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The p53-induced Wig-1 protein: Studies of interaction partners and expression in tumor cells

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ABSTRACT

The tumor suppressor p53 is a critical regulator of life and death in cells. The p53 protein is present at very low levels under normal conditions but accumulates in response to different stress stimuli, such as DNA damage, hypoxia, and oncogene activation. By acting on its targets p53 can lead the cell into various responses ranging from reversible cell cycle arrest to irreversible cell death or senescence.

The Wig-1 gene (for wild type p53-induced gene 1) was first discovered as a p53-induced gene in J3D mouse T lymphoma cells carrying temperature-sensitive p53. The wig-1 gene encodes a zinc finger protein containing three Cys_2His_2-type zinc fingers and a nuclear localisation signal between the second and the third zinc finger. Human Wig-1 is mapped it to chromosome 3q26.3-27. Wig-1 is highly conserved between man, mouse, rat, chicken, frog and fish, particularly the zinc fingers, which are almost perfectly conserved even between man and fish.

Gel shift assay revealed that human Wig-1 binds a 100 bp dsRNA probe with high affinity compared to ssRNA and DNA-RNA hybrids. We were able to immunoprecipitate dsRNA from Saos-2 cells expressing Tet-regulated Wig-1 using a dsRNA-specific J2 antibody, demonstrating that Wig-1 binds endogenous dsRNA in living cells. However, Wig-1 harboring mutations in either the first or second zinc finger were not able to bind dsRNA. Thus, both the first and the second zinc finger are necessary for binding in living cells. A colony formation assay show that the first and second zinc finger are important for Wig-1-mediated growth inhibition. Interestingly, Wig-1 binds a 22-mer dsRNA with miRNA-like features, but not a control probe with 5' overhangs.

We generated a Saos-2 cell line expressing Flag-tagged human Wig-1 under the control of tetracycline, and showed that Wig-1 does not induce significant cell cycle arrest or apoptosis in these cells. Using these cells we also identified two Wig-1-binding proteins: RNA Helicase A (RHA) and hnRNP A2/B1, both of which are involved in RNA processing at different levels. RNase treatment of the cell extracts abolished the binding between Wig-1, RHA and hnRNP A2/B1, demonstrating that these interactions are dependent on RNA. Wig-1 harboring mutations in the first or second zinc finger did not immunoprecipitate RHA or hnRNP A2/B1, confirming that the interaction occurs via RNA. Finally, knockdown of Wig-1, hnRNP A2/B1 or both of these simultaneously had similar growth inhibitory effect on cells in a WST-1 proliferation assay, suggesting that they are involved in the same pathway.

Gain within the chromosomal region 3q, where Wig-1 is located, is a common feature of cervical carcinoma, and is also linked to the transition from an in situ tumor to invasive carcinoma. To address wether a 3q gain in cervical cancers involves the Wig-1 gene at 3q26, we evaluated eight established cervical cancer cell lines. We could show that Wig-1 is not a main target for the frequent gains and amplifications in 3q seen in cervical cancer cells. However, Wig-1 mRNA and protein levels were found to be relatively lower in HPV positive cervical carcinoma cells independently of p53 protein levels. This suggests that Wig-1 expression is at least in part independent of p53, and raises the interesting possibility that HPV somehow influences Wig-1 expression, directly or indirectly.

In conclusion, this thesis provides important clues to the cellular function of Wig-1, by demonstrating dsRNA binding in cells, identification of the protein partners RHA and hnRNP A2/B1 and revealing a possible correlation between Wig-1 expression and HPV in cervical carcinoma.

LIST OF PUBLICATIONS

I. Cristina Méndez Vidal, **Magdalena Prahl**, Klas G. Wiman
The p53-induced Wig-1 protein binds double-stranded RNAs with structural characteristics of siRNAs and miRNAs.
FEBS Letters, Volume 580, Issue 18, 7 August 2006, Pages 4401-4408

II. **Magdalena Prahl**, Anna Vilborg, Carina Palmberg, Hans Jörnvall, Charlotte Asker, Klas G. Wiman
The p53 target protein Wig-1 binds hnRNP A2/B1 and RNA Helicase A via RNA.
FEBS Letters, Volume 582, Issue 15, 25 June 2008, Pages 2173-2177

III. Srinivasan R. Thoppe, **Magdalena Prahl**, Fredrik Hellborg, Mikael Lerner, Biying Zheng, Nohamin Tamyalew, Svetlana Lagercrantz, Dan Grandér, Klas G. Wiman, Catharina Larsson, Keng Ling Wallin
Association between Wig-1 expression and HPV infection in cervical cancer cells.
Submitted

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ABBREVIATIONS

bp,	Basepairs
6-OHDA,	6-hydroxyldopamine
ADAR,	Adenosine deaminase acting on RNA
ARE,	AU-rich element
ASPP	Apoptosis Stimulating Protein of p53
CDK,	Cyclin Dependent Kinase
CGH,	Comparative genomic hybridization
CLL,	B-cell chronic lymphocytic leukemia
DGCR8,	DiGeorge critical region-8
DRBD,	dsRNA binding domain
dsRNA,	Double stranded RNA
ER,	Endoplasmatic reticulum
hnRNP,	heterogeneous nuclear ribonucleoparticle
HPV,	human papillomavirus
IFN,	interferon
JAZ,	Just Another Zinc finger protein
kb,	Kilobase
kDa,	Kilodalton
MDM-2,	Mouse double minute 2
mRNA,	Messenger RNA
miRNA,	Micro RNA
NLS,	nuclear localisation signal
RHA,	RNA Helicase A
RISC,	RNA-induced silencing complex
siRNA,	Small interfering RNA
ssRNA,	Single stranded RNA
Tet,	Tetracycline
Wig-1,	Wild type p53-induced gene 1
ZF,	Zinc finger

INTRODUCTION

Cancer has existed for a very long time. Cancer was found in fossilised bones and manuscripts of ancient Egypt, and the oldest cancer specimen is dated back to the Bronze Age (1900-1600 BC). However, it is only in our lifetime that we have gained any deeper insight into the nature of cancer.

In order to keep the integrity of a multicellular organism, cells are kept under tight control with regard to growth and survival. Cancer arises when the control fails, by mutation of essential genes. The loss of control is regarded as a multi-step process in which the cell acquires several abilities ultimately leading to autonomous and uncontrolled growth, thus transforming a normal cell into a cancer cell [1]. Six essential alterations in cell physiology are shared by most, if not all, malignant cells: Self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, angiogenesis and metastasis [1]. Cancer research is in many aspects focused on the identification and characterisation of genes causally implicated in cancer, “cancer genes”. [2]

The two main types of genes recognized as playing a role in cancer are *oncogenes* and *tumor suppressor genes*. *Oncogenes* are mutated forms of genes that normally drive cell proliferation. They result from a "gain of function" mutation resulting in their overexpression or unregulated activity. *Tumor suppressor genes* are cellular genes that normally suppress cell growth and proliferation, and are inactivated in cancer. One important cancer gene is the tumor suppressor p53, which is mutated in around half of all human tumors [3].

p53

The tumor suppressor p53 is the center of a complex network that will lead the cell either into cell cycle arrest, apoptosis or senescence, depending on the cellular context and inducing agent (Fig.1). The p53 protein was first discovered in 1979 [4], and since then it has become one of the most extensively studies genes of all times. Its importance is most of all demonstrated by the high frequency of p53 mutations in various types of cancers (www.iarc.fr/p53).

The p53 protein acts as a transcription factor with sequence-specific DNA-binding activity [5]. Bioinformatic studies suggest more than 4000 targets; however the number should be closer to 500-1600 as indicated by studies using chromatin-immunoprecipitation-based techniques [6,7]. By acting on these targets p53 can lead the cell into various responses ranging from reversible cell cycle arrest to irreversible cell death or senescence. The cellular outcome usually depends on the severity of the damage and the cell type.

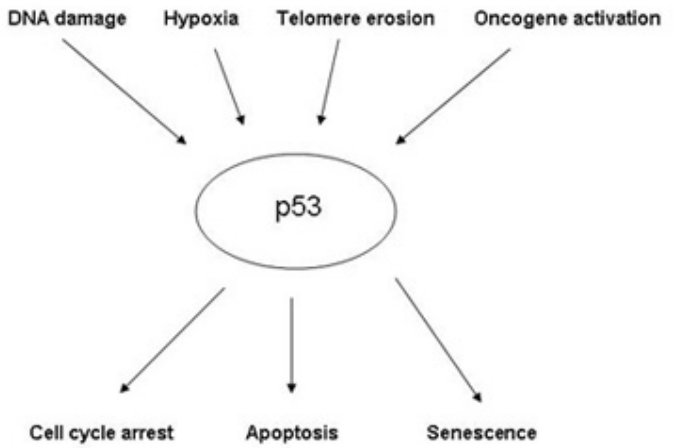

Figure 1. The p53 pathway

p53 regulation and activation

Under normal conditions, the p53 protein levels are quite low due to tight regulation, the most well-known regulator being MDM-2 (mouse double minute 2) [8]. MDM-2 regulates p53 in two ways: (1) MDM-2 binds directly to p53, thereby blocking the transcription activation domain [9], and (2) MDM-2 acts as an E3 ubiquitin ligase, attaching ubiquitin molecules to p53 that act as a signal for degradation by the proteasome [10]. A recent study using knock-out mice carrying a mutation in the RING finger E3 ligase domain of MDM2 reveals that binding to p53 alone is not sufficient to regulate p53 in vivo, suggesting that regulation of p53 by MDM2 occurs mainly by ubiquitylation [11]. Other ubiquitin ligases such as Pirh2 and COP1 have been reported to target p53, however MDM2 is still considered to be the main regulator of p53 [12,13].

When the cell is subject to various stress signals such as DNA damage, hypoxia, or oncogene activation p53 accumulates in the nucleus where it forms tetrameres and is able to act as a transcription factor [14]. Different types of stressors give rise to specific patterns of modifications in the p53 protein. This occurs by way of protein sensors acting as mediators that activate p53 by posttranscriptional modifications resulting in phosphorylation, acetylation, methylation, ubiquitination or sumoylation [15]. These modifications affect the stability and transactivation activity of p53. For instance, phosphorylation of p53 at serine 15 by DNA-PK prevents MDM2 binding and subsequently p53 degradation [16].

Outcomes of p53 activation

How can it be that activation of p53 can leads to such diverse outcomes as either survival cell cycle arrest, DNA repair or apoptosis? And how is the choice made? Promoter selection is crucial in determining the response to p53. Differences in the sequence and spacing of the p53-binding site, the post-transcriptional modifications of p53 and the presence of transcriptional cofactors highly influence the promoter selection and type of response [17].

For example, DNA-damage signals activate kinases such as HIPK2 that phosphorylate p53 at serine 46, and this phosphorylated form is more effective in activating various pro-apoptotic gees such as Noxa, Pidd and Puma [18,19].

The ASPP family (Apoptosis Stimulating Protein of p53) bind and regulate activities of p53. There are three members of this family, all of them encoded by three different genes located on three different chromosomes. ASPP1 and ASPP2 specifically enhance the induction of apoptosis but not cell cycle arrest by p53 [20]. iASSP, on the other hand, binds to p53 and inhibits p53-mediated apoptosis.

Cell cycle arrest

Checkpoint controls throughout the cell cycle ensure that the cell is fit for cell division. This involves detecting and reparing DNA damage. If the DNA is damaged, the cell can go into reversible cell cycle arrest at the G1/S or G2/M transitions. p53 can block cell cycle progression at both these transitions.

P21 is a CDK (Cyclin Dependent Kinase) inhibitor that is induced by p53. p21 binds and inactivates kinases, resulting in Rb hypophosporylation, and cell cycle arrest. Rb negatively regulates the E2F transcription factor, which is a specific activator of S-phase genes. p21 acts on kinases both in G1 and in G2 [21].

The 14-3-3 sigma gene is another p53-induced protein that blocks cells in G2 [22] by inhibiting translocation of cyclin B/CDK1 to the nucleus, thus blocking initiation of mitosis [23]. GADD45 is a p53 target that binds cdc2 and prevents the formation of cyclin B/cdc2 complexes [24].

Apoptosis

When a cell is badly damaged it can pose a threat to the organism. In this case the cell is usually eradicated through activation of an apoptotic cell death program.
Several different cell death pathways can be activated by p53, i.e. the death receptor pathway, the mitochondrial pathway and the endoplasmatic reticulum (ER) pathway.

The death receptor KILLER/DR5 is transcriptionally upregulated by p53 following DNA damage [25], rendering the cell more sensitive to death-receptor ligands. This pathway can activate caspase-8, which can either directly activate effector caspases or cleavage of Bid, which is a negative regulator of the pro-apoptotic Bax protein, in both cases leading to apoptosis [26]

p53 activates transcription of several mitochondrial pro-apoptotic Bcl-2 family members like Bax, Puma and Noxa [27-29]. Bax translocates from the cytoplasm to mitochondria upon activation, thus facilitating the release of cytochrome c. This release enables cytochrome c to form the apoptosome together with Apaf-1 and caspase 9, which leads to activation of effector caspases 3 and 6 and ultimately apoptosis.[26]

p53 can also transrepress a number of anti-apoptotic genes, such as Bcl-2, Bcl-xl and survivin [30-32] Furthermore, p53 can enchance apoptosis by transcription

independent mechanisms. A fraction of induced p53 translocates to the mitochondrial outer membrane early during p53-dependent apoptosis [33]. Moreover, p53 forms complexes with Bcl-xl and Bcl-2, antagonizing the membrane stabilising function of these proteins [34].

Aging and Senescence

Cellular senescence is generally defined as an irreversible state of G1 cell cycle arrest in which cells are insensitive to growth factor stimulation. Replicative senescence is mainly due to shortening of the telomeres, which are protective caps at the end of all eukaryotic chromosomes. The enzyme that maintains telomeres, telomerase, is expressed mainly in stem cells and is absent in somatic cells. Ectopic expression of telomerase in somatic cells results in telomere elongation and abrogation of replicative senescence. [35]

Severe DNA damage activates p53 and usually leads to either apoptosis or senescence. p53 induces senescence via activation of p21. Strong transient expression of p21 induces senescence and subsequently p16 is constituively upregulated to maintain the growth arrest of the senescent cell [36]

Telomere length and aging are inversely correlated: Telomere shortening is a part of the aging process. Several studies in mice indicate that there is a delicate balance between tumor suppressive and age-promoting functions of p53. Telomerase-deficient mice have a decreased life span, show decreased body weight, increased skin lesions and delayed wound healing [37]. Interestingly, some of these phenotypes are rescued by p53 deficiency [38], indicating that p53 contributes to premature aging when activated by short telomeres.

The p53 family

As most cellular proteins, p53 is part of a protein family. The p63 and p73 genes were first discovered in 1997-98 [39,40] p53, p63 and p73 show high similarity in the DNA-binding domain, which allows p63 and p73 to transactivate p53 targets that cause cell-cycle arrest and apoptosis. However, these three proteins are not functionally redundant, as each family member shows distinct phenotypes when knocked out in mice. Mice lacking p63 or p73 predominantly display developmental defect in contrast to p53 knockout mice that show high susceptibility to various tumors [41-43]

p53 in cancer

Around half of all human tumors carry mutated p53, and it is believed that in tumors carrying wild-type p53, the p53 pathway is disrupted by other mechanisms. Inactivation of p53 in tumors mainly occurs through missense mutations, most commonly in the DNA binding domain. The prevalence of p53 mutations varies depending on the type of cancer. Mutations in p53 are quite rare in malignant melanoma, but common in for instance in ovarian and colorectal cancer. (www.iarc.fr/p53)

Wig-1

The Wig-1 gene (for wild type p53-induced gene 1) was discovered in1997 as a p53-induced gene in J3D mouse T lymphoma cells carrying temperature-sensitive p53 [44] The rat homologue, PAG608 was independently identified by Israeli et al [45]. Subsequently the human ortholog of Wig-1 was cloned and mapped to chromosome 3q26.3-27 [46]. Wig-1 is induced upon p53 activation with kinetics similar to those of the classical p53 target p21 and has several p53 DNA binding motifs in its promoter [47], demonstrating that it is a bona fide p53 target gene. Wig-1 is induced in several mouse tissues following whole body irradiation but is also expressed at significant levels in untreated mouse brain, indicating p53-independent expression [44]. Exogenous Wig-1 localises to the nucleus, and in some cells to the nucleolus [46,48], as does endogenous Wig-1 [49].

Wig-1 structure

Wig-1 is a 290/288 (mouse/human) amino protein with an apparent molecular weight of 32 kDa (Fig. 2) [44,46].Wig-1 is a zinc finger protein containing three Cys_2His_2-type zinc fingers and a nuclear localisation signal (NLS) between the second and the third zinc finger. The structure of Wig-1 differs from that of most zinc finger proteins by the long linkers (56-75 aa instead of 8) between the zinc fingers and the unusually long spacing between the histidines in each zinc finger (5 aa instead of 2 to 4). Wig-1 is highly conserved between man, mouse, rat, chicken, frog and fish, particularly the zinc fingers, which are almost perfectly conserved even between man and fish [50].

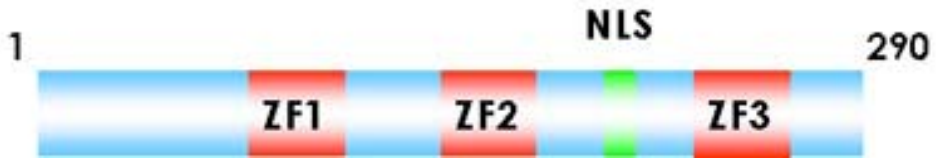

Figure 2. Wig-1 Structure

Wig-1 nucleic acid binding properties

Studies of mouse Wig-1 reveal that it binds double stranded RNA (dsRNA) with high affinity in vitro and in living cells [48]. GST-Wig-1 was able to bind dsRNA in a gel shift assay. The binding could be competed out with cold dsRNA probe, but not a ssRNA probe. Mutant Wig-1 lacking the first zinc finger domain or the first and the second zinc fingers failed to bind dsRNA, whereas mutants lacking only the second or the third zinc finger retained dsRNA binding. This suggests that the first zinc finger of Wig-1 is necessary for its binding to dsRNA. Furthermore, Flag-Wig-1 expressed in NIH3T3 cells could bind poly(I-C) in an immunoprecipitation experiment, while Flag-Wig-1 lacking the first zinc finger domain was unable to bind.

Wig-1 function

Ectopic expression of Wig-1 does not induce cell cycle arrest or massive apoptosis but inhibits colony formation and cell growth over longer time periods [46].
Several reports implicate the rat ortholog of Wig-1, PAG608 in neuronal cell death following ischaemia. [51-53].

Moreover, p53 and Wig-1/PAG608 expression was induced by 6-hydroxyldopamine (6-OHDA) treatment in cathecolaminergic neuronal cell lines [54] Transient transfection of Wig-1/PAG608 induced p53 expression in PC12 cells and mutant Wig-1/PAG608, lacking the first zinc finger domain could inhibit 6-OHDA induced cell death[54].

Furthermore, Wig-1 has been implicated in the regulation of stem cell proliferation, survival and differentiation. Wig-1 is enriched in embryonic, neural and haematopoietic stem cells [55]. Bmi-1 is a transcriptional repressor that controls development by the regulation of cell growth and differentiation genes. Bmi-1 deficient mice develop hypocellular bones and die due to no self-renewal of haematopoietic stem cells. Interestingly, Wig-1 mRNA was upregulated in haematopoietic stem cells lacking Bmi-1. [56]

Finally, Wig-1 knock out mice are embryonic lethal. Wig-1 null embryos die before embryonic day 12.5 (Wilhelm, unpublished results), demonstrating that Wig-1 has a crucial role in embryonic development.

In conclusion, despite extensive research, the cellular function of Wig-1 is basically still unknown. Hopefully, the clues presented here, together with future efforts, will lead us in the right direction.

Proteins with structural similarities to Wig-1

The unusual zinc finger structure in Wig-1 is shared with a small group of dsRNA-binding proteins, i.e. dsRBP-Zfa, JAZ and Hzf (Just Another Zinc finger protein) [57,58].

dsRBP-Zfa is nuclear protein with unknown function that was originally identified in Xenopus [58]. The zinc finger domains in JAZ are not only essential for dsRNA binding but are also required for its nucleolar localization. JAZ has been shown to positively regulate p53 transcriptional activity, thus inducing G1 cell cycle arrest and apoptosis [59].

Another protein with structural similarities to Wig-1 is Hzf. Interestingly, Hzf is a also upregulated by p53 [60]. Upon induction by p53, Hzf binds to the DNA binding domain in p53 and can recruit p53 to the promoters of its cell-cycle-arrest target genes rather than to its pro-apoptotic target genes[61].

dsRNA and dsRNA-binding proteins

Double stranded RNA (dsRNA) can either be exogenous, in the form of replicating viruses or endogenous, such as microRNA (miRNA), hnRNA and primary ribosomal RNA. Many RNA species contain some form of dsRNA structure such as loops or bulges at one or more stages in their life. The largest group of dsRNA-binding proteins are the DRBPs, which contain one or several evolutionarily conserved dsRNA binding domains (DRBD) of 65-68 amino acids. DRBPs are not sequence specific, but interact with A-form double helix RNAs. [62] Since DRBPs do not bind to a specific RNA sequence, they can have very diverse cellular functions and can influence distinct cellular processes including cell growth control, gene expression and RNA localisation.

Interferon response to dsRNA

Cells respond to dsRNA by activating the interferon (IFN) system, as a part of an innate immune response [63] In response to dsRNA, the IFN-inducible protein kinase PKR changes conformation and can phosphorylate the alpha subunit of eukaryotic intiation factor 2 (eIF-2α) and other substrates, thus inhibiting protein translation [64]

RNA editing - ADARs

ADARs (adenosine deaminase acting on RNA) is a family of RNA editing enzymes that substitute (A) by (I) in cellular mRNA and viral dsRNA targets [65] Deamination of A to I results in unstable RNA products prone to strand separation [66]. In the case of viral RNA, the end result may be removal of the dsRNA by endonucleases that recognise hyperedited dsRNA species [67].

hn RNP A2/B1

hnRNPs (heterogeneous nuclear ribonucleoparticles) are a group of proteins that form complexes with pre-mRNAs in the nucleus. They shuttle between the nucleus and the cytoplasm. In the nucleus, hnRNPs participate in processes such as transcriptional regulation, maintenance of telomere length, alternative pre-mRNA splicing and pre-mRNA 3' end processing. In the cytoplasm, hnRNPs can regulate mRNA localization, translation and turnover [68].

hnRNP A2 (36 KDa) and B1 (38 KDa) are derived from two transcripts from the same gene and differ by only 12 amino acids, due to the presence of exon 2 in the B1 transcript. [69]. Several studies have suggested that hnRNP A2/B1 have a function in telomere binding, the B1 transcripts having a higher affinity [70-72]. Furthermore, targeting of hnRNP A2/B1 promotes cell death in transformed cells but not in mortal cells [73], also supporting a role for these proteins in telomere maintenance. The levels of hnRNPs A1, A2 and B1 were found to be modulated specifically during the cell cycle of Colo16 cells, and furthermore, suppression of hnRNP A2 caused a non-apoptotic inhibition of cell proliferation [74]. Another report showed that hnRNP B1 interacts with the DNA-dependent protein kinase (DNA-PK) complex and in fact could inhibit this complex, thus slowing down DNA repair and possibly stimulating tumor progression [75].

hnRNP A2 binds AU-rich elements (AREs) in the 3'UTR of mRNAs [76,77]. These elements are known to target mRNAs for degradation by shortening of the poly(A) tail, and are commonly present in mRNAs that are subject to rapid degradation, like cytokines, cyclins and proteins involved in cell proliferation, such as p53 [78].

hnRNP A2/B1 overexpression is an early marker for several cancer types, and a recent microarray study reveals that many of its downstream targets are involved in regulation of the cell cycle and cell proliferation [79].

RNA Helicase A

RNA Helicase A (RHA, DHX9, Nuclear DNA Helicase II (NDHII)) is a 142 KDa nuclear helicase (reviewed in [80]), that unwinds dsRNA in a 3' to 5' direction. RHA is involved in diverse cellular processes such as transcription regulation and RNA processing at different levels.

In the nucleus, RHA has been shown to bind CBP [81] and NF-κB [82], thus stimulating transcription. One report provides evidence that RHA can be recruited to PML nuclear bodies upon IFNα treatment [83], further supporting a transcriptional role for the RHA protein. Another function for RHA involves ADAR. RHA acts together with this enzyme to coordinate editing and splicing of the glutamate receptor pre-mRNA [84]. Intriguingly, RHA is necessary for translation of selected mRNAs by recognising a 5' post-transcriptional control element [85]. Similarly to hnRNPs, RHA can also be found in the cytoplasm. Following transcriptional inhibition RHA was translocated to the cytoplasm and was demonstrated to bind mRNAs as well as pre-mRNAs [86]. Furthermore, RHA can interact with siRNA, Ago2, TRPB and Dicer, thus functioning in the RNA interference pathway [87].

RNA interference

RNA interference is maybe the most exciting discovery of this decade, having huge impact on many research fields. RNA interference is a process where short dsRNA structures induce homology-dependent gene silencing at the post-transcriptional level [88]. The so-called miRNA (micro RNA) are expressed from intergenic regions (60%) or introns of protein-coding genes or other transcriptional units [89] and there are currently over 500 reported miRNA in the human genome [90]. miRNAs are crucial for a number of cellular functions including development, cell proliferation, differentiation, oncogenesis and immune function.

Mechanism

miRNA (micro RNA) are transcribed as 5' capped and polyadenylated pri-miRNAs which can be several kilobases long. In mammalian cells pri-miRNA are cleaved by the RNase III enzyme Drosha in complex with DGCR8 (DiGeorge critical region-8) into 65-75 nt stem-loop structures with two-base 3' overhangs and 5'- phosphate groups [88]. The resulting pre-miRNA is translocated from the nucleus to the cytoplasm by the RanGTP- dependent exportin-5 protein [91]. In the cytoplasm, these precursors are processed into 21-23 nucleotide long dsRNA structures by ATP-dependent cleavage by the RNase III enzyme Dicer [92].

After cleavage by Dicer, the dsRNA is unwound by helicases and one strand is loaded onto Ago2 [93]. This is the mature miRNA, which is antisense to its target. The relative stability of the 5' or 3' end determines the selection of the guide strand [94]. At this point Dicer and TRBP leave the complex and other proteins associate with the Ago2-miRNA complex to form the RISC (RNA-induced silencing complex). The gene silencing can now occur by different mechanisms depending on the level of complementarity to the target mRNA. In case of perfect complementarity, Ago2 cleaves the target. Incomplete complementarity results in translational inhibition either blocking the interaction between eIF4E and the 5' cap of the mRNA or dissociation from the ribosome or decapping or deadenylation of the mRNA by unknown mechanism [88].

miRNA targets

The miRNA targets are usually the 3'UTR of mRNAs, a single miRNA may bind as many as 200 different targets. Target recognition by miRNA is quite complex, although there are some common principles. Usually the 5' region of the miRNA is perfectly complementary to the target sequence, a 7-8 base pair region starting from either the first or the second base at the 5' end of the miRNA is referred to as the "seed region". Base pairing at the 3' end of the miRNA appears to be weaker [95].
Several attempts have been made to develop algorithms that will prevent such targets. The experimental validation is still lagging behind, making it difficult to assess the accuracy of these algorithms [96].

miRNA in cancer

Around 50% of all identified human miRNAs are located in fragile sites, in minimal regions of loss of heterozygocity, minimal regions of amplifications and common breakpoints associated with cancer [97]. This indicates that miRNAs have a critical role in cancer progression, and can act both as tumor suppressors and as oncogenes.

The two clustered miRNA genes, *mir-15a* adn *mir-16-1* are often deleted or downregulated in B-cell chronic lymphocytic leukemia (CLL) [98]. *miR-15a* and *miR-16-1* were shown to negatively regulate Bcl-2 [99], the deletion of these miRNA should therefore result in increased expression of Bcl-2, thus promoting tumor cell growth.

The oncogenic *miR-155* is overexpressed in diverse tumor types including B-cell lymphoma, breast, lung, colon and thyroid cancers [100]. Furthermore, transgenic mice with enhanced expression of *miR-155* rapidly develop B-cell malignancy [101].

Recently, it was shown that *miR-34a* is significantly induced by p53 [102] *miR-34a* is directly transactivated by p53 and expression promotes apoptosis. The *miR-34* family has three members, all of which are induced by p53, although *miR34a* is the most significantly induced, leading to cell cycle arrest, apoposis or senescence [103]. The mechanism of action is still unclear, although a recent report identified MYCN as a direct target of *miR-34a* [104]. MYCN is an oncogene that is commonly amplified in neuroblastoma and correlates with poor prognosis [105]

Cervical cancer and HPV

Cervical cancer is the second most common cancer in women worldwide[106], The importance of human papillomavirus (HPV) in the etiology of cervical cancer is well established. Persistent HPV infection causes almost all cases of cervical carcinoma. Data pooled from 11 case-control studies involving nine countries showed that >90% of cervical cancers carry HPV DNA as compared to 13% among the controls [107]. HPV16 and HPV18 are the two most carcinogenic types, being responsible for 70% of cervical cancers [108].

Several small DNA tumor viruses like SV40, andenovirus and HPV have developed protiens that bind and block two central tumor suppressor genes, namely p53 and Rb. The virus first inactivates Rb, allowing the infected cell to enter S-phase and replicate viral DNA, and at the same time p53 is blocked to prevent the cell from entering p53 induced apoptosis as a response to the Rb-inactivation. Later when the cell is full of new virus particles, it will die. When this lytic cycle is somehow distrupted, cancer can occur.

The E6 and E7 proteins are the main oncoproteins of HPV. E6 binds to p53 and blocks p53-dependent apoptosis, while E7 binds to Rb and disrupts its complex formation with E2F transcription factors. [109-112] Normally, Rb and p53 regulate cell growth and survival. Therefore, their mutational or functional inactivation contributes to loss of cell cycle control and evasion from apoptosis, thus allowing cells to enter into continuous proliferation and ultimately into immortalization or transformation [113]. HPV-positive cervical cancers often carry wild-type p53 and Rb genes, but the proteins products are blocked by the viral oncoproteins E6 and E7, respectively. However, in HPV negative cervical cancers both p53 and RB are commonly mutated [114].

AIM OF THE THESIS

The general aim of the thesis is to further characterise the nucleic acid binding properties of Wig-1 and to investigate the protein function by searching for protein partners and studying expression in tumor cells.

SPECIFIC AIMS:

Paper I: To characterise the nucleic acid binding properties of human Wig-1, both in vitro and in living cells.

Paper II: To study the effects of Wig-1 on cell growth and survival and to identify protein interaction partners of Wig-1.

Paper III: To investigate the possible association between HPV, cervical cancer and Wig-1 on DNA, RNA and protein level.

RESULTS AND DISCUSSION

Paper I: The p53-induced Wig-1 protein binds double-stranded RNAs with structural characteristics of siRNAs and miRNAs

Wig-1 is a Zinc finger protein with an atypical structure. It has unusually long linkers (56-75 residues instead of 8) between the zinc fingers and long spacing between the histidines in each zinc finger (5 residues instead of 2 to 4). These structural features are shared with a small group of dsRNA-binding proteins, i.e. dsRBP-ZFa and JAZ, which lack consensus dsRNA-binding domains. dsRBP-ZFa is a Xenopus protein with unknown function [58]. JAZ has been shown to induce apoptosis in NIH 3T3 cells when overexpressed [57], and to positively regulate p53 transcriptional activity by directly binding to the p53 protein [59]. Additionally, JAZ binds Exportin-5 [115], which is a nuclear export receptor for certain classes of dsRNA.

We show by gel shift assay that human Wig-1 binds dsRNA with high affinity compared to ssRNA and DNA-RNA hybrids. We generated a number of zinc finger mutants, both point mutants and deletion mutants, and demonstrated that, as for mouse Wig-1 mouse Wig-1 [48], the first zinc finger is necessary but not sufficient for dsRNA binding in vitro.

We immunoprecipitated dsRNA from Saos-2 cells expressing Tet-regulated Wig-1 using a dsRNA-specific J2 antibody, demonstrating that Wig-1 binds endogenous dsRNA in living cells. Furthermore, we were not able to immunoprecipitate Wig-1 harboring mutations in either the first or second zinc finger. Thus, both the first and the second zinc finger are necessary for binding in living cells.

Next we studied the lenght requierments of the binding. Wig-1 binds a 100-mer and a 50-mer dsRNA probe with the same affinity. Interestingly, Wig-1 binds a 22-mer dsRNA with siRNA-like features, but not a control probe with 5’ overhangs. This binding seems to be sequence independent.

In a colony formation assay in Saos-2 cells, using wild type Wig-1 and two mutants with amino acid substitutions in zinc finger 1 and 2, wild type Wig-1 could inhibit colony formation by approximately 50% and both mutants were impaired in this respect. This demonstrates that the first and second zinc finger are important for Wig-1-mediated growth inhibition.

These data indicate that Wig-1 is involved in miRNA-mediated regulation of cell growth and survival.

Paper II: The p53 target protein Wig-1 binds hnRNP A2/B1 and RNA Helicase A via RNA

The p53 tumor suppressor is a transcription factor that regulates cell fate in response to cellular stress. Activation of p53 can trigger reversible cell cycle arrest or irreversible responses such as apoptosis or senescence, all depending on cellular context and inducing agent. This raises the question whether Wig-1 acts as a downstream effector in one or more of these processes.

To further investigate the function of Wig-1 in cells we generated a Saos-2 cell line expressing Flag-tagged human Wig-1 under the control of tetracycline. Induction of Wig-1 expression by addition of doxycyline caused a significant growth inhibition over a 5 day time period, as determined by the WST-1 proliferation assay. When these cells were cultured for up to 14 days in the presence or absence of Wig-1, no difference in cell cycle distribution or sub G1 population was detected by flow cytometry, indicating that Wig-1 does not induce significant cell cycle arrest or apoptosis.

To gain further clues as to the function of Wig-1 we studied Wig-1-interacting proteins using the Saos-2 Tet-on-wig-1 cells. Wig-1 was induced by doxycycline, immunoprecipitated using a Flag antibody and separated on an SDS-PAGE gel. Coomassie staining revealed several co-precipitating bands. The two most prominent bands, with apparent molecular weights of 140 kD and 36 kD, were cut out, digested with trypsin and analysed by MALDI-mass spectrometry. The resulting peptides were run through a peptide database. The two Wig-1-binding proteins were thus identified as RNA Helicase A (RHA) and hnRNP A2/B1.

Both of these proteins are involved in RNA processing at different levels. hnRNP A2 (36 kDa) and B1 (38 kDa) are derived from two transcripts from the same gene and differ by only 12 amino acids, due to the presence of exon 2 in the B1 transcript. [69]. Targeting of hnRNP A2/B1 promotes cell death in transformed cells but not in mortal cells [73]. The levels of hnRNPs A2 and B1 were found to be modulated specifically during the cell cycle of Colo16 cells, and furthermore, suppression of hnRNP A2 caused a non-apoptotic inhibition of cell proliferation [74].

RNA Helicase A (RHA, Nuclear DNA Helicase II (NDHII)) is a 142 kDa nuclear helicase (reviewed in [80]) that is involved in diverse cellular processes such as transcriptional regulation and RNA processing at different levels. In the nucleus, RHA has been shown to bind to several proteins involved in transcription, such as CREB binding protein (CBP) [81], the breast cancer-specific tumor suppressor BRCA1[116] and NF-κB [82], thereby stimulating their functions. Following transcriptional inhibition RHA is translocated to the cytoplasm where it binds mRNAs as well as pre-mRNAs [86].

We have confirmed the interactions by Western blotting with specific antibodies against RHA or hnRNPA2/B1. RNase treatment of the cell extracts abolished the binding, demonstrating that these interactions are dependent on RNA. Furthermore, co-immunoprecipiation experiments revealed that hnRNP A2/B1 and RNA Helicase A interact in HEK293 cells and that this interaction is also dependent on RNA.

Since the first and the second zinc finger domains of Wig-1 are necessary for binding to RNA in living cells, we tested wether Wig-1 point mutated in either of the zinc fingers could bind hnRNP A2/B1 and/or RHA. Neither of the mutant co-immunoprecipitated with RHA or hnRNP A2/B1. This confirms that the interaction between these three proteins occurs via RNA.

Knockdown of Wig-1, hnRNP A2/B1 or both of these simultaneously by siRNA in HCT116 colon carcinoma cells had similar growth inhibitory effect on cells in a WST-1 proliferation assay, suggesting that they are involved in the same pathway.

These results provide a link between the p53 tumor suppressor pathway and RNA processing via hnRNP A2/B1 and RNA Helicase A.

Paper III: Association between Wig-1 expression and HPV infection in cervical cancer cells

Cervical cancer is the second most common cancer affecting women worldwide, and in developing nations it is the most common [106]. The importance of human papillomavirus (HPV) in the etiology of cervical cancer is well established. In one report, data pooled from 11 case-controls studies involving nine countries reported that >90% of cervical cancers carry HPV DNA as compared to 13% among the controls [107].

The human Wig-1 gene is located in 3q26.32 [46] and encodes a 290 amino acid nuclear zinc finger protein that is highly conserved from fish to man [50]. Ectopically expressed Wig-1 inhibits colony formation of tumor cells, suggesting that it has tumor suppressor properties [46]. On the other hand, the observation that Wig-1 is amplified and over-expressed in squamous cell carcinoma cells, is consistent with an oncogenic function [117]

Gain within the chromosomal region 3q, where Wig-1 is located, is a common feature of cervical carcinoma, and is also linked to the transition from an in situ tumor to invasive carcinoma [118,119]. To address wether a 3q gain in cervical cancers involves the Wig-1 gene in 3q26, we have evaluated eight established cervical cancer cell lines (Ca Ski, ME-180, MS751, SiHa, SW 756, C-4I, C-33A, and HT-3) and an osteosarcoma cell line (Saos2) for cytogenetic alterations focusing on regional alterations encompassing 3q in relation to the Wig-1 gene locus. Furthermore, Wig-1 gene status and and its expression were determined in relation to the HPV status of these cell lines.

The CGH profiles and Wig-1 locus copy number studies showed that Wig-1 is not a main target for the frequent gains and amplifications in 3q seen in cervical cancer cells. This is well illustrated by the fact that Wig-1 is located telomeric of the commonly amplified 3q23-24 interval in MS751 and SiHa, as well as centromeric of the amplified 3q27-ter interval in ME-180. Similarly, these 3q amplifications detected by CGH were not accompanied by Wig-1 locus amplifications as determined by Southern or FISH.

All cell lines in this study lack functional p53, due to either degradation by E6 or inactivating p53 mutations [111,120-123]. We have confirmed the expression pattern of p53 by Western blot. When compared to the expression of Wig-1, an association between HPV infection status and mRNA expression level of Wig-1 was evident. Using quantitative RT-PCR the Wig-1 expression appeared to be higher in the three HPV negative cell lines (mean 7.8, range: 5.5 - 11.8) than in the HPV positive lines (mean 4.1, range: 2.7 – 5.9) (Table 2). The same three lines showed strong Wig-1 mRNA expression by Northern blotting. In contrast the HPV positive cells only exhibited detectable /moderate or no Wig-1 expression by Northern blot analysis. Similar expression pattern was seen at the protein level, where Wig-1 protein levels were higher in the HPV negative cell lines C33-A and Saos2, although not in HT3,

compared to the HPV positive cell lines. Furthermore, the expression pattern of Wig-1 did not correlate with p53 protein levels, which were high in C33-A and HT-3 carrying mutant p53 and absent in the p53 null Saos2 line.

Wig-1 mRNA and protein levels were found to be relatively lower in HPV positive cervical carcinoma cells. This suggests that Wig-1 expression is also controlled by p53-independent mechanisms, and raise the interesting possibility that HPV somehow influences Wig-1 expression, directly or indirectly. However, we found elevated expression of Wig-1 mRNA and/or protein in most of the cell lines tested. This finding and the observed difference in mRNA levels between HPV negative and HPV positive cell lines irrespective of functional p53 indicates that Wig-1 expression is at lest in part independent of p53.

CONCLUSIONS

Paper I: The p53-induced Wig-1 protein binds double-stranded RNAs with structural characteristics of siRNAs and miRNAs

- Human Wig-1 binds dsRNA with high affinity compared to ssRNA and DNA-RNA hybrids.
- Wig-1 binds endogenous dsRNA in living cells.
- Both the first and the second zinc finger are necessary for binding to dsRNA in living cells.
- Wig-1 binds a 22-mer dsRNA with miRNA-like features.
- The first and second zinc finger are important for Wig-1-mediated growth inhibition.

Paper II: The p53 target protein Wig-1 binds hnRNP A2/B1 and RNA Helicase A via RNA

- Wig-1 does not induce significant cell cycle arrest or apoptosis.
- Wig-1 binds RNA Helicase A (RHA) and hnRNP A2/B1 in living cells and these interactions are dependent on RNA.
- hnRNP A2/B1 and RNA Helicase A interact via RNA in HEK293 cells.
- Wig-1 point mutated in either the first or the second zinc finger could bind not hnRNP A2/B1 and/or RHA.
- Knockdown of Wig-1, hnRNP A2/B1 or both of these simultaneously had similar growth inhibitory effect on cells in a WST-1 proliferation assay, suggesting that they are involved in the same pathway.

Paper III: Association between Wig-1 expression and HPV infection in cervical cancer cells

- Wig-1 is not a main target for the frequent gains and amplifications in 3q seen in cervical cancer cells.
- Wig-1 mRNA levels were found to be relatively lower in HPV positive compared to HPV negative cervical carcinoma cells.

- Wig-1 protein levels were found to be relatively lower in HPV positive compared to HPV negative cervical carcinoma cells.

- Wig-1 mRNA and protein expression was upregulated in several cervical cancer cell lines.

FUTURE PERSPECTIVES

This thesis provides important clues to the cellular function of Wig-1, by demonstrating dsRNA binding in cells, identification of the protein partners RHA and hnRNP A2/B1 and revealing a possible correlation between Wig-1 expression and HPV in cervical carcinoma. We provide a link between p53 and RNA processing, which could have a huge impact on the p53 field. What is the biological relevance of the interaction between Wig-1, hnRNP A2/B1 and RNA Helicase A? Which (groups of) RNA does Wig-1 bind in living cells? These are important questions for future research in the Wig-1 field. When answered, they should most probably reveal the true function of Wig-1.

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