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Viral elements inducing tumor-related apoptosis

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Citation

Kooistra, K. (2007, October 11). *Viral elements inducing tumor-related apoptosis*. Leiden Institute of Chemistry (LIC), Faculty of Science, Leiden University. Retrieved from <https://hdl.handle.net/1887/12375>

Version: Corrected Publisher's Version

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**TT-VIRUS-DERIVED APOPTOSIS-INDUCING
PROTEIN (TAIP) INDUCES APOPTOSIS
PREFERENTIALLY IN HEPATOCELLULAR
CARCINOMA-DERIVED CELLS**

CHAPTER 7

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J. Gen. Virol. (2004) 85: 1445-50

ABSTRACT

TT virus (TTV) is widespread among the global population. Its pathogenic nature is still unclear but TTV seems to be more prevalent in cases of hepatitis than in healthy individuals. TTV harbours similarities to chicken anaemia virus (CAV). Here, the apoptotic potential of a putative TTV-derived 105 aa protein and of the main apoptosis-inducing agent of CAV, Apoptin, is compared. As the putative protein induced apoptosis in various human hepatocellular carcinoma (HCC) cell lines, it was named TTV-derived apoptosis-inducing protein (TAIP). The apoptotic activity of TAIP in HCC lines was comparable with that of Apoptin. Conversely, unlike Apoptin, TAIP induced only low-level apoptosis in several non-HCC human cancer cell lines. The data suggest that TAIP acts in a different way to Apoptin as it is selective to a certain degree for HCC lines. This activity of TAIP, coupled with the heterogeneity of TTV isolates, may help to explain the variable reports of TTV pathogenicity.

SHORT COMMUNICATION

TT virus (TTV) was discovered by representational difference analysis of sera from non-A to non-G post-transfusion hepatitis patients (Nishizawa *et al.*, 1997; Okamoto *et al.*, 1998). Epidemiological studies have shown that TTV is genetically variable and widespread in the general human population and, furthermore, that TTV-like viruses are widespread in chickens, pigs, cows and sheep (Bendinelli *et al.*, 2002; Leary *et al.*, 1999; Okamoto *et al.*, 2002).

TTV can infect not only hepatocytes but also extrahepatic tissues (Zhong *et al.*, 2002; Suzuki *et al.*, 2001), although most of its replication appears to occur in the liver. Most of the literature shows that the infection of TTV seems to have few if any pathogenic effects (reviewed, for example, by Bendinelli *et al.*, 2002), but in certain cases it seems that TTV can still damage liver tissues (Hu *et al.*, 2002). In addition, various reports have described that, although TTV does not seem to contribute to the development of human hepatocellular carcinoma (HCC) from chronic liver disease, it may be a risk factor for the development of HCC in patients with type C liver disease in the F4 stage (Yoshida *et al.*, 2000; Pineau *et al.*, 2000; Tagger *et al.*, 1999). The irregular regeneration of hepatocytes in TTV-positive patients was significantly higher than that in TTV-negative patients (Moriyama *et al.*, 2001), and Camci *et al.* (2002) described a high prevalence of TTV in cancer patients. TTV harbours a circular single-stranded DNA genome, and although there are significant differences, there are also striking similarities to chicken anaemia virus (CAV) in its genomic organization (Fig. 1a; Miyata *et al.*, 1999; Mushahwar *et al.*, 1999; Erker *et al.*, 1999; Noteborn *et al.*, 1991, 1999). Since CAV encodes a protein that is

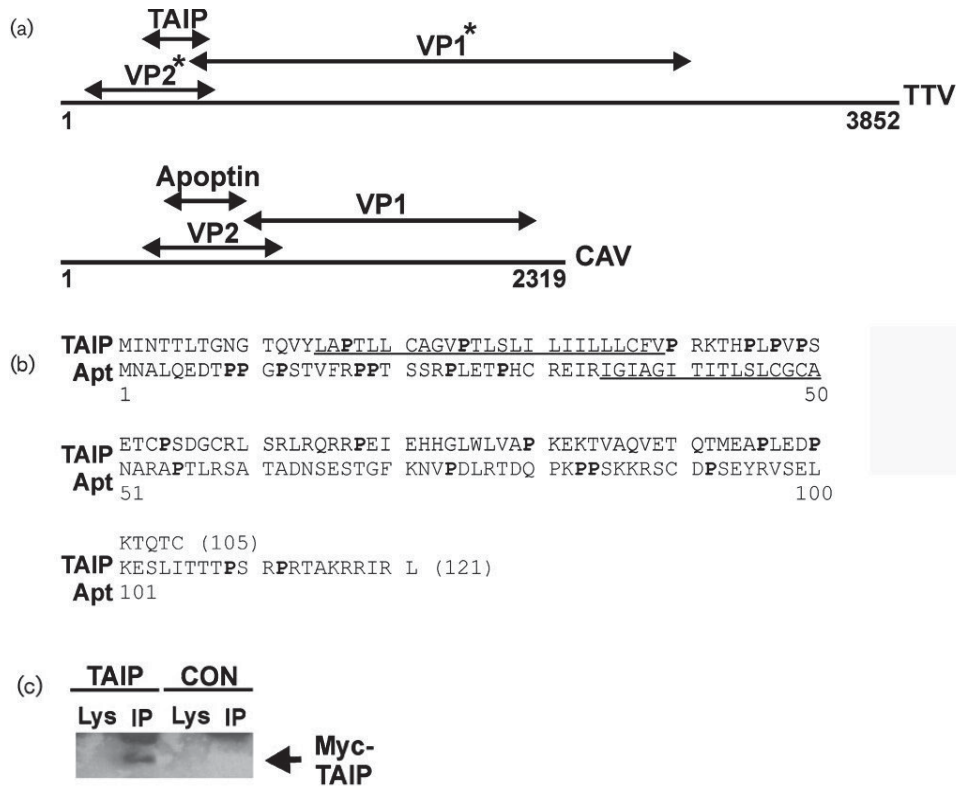


Figure 1. (a) Schematic representation of the TTV and CAV genome organization. The asterisk indicates the putative status of TTV VP1 and VP2. (b) Amino acid sequence of TAIP and Apoptin. The proline residues are shown in bold and hydrophobic amino acids stretches are underlined. Apt, Apoptin. (c) Proteins from transfected cells were separated by 15% SDS-PAGE followed by Western blotting. The figure shows an ECL representation of the Western blot of COS-1 lysates (Lys) and immunoprecipitations (IP) after transfection with the TAIP-encoding plasmid pCMV-TAIP (TAIP) or mock transfection (CON). The arrow indicates the Myc-tagged TAIP product visualized by 9E10 antibodies directed against the Myc tag of TAIP. The shadow at the top of the exposure is derived from the light chain of the precipitating (9E10) antibody.

uniquely apoptotic in cancer cell lines, we were interested in whether TTV contained a similar entity. A detailed comparative analysis of the putative TTV ORF 3 encoding 105 aa (Miyata *et al.*, 1999; GenBank accession no. AB008394) with Apoptin showed that, although their overall sequence homology seemed limited, intriguing similarities existed both in sequence (Fig. 1b) and predicted structure (not shown).

To study the possible apoptotic activities of this putative protein, which we named TTV-derived apoptosis-inducing protein (TAIP), the expression vector pCMV-TAIP was constructed. The production of the required 0.36 kb DNA fragment was carried out by The Midland Certified Reagent Co. (Midland, TX, USA). This construct encoded the complete 105 aa of TAIP with an N-terminal Myc tag (aa EQKLISEEDL) to enable recognition of the TAIP by means of the Myc tag-specific antibody 9E10 (Gebbinck *et al.*, 1997).

The synthesis of the Myc-tagged TAIP was first analysed by transfection of COS-1 cells with plasmid pCMV-TAIP (Van der Vorm *et al.*, 1994; Danen-Van Oorschot *et al.*, 2000). Two days after transfection, the cells were lysed and Western blot analysis was carried out using antibody 9E10. The COS-1 cells transfected with pCMV-TAIP were shown to synthesize a specific Myc-tagged product with the expected size of approximately 15 kDa. Control lysates of mock-transfected COS-1 cells did not contain a protein product reacting with 9E10 (Fig. 1c).

Next, we examined whether the TAIP harboured apoptotic activity in human HCC cells and compared this with the effects of either Apoptin or LacZ as positive and negative controls, respectively. The human HCC-derived cell lines HepG2 (p53⁺), HUH-7 (p53 mutant) (both from ATCC) and Hep3B (p53⁻; Puisieux *et al.*, 1993) were transiently transfected with pCMV-TAIP encoding the Myc-tagged TAIP, pCMV-Apoptin encoding Apoptin and pcDNA3.1-MycHis-LacZ encoding the LacZ protein (Zhuang *et al.*, 1995; Danen-van Oorschot *et al.*, 2000). Two and 5 days after transfection, the cells were screened for the production of the specific transgene by indirect immunofluorescence (Nebenborn *et al.*, 1990). Apoptosis was analysed by nuclear DNA staining with DAPI (Telford *et al.*, 1992). Two days after transfection, the putative TAIP was mainly detected in the cytoplasm, whereas Apoptin was mainly found in the nucleus of the transfected HCC lines. The negative control LacZ protein was present in the cytoplasm (Fig. 2a). Five days after transfection, all three analysed HCC cell lines transfected with the TAIP transgene as well as those expressing Apoptin underwent high levels of apoptosis (Fig. 2b), whereas the cells expressing LacZ only underwent a low level of cell death. These data clearly show that TAIP can induce apoptosis in human HCC cells. Furthermore, the fact that TAIP was able to kill Hep3B cells indicated that TAIP, like Apoptin, induces apoptosis in a p53-independent way.

To characterize the apoptotic activity of TAIP in more detail, two human osteosarcoma cell lines, Saos-2 (p53⁻) and U2OS (p53⁺; both described in Diller *et al.*, 1990), and a human small-cell lung carcinoma cell line, H1299 (ATCC), were transiently transfected with plasmids encoding TAIP, Apoptin or the negative-apoptosis control LacZ as above. Immunofluorescence assays showed that, 2 days after transfection, TAIP was located

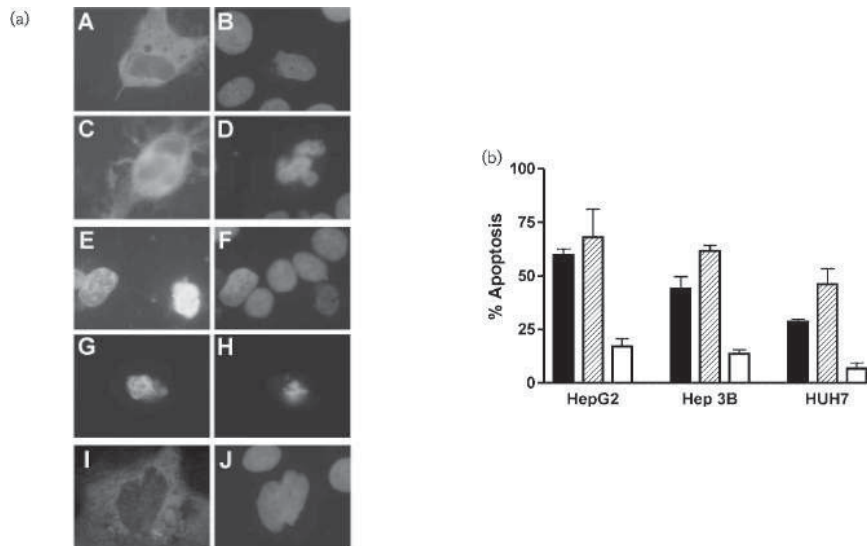


Figure 2. (a) Indirect immunofluorescence of human HCC-derived HUH-7 cells transfected with plasmid pCMV-TAIP encoding the putative TAIP (A-D), with plasmid pCMV-Apoptin encoding the CAV-derived Apoptin (E-H) or with pcDNA3.1-MycHis-LacZ encoding the negative apoptosis control protein LacZ (I, J). The cells were fixed and stained using specific antibodies and DAPI. The panels show the specific antibody staining (left panels) and DAPI staining (right panels). (A), (B), (E), (F), (I) and (J) show intact cells, whereas (C), (D), (G) and (H) represent apoptotic cells. (b) TAIP-versus Apoptin-induced apoptosis activity in human HCC cells 5 days after transfection. The percentage of transgene-positive cells that stained abnormally with DAPI is given as a relative measure of apoptosis in the HCC cell lines HepG2, Hep3B and HUH-7 containing TAIP (solid bars), Apoptin (hatched bars) or negative control LacZ (open bars). Results are the means of at least two independent experiments. In each experiment at least 100 transgene-positive cells were examined for their apoptotic status.

mainly in the cytoplasm, similar to the distribution found in HCC cells (Fig. 3a). Apoptin and LacZ were also found to be distributed in the cells in a similar way to HCC lines (data not shown). Five days after transfection, the DAPI pattern of the majority of the Apoptin-positive cells was weak and irregular, indicating that these cells had undergone apoptosis. TAIP-expressing cells also appeared to undergo apoptosis compared with LacZ-transfected cells, but to a lesser extent than with Apoptin (Fig. 3b).

A two-way analysis of variance (ANOVA) using the data from each individual transfection showed that there was a statistically significant interaction between cell type and

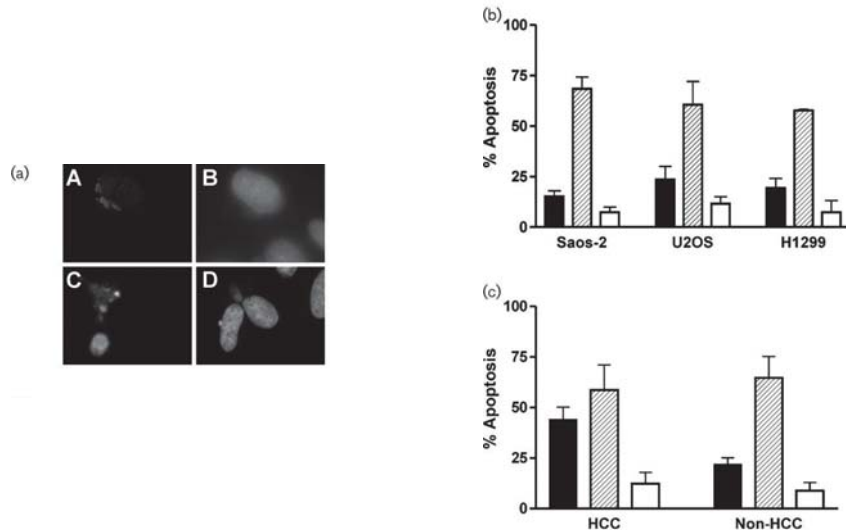


Figure 3. (a) Indirect immunofluorescence of human Saos-2 cells transfected with plasmid pCMV-TAIP encoding the putative TAIP. The cells were fixed and stained with antibody 9E10 detecting Myc-tagged TAIP (A, C) or with DAPI showing intact (B) or apoptotic (D) DNA. (b) TAIP- versus Apoptin-induced apoptosis in human non-HCC cells. The percentage of transgene-positive cells that were stained abnormally by DAPI is given as a relative measure for apoptosis in the non-HCC cell lines Saos-2, U2OS and H1299 containing TAIP (solid bars), Apoptin (hatched bars) or negative control LacZ (open bars). Results are the means of at least two independent experiments. In each experiment at least 100 transgene-positive cells were examined for their apoptotic status. (c) Comparison of mean apoptosis induction (\pm SD) by TAIP (solid bars), Apoptin (hatched bars) or LacZ (open bars) expression in HCC versus non-HCC cells using the data from Figs 2(b) and 3(b).

transfection effect. The response of the HCC cells was significantly different compared with non-HCC cells when TAIP was used ($P < 0.001$, data not shown). When results from HCC and non-HCC cells were considered separately, each group was homogeneous using the same test, indicating that a difference existed between groups rather than between individual cell lines. No significant cell type-specific differences in the response to either Apoptin or LacZ were found. We further investigated this phenomenon using the mean death rates for each group (Fig. 3c). A *t*-test showed that, in the HCC group, the observed apoptosis for TAIP ($P = 0.029$) and Apoptin ($P = 0.003$) were significantly higher than in LacZ-expressing cells. Furthermore, within the HCC group there was no significant difference between the cell death percentages induced by TAIP and Apoptin ($P > 0.28$). In the non-HCC group, there was also a significantly higher

amount of apoptosis induced by TAIP ($P < 0.002$) and Apoptin ($P < 0.0001$) when compared with LacZ-induced apoptosis. However, in non-HCC cells TAIP was significantly less able to induce apoptosis when compared with Apoptin ($P < 0.0001$).

The difference in efficacy for TAIP-induced death in HCC versus non-HCC lines is probably not caused by a technical or cell culture difference, since no similar difference was observed in the other transfections. In conclusion, TAIP seems to induce apoptosis preferentially in HCC cells, unlike Apoptin, which induces high levels of apoptosis independent of the tumour origin.

The original hypothesis that TTV causes cryptogenic hepatitis has not been borne out (Bendinelli *et al.*, 2002). On the other hand, it has been shown that, in particular cases, TTV seems to be involved in liver damage (Hu *et al.*, 2002). Here, we have reported that the putative TTV-derived TAIP induced cell death in three liver-derived human cell lines but significantly less in three non-liver-derived cell lines. First indications from experiments using normal rat hepatocytes microinjected in the nucleus with plasmids encoding TAIP, Apoptin or the non-apoptotic LacZ protein indeed showed that neither Apoptin nor TAIP induced apoptosis in these cells (data not shown). Although this is interesting, further experiments with, for example, normal human liver cells will be required before concluding that TAIP causes cell death in a tumour-specific manner, similar to Apoptin (Noteborn, 2002).

Besides genomic homologies between CAV and TTV (Miyata *et al.*, 1999; Bendinelli *et al.*, 2002), we have provided evidence here that at least one TTV strain also encodes a potential apoptotic activity, which has some characteristics reminiscent of Apoptin. Only two TTV isolates have been reported with 100 % homology to TAIP, the source of our TAIP sequence, TA278, and VT 416, both type 1 genotypes. A further eight genotypes (all type 1) showed 90-99% homology at the protein level, but until the relevant experiments have been performed we cannot know whether even single amino acid variants contain the same activity as TAIP. This restricted distribution might indicate that TAIP (activity) is not essential for TTV or that it may be responsible for some strain-specific cytopathogenic effect. In addition, there may be tissue-specific effects of TTV infection, since in the limited cases where pathology has been observed this was always restricted to the liver of TTV-infected individuals (e.g. Hu *et al.*, 2002), despite some reports of replication in additional sites in the body (Xiao *et al.*, 2002; Bando *et al.*, 2001). This may be relevant to the finding by Kamahora *et al.* (2000), who found expression of only three transcripts when full-length TTV was transfected into COS cells, none of which resembled TAIP. However, if TTV-related pathology is restricted to liver, it seems possible that cell-type/tissue-specific additional transcripts may exist. In light of the possibility of tissue- and/or strain-specific pathology, it seems important to identify more complete TTV coding sequences and to sample several

tissues in order to characterize its pathogenic potential adequately. In addition, our results do not exclude the possibility that further (putative) ORFs of newly identified TTV isolates may also induce apoptosis (Peng *et al.*, 2002). It is even possible that other TTV proteins harbour (further) apoptotic potential, analogous to the CAV protein VP2 (Noteborn, 2002). Finally, it might be of interest to determine whether the TTV-like mini virus, which is an intermediate between CAV and TTV (Tahahashi *et al.*, 2000; Hino, 2002), harbours apoptotic activity.

TAIP and Apoptin induced p53-independent apoptosis; however, unlike Apoptin, TAIP only induced low levels of apoptosis in non-HCC lines. This apparent contradiction may be explained by a characteristic phenomenon of Apoptin - a tumour-specific phosphorylation site at Thr-108 (Rohn *et al.*, 2002). Asabe *et al.* (2001) described a TTV ORF3 gene, not homologous to TAIP, which generates two variants of a protein with a different serine-phosphorylation state, similar to the hepatitis C virus non-structural protein. It might well be that small TTV-derived proteins derived from this ORF3 gene and possibly also TAIP are regulated by a specific kinase, as is the case for Apoptin. TAIP might be more active in cells that contain such a kinase. To test this, one has to examine the phosphorylation state of TAIP, for example, in HCC versus normal liver cells.

Apoptin has been shown to be a promising anti-cancer gene therapeutic agent in various animal models (Pietersen *et al.*, 1999; Van der Eb *et al.*, 2002). Our initial TAIP studies suggest that TAIP shows potential as a basis for treatment of HCC tumours. Furthermore, TAIP promises to be another agent with which to dissect (disturbed) tumour-specific cell death pathways, as it appears to have some specificity in which tumours it kills preferentially. Finally, the heterogenic nature of TTV, coupled with the potentially different pathogenic sequences, such as TAIP, indicate that it may be of clinical relevance which TTV strain has actually infected the patient.

ACKNOWLEDGEMENTS

This work was supported by grants from the Netherlands Ministry of Economic Affairs and Schering AG, Berlin, Germany.

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