresponding to their respective truncation mutation and thus run at different sizes on a SDS-polyacrylamide [7]. We used three antibodies Ab3, Ab5 and CM2B4 to immunoprecipitate MCV LT, all three of which pulled down BRD4 (Fig. 1A). All the three antibodies used have different sensitivities and specificities besides binding different epitopes in MCV T antigen [9] [10]. While Ab5 binds to the both LT and sT, Ab3 and CM2B4 are specific to LT and interact with its Exon 2 region [10] [11]. IP using BRD4 antibody however, showed a very weak LT interaction. Wang *et al.* have previously shown that BRD4-LT interaction is facilitated through the N-terminal region (156-284 aa) of MCV LT, which is retained in the tumor antigens [3]. These assays corroborate the interaction, however in the tumor context, where truncated LT is endogenously expressed.

To further validate this interaction we investigated whether MCV LT co-localizes with BRD4 in Merkel Cell Carcinoma cells. MKL-1 were immunostained with antibodies against MCV LT (CM2B4) and BRD4 protein (Fig. 1B). Since MCV positive MCC cell lines grow as suspension cells that clump together, before performing the immunofluorescence we treated them with 2 mM EDTA followed by gentle pipetting to break the clumps and get single cells that adhered to poly-L-Lysine coverslips. Immunofluoroscence analysis revealed that MCV LT antigen did co-localize with BRD4 protein in nucleus, although weakly at only an average 7.8 % with a correlation coefficient of 0.36 (coefficient range 4.4 to 11.9).

These results validate that BRD4 interacts with truncated (tumor) MCV LT antigen. Although, BRD4's interaction with full-length MCV large T antigen aids in viral replication, we were unclear as to why BRD4 would interact with truncated LT in Merkel tumor cells.

To address this, we next studied the implication of this interaction with MCV transcription. We thereby performed luciferase reporter assays in U2OS cells by overexpressing the NCCR driving firefly luciferase in the presence of different plasmid combinations of viral T antigens and BRD4. We found that full length LT, along with sT antigen, significantly increased ($p<0.0001$) luciferase activity in the presence of BRD4, however the truncated LT antigens or sT alone did not (Fig. 1C). T antigen expression was validated by immunoblotting figure 1D.

Kwun *et al* and others [12] [13] [14] [15] [16], have previously shown that full-length LT drives viral replication by binding to the origin of replication. sT also contributes to viral replication by forming a complex with LT and stabilizing it [12] [15] [17].

In our experiments we included the entire NCCR region containing both, the origin of replication and the viral promoter, as opposed to only the viral origin region in other studies [12] [15] [17]. Hence, the reason for higher luciferase activity in the condition expressing BRD4 along with full length LT and sT could be explained by increased replication of the NCCR plasmid. Cheng *et al.* [10] and Borchert *et al.* [18] have not found any evidence for MCV tumor LT's direct interaction with DNA. Hence, MCV truncated LT probably interacts with BRD4 to regulate BRD4's DNA binding and gene regulation. One such important region may be the viral promoter itself. To further test the implications of this interaction, ChIP studies of BRD4 in MCC cell lines, specifically on the viral promoter and enhancer regions will be valuable.

Conclusions

We show that Merkel cell polyomavirus's truncated (tumor) LT antigen, expressed in Merkel tumor cells, also interacts with BRD4, in the absence of virus replication in Merkel tumors.

Limitations

We were limited by the antibodies we had to detect MCV large T and BRD4. BRD4 is a large protein and doing western blotting and immunoprecipitations with the same

antibody was challenging. Also we used only 2 MCC cell lines (MKL1 and MS-1) both of which grow in suspension as large clumps. For the Immunofluoresence experiments we had to treat these clumps with EDTA to disperse them as single cells and then attach them to slides for staining purposes.
While we showed that the truncated tumor T interacting with BRD4 was unable to increase viral transcription, nonetheless we used a luciferase reporter assay. It is still likely that truncated LT's interaction with BRD4 is important for its transcription regulation function and further studies could test this via ChIP-PCR analysis and other direct investigations.

Additional Information

Methods and Supplementary Material

Please see https://sciencematters.io/articles/201811000004.

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Ethics Statement

Not Applicable.

Citations

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