Abstract. Medulloblastoma is the most common malignant brain tumor in childhood. The most prevalent chromosomal abnormalities are isochromosome 17q and loss of 17p, the location of the tumor suppressor gene p53. Mutations in the p53 gene in medulloblastoma are relatively infrequent but have recently been correlated to poor prognosis. Furthermore, the p53 gene encodes nine different isoforms, which may have a profound impact on p53 tumor suppressor activity. Nine medulloblastoma primary biopsy samples, six cell lines from medulloblastoma, and one from a supratentorial PNET, and a medulloblastoma xenograft, along with human brain and visceral tissues, were analyzed by Western blotting, using monoclonal p53 antibodies against two regions in the N-terminus or the central domain. Medulloblastoma primary tissue and xenografts present low molecular weight proteins recognized by both N-terminal p53 antibodies that are absent in all cell lines including the one used for xenografts. Normal visceral organs display short forms of p53, and low levels of canonical p53. Normal brain structures, including cerebellum, contained only canonical size p53 at high levels. In conclusion, our results indicate that the presence of p53 isoforms may play a functional role in medulloblastoma. The observed differences in their presence in cell lines and derived xenografts, suggest that p53 should be investigated in in vivo models rather than in cell lines.

Introduction

Medulloblastoma (MB) is the most common malignant brain tumor in children. The tumor, and the current treatment modalities, in particular surgery and irradiation (frequently in combination with chemotherapy), cause adverse effects to the majority of long-term survivors. MB therefore represents an important therapeutic challenge which requires novel approaches based on current biological knowledge.

The WHO classifies 5 different variants of MB based on histopathological characteristics (1). The treatment of MB is performed by surgical resection, craniospinal radiation and chemotherapy. The 5-year progression-free survival after treatment is around 80% (2). However, even if the morbidity due to the aggressive therapy gradually has been reduced, it is still a substantial problem (3).

Together with clinical factors (e.g. subtotal tumor resection) there are molecular features of the tumor, like tyrosine kinase receptor ERBB2 overexpression and loss of genetic material at chromosome 17p that indicate a poor chance of survival (4,5). The majority of MBs are sporadic, but familial predisposition related to germline mutations exists (6). Familial MBs have assisted in the identification of relevant pathways for sporadic MB, such as in the Turcot syndrome with APC mutations involving the Wnt/Wingless pathway (7), and the Gorlin syndrome which is associated with mutations in the PTCH1 gene affecting the Sonic hedgehog pathway (8).

Mutations of the TP53 tumor suppressor gene have been shown to occur as an N-terminal truncated version denoted p47, Δ40p53 or ΔN-p53 (13-16). This protein...
is generated by the employment of an alternative translation start site. The direct consequence of this is a protein with an apparent size of 47 kDa, lacking the N-terminal transactivation and Mdm-2 binding domain of p53, but which can form heterotetramers with full length p53 and interfere with p53 function (13,14). p47 may act as a ‘cell cycle facilitator’ through its capacity to negatively regulate p53 transactivation. The human p53 gene can encode at least nine different p53 protein isoforms: p53, p53ß, Δ133p53, Δ133p53ß and Δ133p53ßy are produced by alternative splicing of the intron 9, and alternative usage of the alternative promoter in intron 4, and Δ40p53 (p47), Δ40p53ß and Δ40p53ßy - produced by alternative splicing of intron 9, and alternative initiation of translation or alternative splicing of intron 2 (14).

p53 isoforms have been described in breast cancer, acute myeloid leukemia, neuroblastoma, and in squamous cell carcinoma of the head and neck (17-21). In leukemia the appearance of a low molecular weight form of p53 is inversely correlated with the response to induction chemotherapy (17). Despite all the experimental evidence showing the importance of p53 in preventing carcinogenesis, it is difficult in clinical studies to link p53 status to cancer treatment and clinical outcome. The p53 isoforms may have profound impact on our understanding of p53 tumor suppressor activity (20). In this study we used different specific anti-p53 antibodies to evaluate p53 status and variants in patients with MB, in cell lines and xenografts.

Materials and methods

Ethics statement. Human postmortem tissue was obtained with approved ethical permission by the Stockholm Regional Ethics Review Board. Prospective written informed consent was obtained by the donors. The HIT study protocol, including the preservation and scientific analysis of medulloblastoma tumor tissue, was approved by the ethics committee of the University of Wuerzburg. Written informed consent was obtained from legal representatives of all patients before registration.

All animal experiments were approved by the Northern Stockholm Regional Ethics Committee for animal research (approval N234/05) in accordance with the Animal Protection Law (SFS1988:534), the Animal Protection Regulation (SFS 1988:539), and the Regulation for the Swedish National Board for Laboratory Animals (SFS1988:541).

Tissue preparation. Nine samples of MB (8 classic, 1 desmoplastic) were collected at the Department of Neuropathology, University of Bonn Medical Center, Germany, and frozen at -80°C. The frozen sections from the MB samples contained at least 80% tumor tissue. Normal human tissue samples dissected from postmortem human brains and visceral organs were obtained at autopsy and immediately frozen. Frozen tissue samples were kept on dry ice during tissue weight measurement and crushing. Crushed, powdered tissue samples were kept at -80°C. Human MB xenograft samples from mice were dissected and immediately frozen. All samples were stored at -80°C.

Cell lines and cell culturing. Six MB cell lines - D283Med (22), D324Med (23), D425Med and D458Med (24), MEB-MED8A (25), UW228-3 (26), and one supratentorial PNET, PFSk-1 (27), were used in the experiments for evaluating the p53 status by Western blotting, D283Med, D324Med, D425Med, D458Med and MEB-MED8A cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), supplemented with 10-20% of fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin - 100 μg/ml streptomycin) in an atmosphere of 5% of CO2. PFSk-1 was maintained in RPMI and UW228-3 in DMEM:F12 (Invitrogen), supplemented with 10-20% of FBS and antibiotics (100 units/ml penicillin - 100 μg/ml streptomycin) in an atmosphere of 5% of CO2.

Xenografts. Subcutaneous MB xenografts were obtained by transplanting the human D283Med MB cells in mice. Four to eight-week old female NMRI nu/nu mice (Taconic Laboratories, Ejby, Denmark) were maintained in groups of 5 per cage and were given sterile water and food ad libitum. Each NMRI nu/nu mouse was s.c. injected in the flank with 20x10⁶ D283MED MB cells. Tumors were measured every day and tumor volume was calculated as (width² x length x 0.44). After 9 days, the tumor volume was approximately 0.2 ml.

Plasmid and transfection. Positive control for full-length p53 was produced by transfecting a p53 expression plasmid into Hep3B cells, which are negative for p53. The same cell line was used for producing the p53 N-terminal truncated p47 positive control. The p53 and p47 expression plasmids were kindly gifts from Dr Pierre Hainaut, Lyon, France. Hep3B cells were passaged in DMEM, 10% FBS, 1% antibiotics to 60-70% confluence before transfection. The cells were transfected with either a p53 or p47 expression plasmids using Lipofectamine Plus in Opti-MEM medium (Invitrogen) according to the manufacturer’s recommendations.

Preparation of cell/tissue extracts. Buffers were supplemented with 5X protease inhibitors, Complete™, (Boehringer Mannheim) and 5 mM of the proteasome inhibitor MG132 (Calbiochem). All procedures were carried out on ice. Frozen tissue samples were crushed on dry ice and boiled in prewarmed SDS extraction buffer (0.45 M Tris-HCl, pH 8.5, 2.5% glycerol, 4% SDS, 0.5 mM DTT) and 5X protease inhibitors for 5 min, sonicated, centrifuged, and protein extracts were aliquoted and kept at -80°C until use. Protein concentration was determined with the DC protein assay (Bio-Rad Laboratories). Aliquots of tissue extracts (25-60 μg protein) were heated for 5 min at +95°C in the presence of 5 mM 8-mercaptoethanol and separated by SDS PAGE on 10% Tricine gels.

Western blot analysis. Equal amounts of cell/tissue protein extracts were separated by SDS-PAGE on 10% Tricine large (13x15 cm) gels (Acrylamide, Gel buffer, 100% Glycerol, dH₂O, 10% ammonium persulfate, TEMED, Bio-Rad Laboratories). Proteins were electrotropherated onto a nitrocellulose membrane (Schleicher and Schuell) at 4°C and stained by MemCode Reversible Protein Stain kit (Pierce). After destaining and blocking with 5% non-fat dry milk in Tris-buffered Saline Tween-20 (TBST), the membranes were incubated with monoclonal anti-p53-antibodies directed against
the pH3 N-terminus: Ab1801 (1:50 dilution, Calbiochem) and AbDO-7 (1:250 dilution, Dako), or the pH3 central domain, Ab240 (1:80 dilution, Calbiochem). As a cytosolic marker, a monoclonal antibody against Ï€-enolase was used (NSE-P2, 1:1000, Santa Cruz Biotechnology). The membranes were washed with Tris-Buffered Saline Tween-20 (TBST) and subsequently probed with a horseradish peroxidase conjugated goat anti-mouse immunoglobulin antibody (Calbiochem) at 1:2000 dilution, developed by ECL (Amersham) according to the manufacturer's instructions and exposed for 1-10 min on Hyperfilm-ECL (Amersham).

For reprobing, membranes were incubated with stripping buffer (62.5 mM Tris-HCl, 2% SDS, 100 mM ß-mercaptoethanol, pH 6.7) for 20 min at 55˚C, blocked for 30 min with 5% non-fat dry milk in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween-20 buffer, and probed with the goat anti-mouse immunoglobulin antibody (dilution 1:2000). Quantification of Western blots was performed by phosphoimager using Image Gauge Ver.3.11 software (Fuji Photo Film Co. Ltd., Japan).

Results

The expression of different p53 isoforms in human cancers may explain the inconsistencies in the published findings on the prognostic role of p53. The finding that the p53 gene can produce a number of different transcripts prompted us to investigate the presence of protein variants of p53 in solid tumors and to compare this with human brain regions and visceral tissues. This was done by analyzing a number of tumor samples using Western blot with three different antibodies specific for different domains of the protein (Fig. 1).

Figure 1. Human p53 protein structure and antibodies used in the present study. The extreme N-terminus (1-42aa) contains the acidic transactivation domain and the MDM2 protein binding site. It also contains the Highly Conserved Domain I (HCD I). Region 40-92aa contains series of proline residues that are less evolutionarily conserved, and a second transactivation domain. The central region (101-306aa) - the target of 90% of p53 mutations found in human cancers - contains the DNA binding domain. It contains HCD II to V. The oligomerization domain (307-355aa, TET) consists of a ß-strand, followed by an Ï€-helix necessary for dimerization. This oligomerization domain also contains a nuclear export signal (NES). The C-terminus of p53 (356-393aa) contains 3 nuclear localization signals (NLS) and a non-specific DNA binding domain that binds to damaged DNA. This region is also involved in the downregulation of DNA binding of the central domain. NEG is the C-terminal negative regulatory domain.

AbDO-7 recognizes aa 1-45 in the extreme N-terminus, Ab1801 recognizes aa 46-55 in the N-terminus, and Ab240 binds to aa 213-217 in the central domain.

p53 variants vary in medulloblastoma tumors and xenografts.

The expression of p53 in clinical MB samples, cell lines and xenografts was examined by Western blot using antibodies against the N-terminus of p53. Detection of a band consistent with the expected full length canonical MW of wild-type p53 (FL) was found in the majority of the samples (Fig. 2) using the antibody Ab1801 (directed to aa 46-55). The expression level of canonical p53 was variable between the different MB primary tumor samples. In addition, a protein species with an apparent size of 22 kDa was present at varying levels in all MB tumors. The levels of the 22 kDa protein did not proportionally correlate with the amount of the FL p53 (Fig. 2). Neither the control p53 generated from the transfected p53 canonical plasmid construct, nor the fetal cerebellum displayed the 22 kDa protein. In some of the MB samples, variable amounts of larger species of p53 with unknown function were also apparent (Fig. 2).

For reprobing, membranes were incubated with stripping buffer (62.5 mM Tris-HCl, 2% SDS, 100 mM ß-mercaptoethanol, pH 6.7) for 20 min at 55˚C, blocked for 30 min with 5% non-fat dry milk in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween-20 buffer, and probed with the goat anti-mouse immunoglobulin antibody (dilution 1:2000). Quantification of Western blots was performed by phosphoimager using Image Gauge Ver.3.11 software (Fuji Photo Film Co. Ltd., Japan).

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Figure 2. p53 status in medulloblastoma (MB) samples, detected by Ab1801. p53 expression was analyzed in cerebellum and MB samples from 9 different subjects and protein extracts (50 μg) were loaded on 10% SDS-PAGE high resolution gels. Arrows indicate full-length p53 (upper arrow) and its short form ~22 kDa (lower arrow) as detected by antibody Ab1801. Positive control for full-length p53 was produced by transfecting a p53 expression plasmid into the p53 deficient Hep3B cell line.
previously reported as being a regulatory factor for p53, we transfected the p53 negative Hep3B cells with p47 in an expression vector and included this in the panel of MB cell lines. Western blot analysis revealed that the Ab1801 anti-body is able to recognize p47, while the AbDO-7, which is directed to the very end of the N-terminus of p53, does not (Fig. 3A). This served as a control for the p47 truncation being only in the first 45 amino acids of the N-terminus of p53. The various sizes of p53 in MB cell lines appear to be very similar irrespective of antibody used (Ab1801 or AbDO-7; Fig. 3). We therefore conclude that truncation of the very N-terminus does not explain the variable and shorter p53 species in MB cell lines. No 22 kDa species of p53 were detected in MB cell lines.

In order to investigate whether the tumor growth environment of MB might affect the p53 isoforms, the cell line D283Med that displayed a p53 species with a slightly smaller apparent migration than FLp53 when grown in culture, was implanted into nude mice to generate subcutaneous xenografts. Western blot analysis of the xenografts, using the Ab1801 antibody, revealed predominantly a protein that corresponds to FLp53. In addition to this, a weak band corresponding to 22 kDa was also evident (Fig. 4). Thus, a smaller p53 protein isoform was present, albeit less abundant than FL p53, both in MB tumor samples (Fig. 2) and in xenografts (Fig. 4) obtained from a cell line (Fig. 3) which did not express this isoform. Thus, the p53 protein is produced in isoform(s) in MB tumor tissue and xenografts, not observed in the corresponding MB cell lines.

As the p53 isoforms might have different biological activities, we investigated whether p53 isoforms are differentially expressed in normal human tissues as was observed in MB. Human brain tissue from cerebellum and temporal cortex, which both revealed only FLp53, was compared with different human organs, heart, lung, liver, intestine and testis using the N-terminus-specific Ab1801 and the central DNA-binding domain-specific Ab240 antibodies. In normal visceral human organs, several short forms of p53 were detected using both antibodies (Fig. 5A and B). The expression of the low molecular weight forms was variable between the different human tissues and was often co-expressed with the FLp53. In lung tissue, the two antibodies gave similar band patterns with two low molecular weight bands, while in heart, intestine, liver and testis, only one of the low molecular bands was visible using Ab1801 (Fig. 5). In visceral tissues, FLp53 was generally expressed at low levels.

Subcellular localization of p53 in brain tissue. The nuclear localization of canonical p53 is essential for its tumor suppressor function. The p53 protein may, however, have different subcellular localizations and therefore distinct activities. In order to investigate this in the normal human brain, we analyzed nuclear and cytosolic fractions of cerebellum since this is the origin of MB, and temporal cortex, as a brain control tissue. These brain structures were assessed with Ab1801, using γ-enolase as a cytosolic neuronal
marker. Two patterns were found: in the temporal cortex, p53 was completely absent in the cytosol, while cerebellum contained the p53 protein in both the nucleus and cytosol (Fig. 6). MB tissues could not be used for subcellular analysis.

Discussion

As p53 isoforms may bind differentially to promoters, modulate p53 target gene expression and p53-mediated apoptosis, and provide regulatory effects of canonical p53, this can explain the difficulties to link p53 status to the biological properties and drug sensitivity of human cancer (20). The aim of the present study was to characterize the p53 isoform status in MB in comparison with normal brain and visceral tissues. Unlike many other cancers, MB cells usually express wild-type p53 and mutational inactivation is relatively rare. We report here the presence of low molecular weight p53 forms (LMWFs) in MB samples, both in solid tissue tumors and in MB xenografts (Fig. 2 and 4). Since none of the tested MB cell lines displayed LMWF p53 (Fig. 3), it is possible that truncated forms, e.g. the 22 kDa variant found in tumors and xenografts, might be produced in non-tumor cells surrounding the tumor cells. Alternatively, variants of p53 may be present in the same cell as the canonical p53, induced by the tumor environment. Although this has not been addressed in this study, the presence of short forms might lead to interactions that could affect its biological activity as was shown for the truncated p53 form, p47, which act in a dominant-negative manner towards FLp53, both inhibiting p53 transcriptional activity and p53-mediated apoptosis (14,20). The expression of various isoforms may therefore partly account for aberrant functions of p53 in MB, as was shown in melanoma where Δ40p53 and p53ß were shown to form complexes with p53 (28). The proportion of p53 isoforms relative to the wild-type protein may thus be important for regulating p53-dependent transcription in MB. p53 protein isoforms are less abundant than FL p53 both in MB solid tissue samples and xenografts. However, p53 undergoes multiple post-translational modifications (29,30) and specific modifications may be targeted to distinct p53 isoforms to give specific biological effects. In addition to low molecular weight form p53, modifications leading to larger p53 species are indeed suggested in this study, both in MB tumor tissue as well as in visceral organ tissue (Figs. 2 and 5). As in many other tumors, MB is formed by a heterogeneous population of cells that may express p53 at various levels in different cells, and the ratio between FLp53 and its variants might vary as well. In MB cell lines, which can be considered substantially more homogeneous, p53 LMWFs were not detected, although some variability around 53 kDa was evident (Fig. 3). Some of these forms displayed slightly smaller apparent size than 53 kDa. Since even the antibody AbDO-7, which specifically recognizes the N-terminus (1-45 aa), recognizes these molecular forms, any presence of smaller sizes must be accounted for by either C-terminus truncation or differential splicing, leading to faster migration on the gel of the protein product. None of the MB cell lines displayed a form co-migrating with p47.

In the normal human tissues, different p53 forms were found in a tissue-dependent manner (Fig. 5), suggesting that the internal promoter/alternative splicing of p53 can be tissue-specifically regulated. This may lead to different transcriptional activity in response to different types of cellular stress. It is also notable that in some tissues (e.g. liver, lung, intestine, heart, testis) the protein levels of the LMWFs exceed that of the FLp53. With the exception of heart and...
intestine, in visceral tissues FLp53 was expressed at relatively low levels, consistent with p53 being expressed at low levels in normal cell unless activated and stabilized by DNA damage (31). We also noted that in the intestine, liver and lung, FLp53 was not detected by the Ab1801 antibody, and in the liver also Ab240 did not recognize FLp53. We do not know the reason for this but it is possible that tissue-specific modifications in the N-terminus epitope prevent Ab1801 from binding. Degradation is not likely since both the intestine and lung display FLp53 using Ab240. The complex p53 patterns in visceral tissues (Fig. 5) underscore the need for more thorough investigations using several antibodies as well as functional studies.

FLp53 is multifunctional and is found as a nuclear protein (20), but may enter the cytosol during cellular stress (32-35). If p53 isoforms have different subcellular localizations, this might be relevant for their various biological activities. LMWF of p53 were indeed shown to be abnormally localized in melanoma, where the endogenously expressed p53 isoforms may have differential localization between the nucleus and the cytosol of melanoma cells, compared with the wild-type p53 (28). The different p53 subcellular localization shown in the present study, even between the two different brain structures, cerebellum and temporal cortex (Fig. 6), may reflect differences in protective mechanisms in the different parts of the brain which may be involved in the pathogenesis of MB.

Taken together, our results suggest that the presence of p53 isoforms and their proportion to the expression of the full-length protein may be involved in regulating p53-dependent transcription. One important conclusion of this study is that since differences in the presence of p53 isoforms were found between cell lines and their derived xenografts as well as with MB tissue, it is suggested that p53 should be investigated in in vivo models rather than in vitro in cell lines.

The selection of antibodies to be applied in quantitative p53 assays is important to assess various p53 isoforms, the proportion of which may hint at biological activity. This selection may establish specificity for subsequent clinical prognosis. The prediction of outcome in MB would permit allocation of patients with poor survival prognosis to appropriate aggressive therapeutic regimen, while reducing therapeutic morbidity in patients with better prognosis.

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